GENETIC AND BIOCHEMICAL STUDIES OF THE CYSTEINE - METHIONINE SERIES OF MUTANTS IN *NEUROSPORA CRASSA*

Thesis by

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In Partial Fulfillment of the Requirement

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1954

ACKNOWLEDGEMENTS

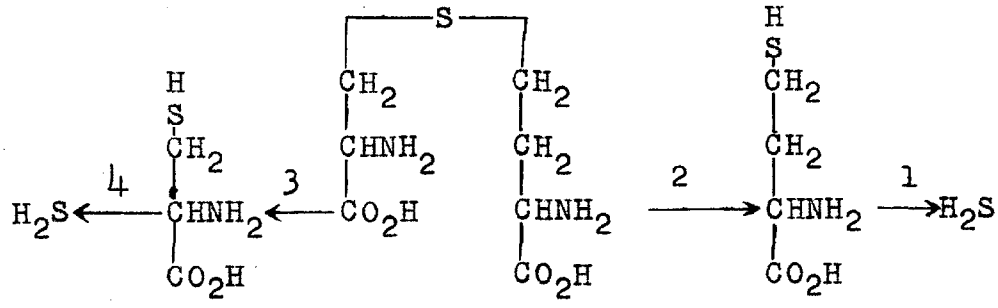
The author acknowledges with gratitude the advice and guidance of Dr. N. H. Horowitz.

The author also wishes to express his appreciation of the assistance received from Dr. M. Fling and Dr. J. Dubnoff as well as from the Biology Department in general.

The McCallum Foundation has provided financial assistance which greatly assisted in the completion of this work.

ABSTRACT

1. The following enzyme activities were studied in extracts of wild type and mutant Neurospora:



cysteine cystathionine homocysteine

Activity 2 was shown to be absent in a homocysteineless mutant and activity 3 was shown to be absent in a cystathionineless mutant. It was found that a suppressor, obtained by Giles, which causes these mutants to grow on minimal, returns enzyme activity to the mutants. Each activity is shown to be catalyzed by a different enzyme.

2. Evidence is presented which indicates that a Neurospora cystathionineless mutant can synthesize cystathionine from methionine without the intervention of cysteine.

3. Mutants which are blocked between thiosulfate and cysteine are shown to grow on elemental sulfur and H_2S.

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I. INTRODUCTION

The close relationship between genes and the chemistry of the cell was recognized soon after the rediscovery of Mendel. In 1909 Garrod published a review of human diseases that appeared to be of genetic origin. Of these, the study of alcaptonuria is recognized as the first biochemical genetical investigation. Garrod summarized data which indicated that the urinary secretion of homogentisic acid caused the blackened urine by which diseased individuals were recognized. At the same time, he accepted the interpretation of Bateson that the disease behaved in inheritance as if it were due to a simple Mendelian recessive factor, and further proposed that the disease was the result of the absence of an enzyme which could break down homogentisic acid (1).

Studies on flower color (2) and Drosophila eye color (3) have also been interpreted as indicating a one-to-one relationship between genes and single chemical reactions in the cell. By this time advances had also been made in biochemistry and enzymology which permitted closer examination of the relationship of genes and metabolism. These advances were in enzymology, in nutrition and in the recognition that biosynthetic reactions proceed through series of intermediates.

Neurospora was selected by Beadle and Tatum as an organism which would facilitate biochemical genetical investigations (4). In Neurospora a short time (10 to 16 days) is required to complete a cross and a large amount of haploid material for biochemical work can be easily obtained.

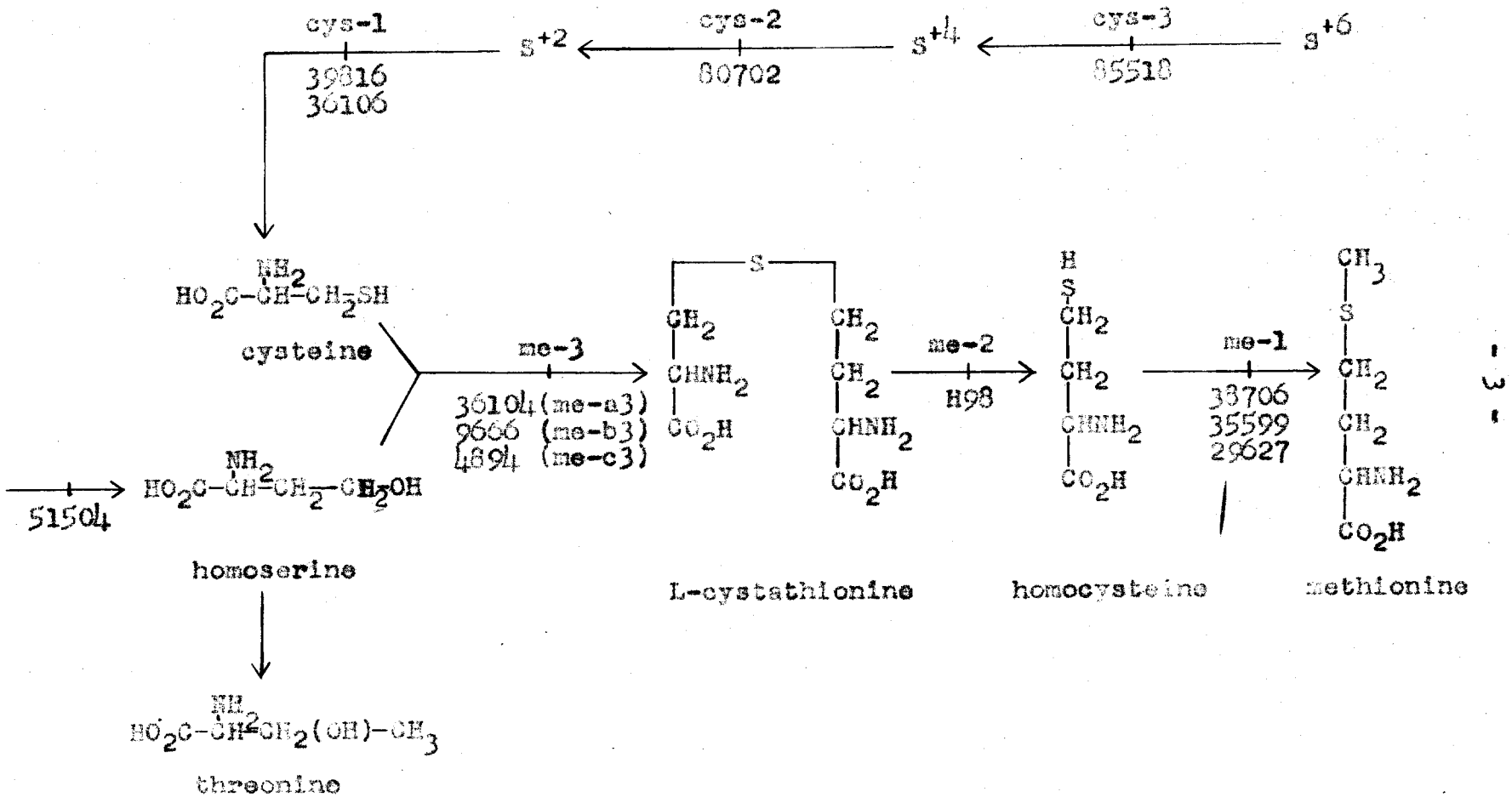
The discovery that the single gene nutritional mutants of Neurospora commonly have but a single nutritional requirement not only demonstrated that genes control the essential chemical activities of the cell but indicated that the primary function of the gene was to supply a single enzyme activity to the cell (5). This has come to be known as the one gene - one function or one gene - one enzyme hypothesis.

At the time this work was begun, there were very few data reported from in vitro systems which were designed to obtain information about the one gene - one enzyme hypothesis. Mitchell and Lein had reported that a tryptophaneless mutant of Neurospora had no detectable tryptophane-synthesizing activity in vitro, although such activity was readily observed in wild type strains and other mutants (6). Wagner had reported that a pantothenic acid-requiring mutant under certain in vitro conditions was as active in the synthesis of pantothenic acid as was wild type (7). There were also reports that a number of genes affect the activity of a single enzyme, as with tyrosinase in Glomerella (8). At least six nonallelic genes were shown to affect tyrosinase activity of crude extracts of the mutants.

The methionineless mutants of Neurospora offer favorable material for in vitro experiments relating to the one gene - one enzyme hypothesis, since the genetic relationship and nutritional requirements of these mutants have largely been determined. The biosynthetic pathway and principal mutants concerned are indicated in figure 1.

Buss has shown that strains H98, 4894, 9666 and 36104

Figure 1



The Pathway of Methionine Synthesis in Neurospora (9, 10, 17, 18)

are single gene, nonallelic mutants (9). Phinney has shown that 38706 is a single gene mutant, nonallelic to H98 and 36104 (10), and Gershowitz has shown that mutants 35599 and 29627 are non-allelic (11).

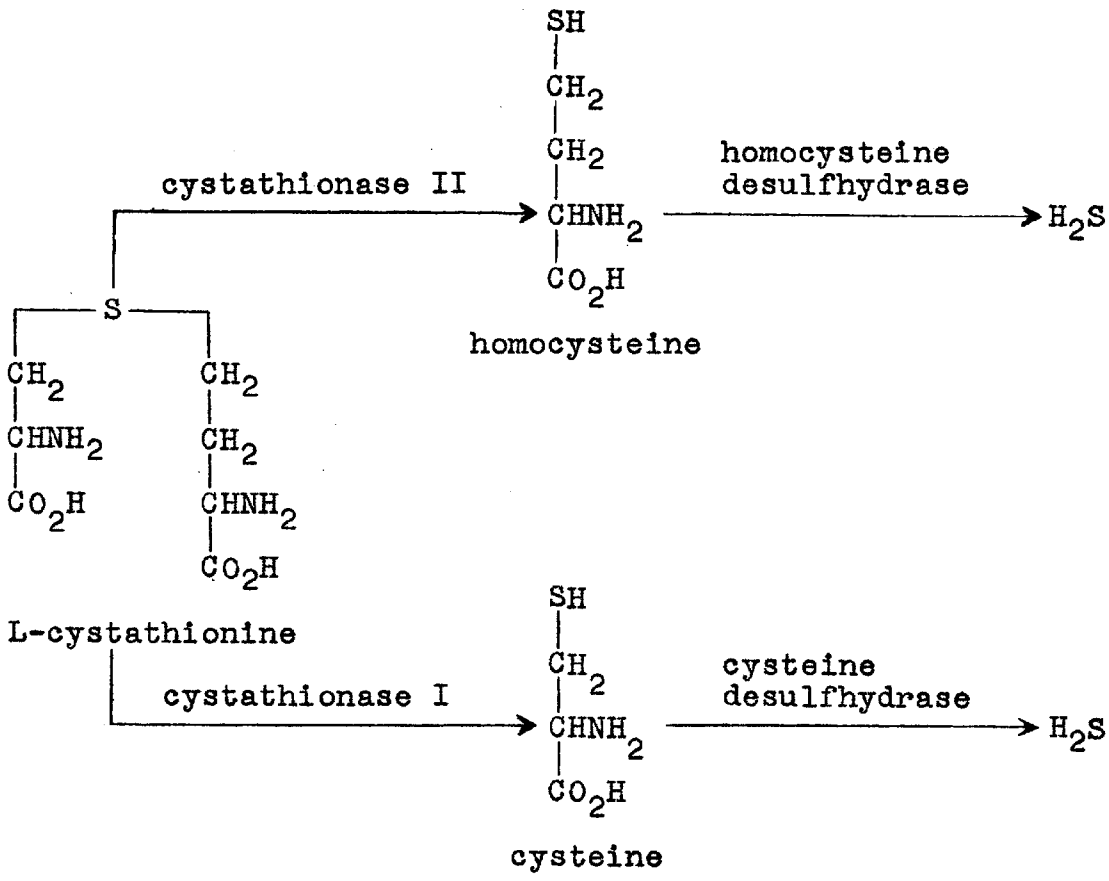
In a series of investigations Horowitz and co-workers have established the nutritional requirements of the mutants. It was found that of the nutritional mutants obtained after the treatment of Neurospora with mutagenic agents, those requiring sulfur compounds could be subdivided into those which would grow on cysteine and those which would not grow on cysteine, but required methionine. On this basis the mutants are divided into cysteineless (cys) and methionineless (me) classes. The cysteineless mutants can be further subdivided into three classes (cys-1, cys-2 and cys-3) on the basis of the level of reduction of the sulfur compound to which they will respond. It was found that one class of methionineless mutants, me-1, would not grow on homocysteine but responded only to methionine. This class of mutants is apparently blocked in the methylation of homocysteine. The isolation of L-cystathionine from H98, a me-2 strain, followed the observation that crude extracts of this mutant fed me-3 mutants. The L-cystathionine isolated permitted a further subdivision of the methionineless mutants into me-2 and me-3 strains, since it was shown to support the growth of me-3 and cys-1 strains, but not me-2 or me-1 strains (10). This was the first isolation of cystathionine from a biological source. L-cystathionine was previously implicated in transulfuration in mammals by Brand et al. (12) and by

DuVigneaud and collaborators who found by nutritional and enzymatic experiments using rats that the sulfur of homocysteine was transferred to cysteine through cystathionine (13 - 16). These results demonstrated that the in vivo route of transulfuration in Neurospora and mammals involves the same intermediates. However, the pathway used by Neurospora is reversed in mammals.

Teas discovered that a single gene mutant strain which had a nutritional requirement for both threonine and methionine would grow on homoserine alone. This suggests that homoserine contributes to both methionine and threonine synthesis and that the carbon chain of homoserine forms the 4 carbon portion of cystathionine and methionine as well (17). The isolation of threonine and homoserine from mutant No. 9666, by Fling and Horowitz, supports the interpretation of the double role of homoserine, since this mutant would be expected to accumulate cystathionine precursors or compounds closely related to these precursors (18).

From the enzymes related to methionine synthesis, the cystathionine cleavage enzymes were selected for study in wild type and mutant strains. When crude extracts of wild type Neurospora were tested for cystathionine cleavage, the activities indicated in figure 2 were observed. It was shown

Figure 2



that cystathionase II activity could not be detected in a me-2 strain, that cystathionase I activity could not be detected in a me-c3 strain and that each activity is due to a separate enzyme. It was also shown that the effect of a suppressor which suppressed both strains is to restore enzyme activity to the appropriate mutant.

II. MATERIALS AND METHODS

A. Strains Used

The strains of Neurospora crassa used in this work are listed in table 1. Cultures were maintained on agar slants of complete medium (10).

B. Chemicals

L-cystathionine was isolated from cultures of mutant No. H98 by the method of Horowitz (10).

Tris-hydroxymethylaminomethane (Tris) was recrystallized twice from H_2O before use as a buffer.

Elemental sulfur (flowers of sulfur and roll sulfur) was repeatedly washed with sterile glass-distilled water to remove material which precipitated with Ba^{++}. The washed sulfur was kept in vacuo over $CaCL_2$.

All other compounds were from commercial sources, except the following compounds furnished through the courtesy of the persons noted.

L-cystathionine-synthetic	Dr. N. H. Horowitz
Pyridoxal phosphate (Ca^{++} salt)	Dr. Karl Folkers
α-ketobutyric acid	Dr. J. L. Reissig
DL-homocysteine thiolactone	Dr. M. Fling
DL-homoserine	Dr. M. Fling

C. Culture Methods

Growth was measured by the dry weight of mycelium produced at 25°C in 20 ml of medium. The medium and techniques used are described by Horowitz and Beadle (24). "Sulfur-free"

Table 1

Standard strains and ascospore reisolates of Neurospora crassa used.

Standard Strains	Origin	Nutritional Requirement	Reference
5256A		none	Emerson, Cushing (19)
5297a		none	" "
85518	mustard gas	sulfite	Horowitz (20)
80702A	heavy neutron	thiosulfate	Beadle, Tatum (21)
6125a	X-ray	cystine	" "
34555-H1A	U.V.	"	" "
36103	U.V.	"	" "
36106A	U.V.	"	Shen (22)
39816a	U.V.	"	Horowitz (10)
47409	U.V.	"	Beadle, Tatum (21)
65111A	U.V.	"	" "
71310	U.V.	"	" "
83002	X-ray	"	" "
36104	U.V.	cystathionine	Buss (9)
9666	X-ray	"	" "
4894	X-ray	"	" "
H98A	X-ray	homocysteine	Buss, Horowitz (9, 10)
35599-R1A*	U.V.	methionine	Beadle, Tatum (21)
29627-R1a*	X-ray	"	" "
51504	U.V.	homoserine	Teas (17)

* Reisolated by Mr. Henry Gershowitz

Table 1

(Continued)

Strain	Genotype	Nutritional Requirement	Reference
4894-CL6-9.1a	al_2 su-1	none	Giles (23)
4894-CL1-5a	al_2 su-1 me-c3	"	" "
H98-B203-CL3-12.7	al_2 su-1 me-2	"	" "
D a-3	"	"	This thesis
D 8.1-7*	"	"	"
D 15.1-7	"	"	"
D 8.4-1	al_2 su-1	"	"
D 8.4-6	me-2	homocysteine	"
4894-R1**	me-c3	cystathionine	"

* D refers to cross number (4894-CL6-9.1a x H98A), 8 to perithecium number, 1 to ascus number, 7 to ascospore number.

** Reisolated from the cross 4894-CL1-5a x 5256A.

minimal medium was prepared by replacement of $MgSO_4$ with an equivalent amount of $MgCl_2$. This medium was probably contaminated with sulfur compounds, since it permitted wild type to grow 4 to 22 mg dry weight in 72 hours without the addition of sulfur-containing compounds. Stationary cultures to be used for enzyme preparations were grown for 3 - 9 days in Fernbach flasks containing 300 ml of medium. Aerated cultures were grown for 1.5 - 7 days in 5 gallon Pyrex carboys containing 8 - 16 liters of medium. The medium was supplemented with 37.5 mg of Dl-methionine per liter of medium, except where noted.

D. Enzyme Preparations

Enzymatic activities have been studied in crude and partially purified extracts of Neurospora. Three types of extractions were made (Methods A, B and C). The solutions were kept cold throughout the operations.

Method A: When less than 30 gm wet weight of mycelium was to be extracted, 2 ml of cold distilled water and 0.25 gm of sand were added per gram wet weight of mycelium. The material was ground for 15 minutes in a cold mortar and centrifuged at 800 g for 20 minutes. The supernatant was collected and dialyzed with stirring for 6 - 12 hours against 20 volumes of 0.004 M Tris buffer, pH 8.60. The buffer was changed every two hours.

Method B: With larger amounts, successive 50 gram samples were ground for 10 minutes in a cold mortar with 1 ml distilled H_2O and 0.5 gm sand/gm wet weight. Samples were then combined and ground for 10 - 15 additional minutes. The

suspension was centrifuged at 8,000 g for 20 minutes. The supernatant was decanted and the precipitate re-extracted by grinding in a mortar for 15 minutes with one ml of water per gram wet weight of original mycelium and centrifuged for 15 minutes. The precipitate was discarded. The supernatants from the two extractions were combined and brought to 50% saturation with $(NH_4)_2SO_4$. The solution was allowed to stand for 10 minutes and centrifuged at 10,000 to 20,000 g for 20 minutes. The precipitate was suspended in a minimal amount of water and dialyzed in 0.9 mm tubing with rotation against 3 changes of 2 liters 0.004 M Tris buffer, pH 8.6. After dialysis the material was kept at -16°C for 12 - 24 hours. At the end of this time the suspension was thawed and centrifuged at 15,000 g for 20 minutes. The precipitate was discarded and the supernatant used as the enzyme solution.

Method C: Mycelia to be extracted were frozen with dry ice and the cell walls broken in a blendor. While the material was still partly frozen, 0.25 ml water per gram wet weight was added. The material was centrifuged at 19,000 to 20,000 g for 1 hour and the precipitate discarded. The supernatant was dialyzed for 6 hours against 3 changes of 20 volumes of 0.004 M Tris buffer and was used as an enzyme solution.

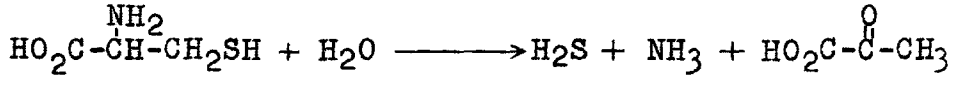
III. THE ENZYMATIC CLEAVAGE OF CYSTATHIONINE

A. Cysteine Desulfhydrase

The principal object of the enzyme experiments was study of cystathionases I and II in Neurospora. Cystathionase I catalyzes the production of cyst(e)ine from L-cystathionine and homocyst(e)ine is an end product of cystathionase II activity, as indicated in figure 2, page 6. However, most of the preparations contained active cysteine desulfhydrase and homocysteine desulfhydrase, since H_2S was produced from cysteine and homocysteine. This is a complication in the determination of cystathionase I and cystathionase II activities, because the immediate origin of H_2S arising in incubations with L-cystathionine as substrate could not be determined. Therefore, the observed properties of the desulfhydrases will be described before presenting experiments directly related to the cystathionases.

Two reviews of cysteine desulfhydrase have been published. The probable end products of cysteine desulfhydrase activity are presented in figure 3 (25) (26).

Figure 3



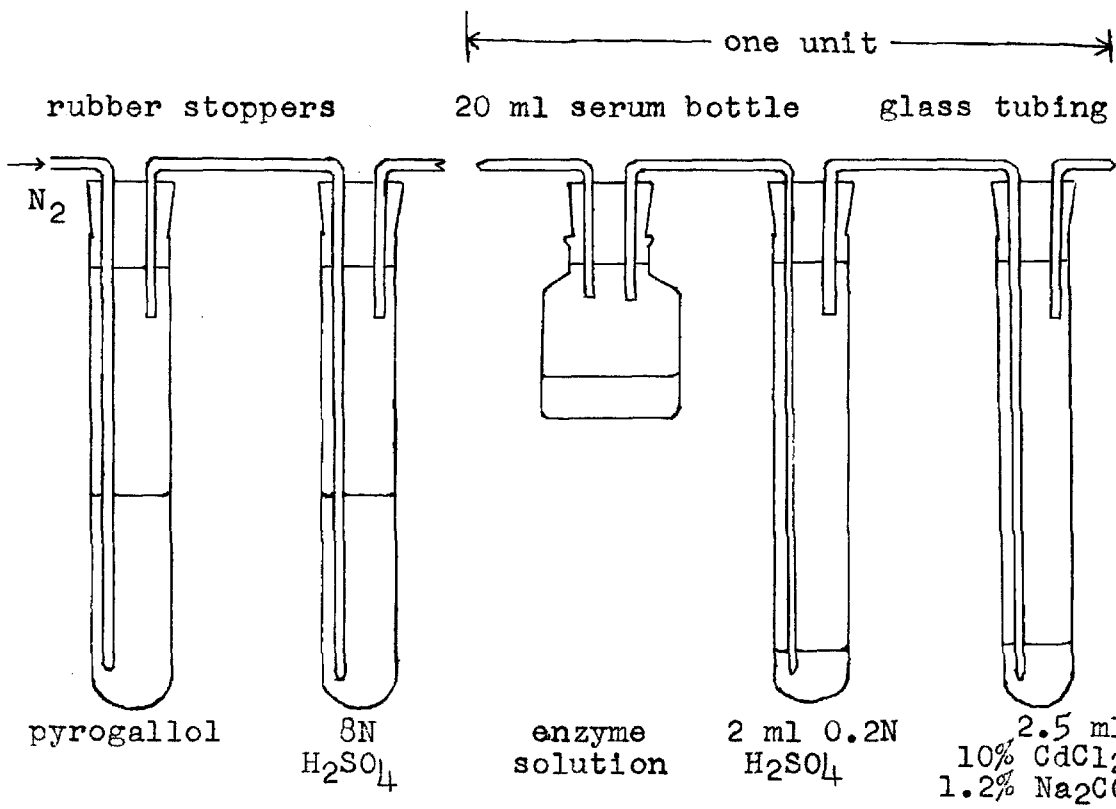
cysteine — pyruvic acid

1. Incubation methods

The apparatus used to detect cysteine desulfhydrase activity is presented in figure 4. Since the gases, H_2S and

NH_3, were enzymatic end products, a continuous stream of N_2 was passed over the enzyme preparations contained in serum bottles in order to recover these products in appropriate trapping agents, $CdCl_2$ for H_2S and 0.2N H_2SO_4 for NH_3. The

Figure 4



solutions in the serum bottles were used to detect nonvolatile products. As many as six enzyme preparations could be used in one series. Each experimental incubation was matched by a control incubation, except that the substrate was omitted.

To stop the reaction, 50% trichloroacetic acid was added to the enzyme preparation to bring the final concentration to 5%. The nitrogen stream was then continued for 30 minutes. If NH_3 was to be determined, an aliquot of the solution was made strongly basic (0.2 ml 5N NaOH per ml solution to be

assayed), placed in a fresh serum bottle and the nitrogen stream continued for 5 hours into a fresh trap of 0.2N H_2SO_4.

2. Quantitative methods

Hydrogen sulfide was determined by the methylene blue method of Fogo and Popowsky (27), except that CdS was the source of H_2S and the volumes they used were decreased tenfold. Hydrogen sulfide was quantitatively recovered from enzyme mixtures to which H_2S in the range of 0.12 to 0.50 μM was added. Less than 0.12 μM could not be determined accurately. Standard H_2S solutions were prepared by collecting H_2S from a commercial tank in freshly boiled glass distilled H_2O. The concentration of H_2S was determined by iodometric titration.

Ammonia was determined by adding aliquots of the 0.2N H_2SO_4 trap solutions to 10 ml calibrated test tubes and diluting to 8 ml; 1 ml of Nessler's solution was added, the volumes made to 10 ml with H_2O and the solutions read in a Klett-Summerson colorimeter after 30 minutes. This method permits quantitative determination of 0.1 - 1.0 μM NH_3. Additional washing of the trap solutions was sometimes necessary to remove interfering substances.

Pyruvic acid was determined by the method of Lu as modified by Elgart and Nelson (28). This method gave quantitative recovery of pyruvic acid added to enzyme preparations, but was unsatisfactory for α-ketobutyric acid. The chromatographic system of Cavallini et al. (29) was used to identify the dinitrophenylhydrazone of pyruvic acid.

With Neurospora, attempts to demonstrate NH_3, pyruvic

acid, alanine or serine as end products of cysteine desulfhydrase activity have been unsuccessful, although ammonia in varying and non-stoichiometric amounts has been found.

3. pH optimum

As seen in table 2, crude preparations of Neurospora cysteine desulfhydrase were most active at pH 7.4 when incubated for 1.5 hours.

4. Pyridoxal phosphate as coenzyme

Braunstein and Azarkh reported that cysteine desulfhydrase activity was low in liver extracts of pyridoxine deficient rats, which suggests that the enzyme requires pyridoxal phosphate as a cofactor, although in vitro activity was not restored by the addition of pyridoxal phosphate (30). However, as seen in table 3, the cysteine desulfhydrase activity of crude undialyzed extracts of Neurospora increased when pyridoxal phosphate was added. Dialysis for 6 hours against 0.004 M Tris buffer, pH 8.2, increased the effect. In two experiments dialysis was continued for eighteen hours. These preparations showed three and fourfold increase of activity after the addition of pyridoxal phosphate. These results are in agreement with those obtained by Kallio, who reported that the activation by pyridoxal phosphate of cysteine desulfhydrase in cell-free extracts of Proteus morganii (31). Binkley reports that he has obtained a crystalline enzyme, "thionase," from rat liver which is specific for cleavage of acids with free α-amino groups and bivalent sulfur on the β or γ carbon (32). This enzyme was reported to require pyridoxal phosphate when djenkolic acid, L-cystathionine and L-allocystathionine were

Table 2

pH versus activity of Neurospora cysteine desulfhydrase prepared from strain 5297a by method C.

L-cysteine·HCl, 1 mg/ml, pyridoxal phosphate 60 /ml, 1 ml 0.2M Tris buffer, 7 ml enzyme. Final volume, 10 ml. Reaction time, 1.5 hours. Activity is expressed as μM H_2S/10 gm wet wt. of mycelium/hour.

pH	Activity
6.9	0.45
7.4	0.57
7.7	0.40
9.2	0.17

Table 3

Pyridoxal phosphate activation of cysteine desulfhydrase before and after dialysis of crude extracts of wild type Neurospora (5256A), prepared according to method A.

L-cysteine·HCl 6 mg/ml, pyridoxal phosphate, 10σ /ml, 7 ml enzyme. Final volume 10 ml, reaction time 3 hours, pH 7.4. Activity is expressed as a μM H_2S/10 gm wet wt. mycelium/ hour.

	Activity	
	- pyridoxal phosphate	pyridoxal phosphate
Undialyzed	0.37	0.58
Dialyzed	0.22	0.41

substrates (33). Tests with cysteine as a substrate were not reported.

Cysteine desulfhydrase from Neurospora is not activated by 0.001M $MgCl_2$ after six or eighteen hours of dialysis. This agrees with the report of Binkley that magnesium ion is not required by "thionase" with cysteine as a substrate. Earlier reports that the enzyme was activated by magnesium ion may reflect antagonism of an inhibitor (32).

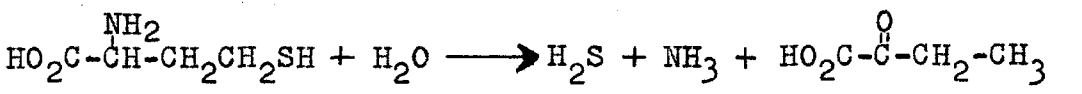
5. Fractionation experiments

Cysteine desulfhydrase activity has been determined in precipitates obtained with $(NH_4)_2SO_4$. The results are presented in table 6, page 22 . Horowitz has found cysteine desulfhydrase activity in preparations containing particles (34). This was confirmed when cysteine desulfhydrase activity was found in washed particle preparations. The distribution of the activity between the particles and supernatant was not determined.

B. Homocysteine desulfhydrase

In 1942 Fromageot and Desnuelle reported the presence of an enzyme producing H_2S from homocysteine in the liver of rats and dogs (35). In 1951 Kallio reported that a cell-free homocysteine desulfhydrase preparation of Proteus morganii produced α-ketobutyric acid, NH_3 and H_2S as enzymatic end products. In addition it was reported that the enzyme required pyridoxal phosphate as a cofactor (31). The probable end products of homocysteine desulfhydrase activity are presented in figure 5. Homocysteine desulfhydrase activity has been found in Neurospora.

Figure 5



homocysteine ∝-ketobutyric acid

1. Methods used

The same incubation and quantitative methods described under cysteine desulfhydrase were also used to detect homocysteine desulfhydrase activity.

2. pH optimum

As seen in table 4, crude preparations of Neurospora homocysteine desulfhydrase were most active at pH 7.9 when incubated 1.5 hours. This is in the pH range used by other workers for the enzyme.

3. Pyridoxal phosphate as coenzyme

Pyridoxal phosphate activates homocysteine desulfhydrase, as seen in table 5. This experiment has been repeated.

4. Fractionation experiments

As seen in table 6 fractionation experiments with $(NH_4)_2SO_4$ indicated that homocysteine desulfhydrase precipitated (or co-precipitated) at a lower $(NH_4)_2SO_4$ concentration than did cysteine desulfhydrase. This suggestion that the desulfhydrases are different enzymes was supported by later experiments.

C. Cysteine Desulfhydrase and Homocysteine Desulfhydrases as Separate Enzymes

The clearest evidence that the desulfhydrases are separate enzymes in Neurospora is the fact that these activities vary independently in different strains. Data are presented in

Table 4

pH dependence of the activity of Neurospora homocysteine desulfhydrase.

The extract was prepared by method C from 5297a. DL-homocysteine was added as the free base to bring the final concentration to 0.8 mg/ml. 6% /ml of pyridoxal phosphate, 7 ml of enzyme and 0.06 M Tris. Final volume 10.0 ml. Reaction time 1.5 hours. Activity is expressed as μM H_2S/10 gm wet wt. mycelium/hr.

pH	Activity
7.1	0.08
7.50	0.29
7.90	0.37
8.4	0.28

Table 5

Pyridoxal phosphate activation of Neurospora homocysteine desulfhydrase.

Enzyme prepared by method C. 0.2 mg/ml DL-homocysteine, 6% /ml pyridoxal phosphate, 8 ml enzyme, final volume 10 ml, pH 7.60, reaction time 2 hours. Activity is expressed as μM H_2S/10 gm wet wt. mycelium/hr.

	Activity	
	-pyridoxal phosphate	pyridoxal phosphate
Dialyzed crude enzyme	0.16	0.29

Table 6

Homocysteine desulfhydrase and cysteine desulfhydrase activities of precipitates obtained by $(NH_4)_2SO_4$ fractionation of extracts from wild type Neurospora.

Enzyme obtained by method B. 6% pyridoxal phosphate/ml, 0.3 mg DL-homocysteine/ml or 0.22 mg. L-cysteine·HCl/ml, 8 ml enzyme. Final volumes 12 ml, pH 7.8. Reaction time 8 hours. Activity is expressed as μM H_2S/10 gm wet wt. mycelium/hour.

Percent saturation ammonium sulfate	Activity Present in Ppt.	
	Homocysteine	Cysteine
0-50	0.50	0.28
50-60	0.12	0.13

table 7. Strains D 8.4-6 and D 8.4-1 had no detectable homocysteine desulfhydrase activity. Conditions of the experiment were such that activities of 0.02 and 0.006 respectively could have been detected. If homocysteine desulfhydrase could produce H_2S from L-cysteine, no enzyme preparation would be expected to be active toward homocysteine alone. It was found that H_2S was produced from homocysteine but not from cysteine when water extracts of acetone powders, prepared from supernatants which contained both enzyme activities, were examined. Eighty percent of the homocysteine desulfhydrase activity was lost during the preparation. It is interesting that water extracts of acetone powders of dog livers were reported to have lost the homocysteine desulfhydrase activity but to have retained the cysteine desulfhydrase activity (35).

In addition to the data presented here, other evidence has been reported concerning the nonidentity of cysteine desulfhydrase and homocysteine desulfhydrase. Kallio has reported that a cell-free preparation of Proteus morganii with enzyme-saturating substrate concentrations produced as much H_2S when cysteine and homocysteine were incubated together as the sum produced when they were incubated separately. This was reported as evidence that the desulfhydrases are different enzymes, since if only one enzyme were involved, no increase in H_2S production would be expected when both substrates were incubated together (31). Binkley has reported that highly purified "thionase" from rat liver can produce H_2S from homocysteine if the enzyme concentration is increased one-

Table 7

Cysteine and homocysteine desulfhydrase activities in different strains and in different preparations from the same strain.

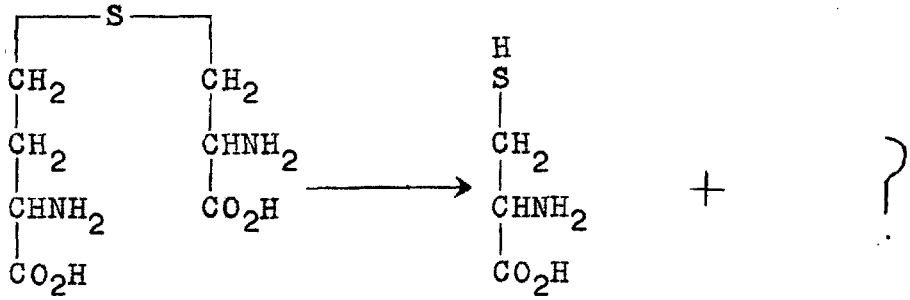
6ø pyridoxal phosphate/ml, 0.04M Tris buffer, pH as indicated. Substrates were L-cysteine·HCl and DL-homocysteine of equal molarity. Activities are expressed as μM H_2S/10 gm wet wt. mycelium/hour.

Strain	Known Genotype	Preparation method	Reaction time, hours	pH	Activity		
					Cysteine	Homocysteine	Homocysteine/ cysteine
5297	wild type	C	1.5	7.1	0.50	0.08	0.16
"	"	C	1.5	7.8	0.38	0.35	0.92
"	"	A	12.0	8.2	0.16	0.33	2.1
5256	"	B	3.0	7.5	0.10	0.17	1.7
H98	me-2	A	12.0	7.6	0.03	0.02	0.7
D8.4-6	me-2	A	3.0	8.1	0.13	0	0
D 8.4-1	su-1	B	2.0	7.4	0.032	0	0
D 8.1-7	me-2 su-1	A	12.0	7.8	0.12	0.12	1.0
4894	me-c3	B	2.0	7.2	0.31	0.10	0.32

hundredfold over that used for cysteine desulfhydrase activity (36). However, Binkley and Hudgins have also reported that cysteine-adapted Proteus morganii has increased cysteine desulfhydrase activity, but not homocysteine desulfhydrase activity and that these enzymes are different (37). The data presented above and the reports of Fromageot, Kallio and, possibly, Binkley suggest that in Neurospora, mammals and bacteria, cysteine and homocysteine desulfhydrases are separate enzymes.

D. Cystathionase I

Cystathionase I catalyzes the cleavage of L-cystathionine to yield cysteine. L-cystathionine was first proposed by Brand



L-cystathionine cysteine

et al. to be the intermediate involved in the transfer of the sulfur of methionine to cysteine by mammals (12). Subsequently Binkley et al. reported the presence of an enzyme in rat liver extracts which cleaved L-cystathionine to produce cysteine (15). In 1951 Binkley also reported separation of the cystathionine-synthesizing and cleaving enzymes, indicating that transulfuration in the rat is a process involving two enzymes (16).

Neurospora extracts catalyze the cleavage of L-cystathionine in two ways (i. e., to cysteine and to homocysteine) (figure 2, page 6). It will be shown that each reaction is

catalyzed by a separate enzyme, cystathionase I and cystathionase II, respectively.

To investigate the relationship between a gene and an enzyme, it is customary to obtain the enzyme from wild type strains in order to provide a standard for comparison. This was the procedure followed in the work to be reported. However, to characterize cystathionase I advantage was taken of the natural separation of cystathionase I from cystathionase II in a homocysteineless mutant (table 12), except where noted.

1. Methods used

The incubation methods used to study cysteine desulfhydrase activity were also used to determine cystathionase I activity. Each experimental incubation was matched by a control, containing enzyme, buffer and pyridoxal phosphate but no cystathionine. L-cystathionine was added to the control solutions at the end of the run. When incubations were continued 10 - 14 hours an additional control, containing boiled enzyme, pyridoxal phosphate, buffer and cystathionine, was used. This indicated that the enzyme activity was not the result of contamination.

The H_2S, NH_3 and keto acid quantitative methods used to study cysteine desulfhydrase activity were also used to detect cystathionase I activity, since these compounds were produced in experimental incubations. The production of H_2S was a complicating factor in the study of cystathionase I activity, since its origin was not determined.

In the experiments described below concerning the

production of H_2S from cystathionine the enzyme preparations contained both cystathionase I and cystathionase II. If H_2S was produced in control incubations, it was subtracted from the H_2S produced in the experimental incubations. This usually gave positive values, but occasionally gave negative values.

The positive values probably resulted from the cleavage of cystathionine to cysteine (or homocysteine) and the secondary desulfhydration of these products, since simultaneous controls to detect cysteine desulfhydrase and homocysteine desulfhydrase indicated that these enzymes were present. The negative values are interpreted to be the result of inhibition of the desulfhydrases by cystathionine or products of cystathionine degradation. Cysteine is probably the source of the H_2S in control incubations, since chromatography has indicated that this amino acid is produced endogenously (possibly by hydrolysis of peptides) during some incubations.

It was found that H_2S production could be minimized by incubation at about pH 8.0 when the enzyme was prepared by method A and incubations continued 10 - 14 hours and at about pH 7.2 or pH 8.0 when the enzyme was obtained by method B and the incubations continued 2 - 4.5 hours. Enzyme extracts prepared by method C have been incubated at pH 7.2 to pH 8.1, as the best pH to avoid H_2S production is not known.

Colorimetric methods used to detect cystathionase I activity included a method reported to be specific for cysteine and a total disulfide method which detects both cysteine and homocysteine. The colorimetric results were confirmed by

chromatography in a number of experiments.

Total disulfides were determined by the method of Kassel and Brand (38) as modified by Dubnoff (39). To oxidize sulfhydryl compounds to disulfides, the solutions to be tested were made basic (pH 7.6 - 8.0) until negative to nitroprusside. Eight N HCl was then added, so that the final pH after all reagents were added would be 5.4 - 6.0. Aliquots of the test and control solution were diluted to 4 ml. One ml of 0.5M Na_3PO_4 was added, followed by 0.5 ml Folins reagent and 0.5 ml 10% Na_2SO_3 adjusted to pH 5.0. The pH was immediately brought to 6.0 ± 0.02 with 1N NaOH and at 20 minutes and at one hour the solutions were read in a Klett-Summerson colorimeter with a red filter. If the one hour readings differed greatly from the 20 minute readings, the results were not used. Since cystathionine may give a slight blue color under these conditions, it was added at the end of the run to the enzyme control solution containing enzyme, buffer and pyridoxal phosphate, but not cystathionine. Mixtures of homocystine and cystine indicate that there is no interference between disulfides with this method. The quantitative range of the method with duplicate determinations was 0.01 - 0.32 μM disulfide. Recovery of sulfhydryl compounds added at either the beginning or end of the incubation were commonly 1% to 20% low. Since this difficulty was not encountered when the enzyme solution was omitted, it is assumed that the loss arises in the protein precipitation step. No difficulty was encountered in recovering disulfides.

Cystine was determined by the method of Sullivan and Hess (40). Color was read in a Klett-Summerson colorimeter with a green filter. Volumes of the solutions described by the authors were reduced by half when it was found that this did not affect the method. All reagents were prepared immediately before use. It has been reported by Dubnoff (39) that homocystine interferes with the color formation. This was confirmed. As the amount of homocystine was increased, the color developed in the presence of constant amounts of cystine decreased, although no quantitative relationship could be established. The greatest suppression observed was 25%, found when 0.5 mg of homocystine was added to 0.1 mg cystine. Deproteinized Neurospora extracts also frequently interfered with the color formation. In a number of experiments, cystine was added to aliquots of experimental and control solutions to detect the extent of the interference. It was also found that duplicate values were closer and nonspecific absorption less when the solutions were allowed to stand for 15 minutes after addition of dithionite and an additional 0.2 ml of aqueous 4% dithionite added immediately before reading. In critical experiments results obtained by the Sullivan-Hess method were qualitatively confirmed by chromatography. The values were used quantitatively when homocystine values were determined, by the subtraction of Sullivan-Hess values from the total disulfide values. When the method was modified as described, the values obtained are regarded as sufficiently reliable to permit this usage.

Chromatographic methods were developed to confirm qualitatively the results obtained by colorimetry. Sulfur amino acids were detected chromatographically before and after oxidation with H_2O_2 by the method of Dent (41). The R_f of the amino acids and derivatives in two developing solvents is seen in table 8. In addition to ninhydrin, disulfides have been detected with the iodoplatinic acid method of Toennies et al. (42). On paper, iodoplatinic acid is pink and bleaches in the presence of bivalent sulfur compounds. The method was modified to use aqueous sprays. It has been found that the chromatograms treated with iodoplatinic acid can be immediately bleached with pyridine, permitting the use of a second spray. In the original method, iodoplatinic acid-treated chromatograms were allowed to stand until all color faded (2 - 3 weeks) before treatment with a second reagent.

Disulfides were also detected with the nitroprusside-cyanide method of Toennies et al. (42).

The cystathionase I activity of the preparations was low. A number of unsuccessful attempts were made to increase the activity. These experiments, described below, relate to cystathionase II as well as to cystathionase I, since both enzymes were present in the preparations and since activity was measured with the total disulfide method alone. The extracts were obtained by method A and the incubations continued 11 to 14 hours, except where noted. In no case was a significant (i.e., 2.5 fold) difference of activity found, as determined on a mycelial wet weight basis.

Table 8

R_f's* of several amino acids in two developing solvents with and without oxidation by H_2O_2.

Amino Acid	Developing Solvent	
	2 n-butanol 1 acetic acid 1 H_2O	6 n-propanol 2 12N HCl 1 H_2O
cystathionine	0.21	0.22
cystathionine H_2O_2		0.13
cystine	0.19	0.19
cystine H_2O_2		0.40
cysteic acid		0.39
homocystine	0.35	0.43
homocystine H_2O_2		0.52
homocysteic acid		0.52
methionine	0.69	0.47
methionine H_2O_2		0.45

* The distance from the origin to the middle of the spot, divided by the distance from the origin to the front.

Preparations made in the presence of sequestrene at pH 7.1 were low in activity in a single experiment. Magnesium or trace element solutions had no detectable effect on dialyzed preparations. Heating for 5 minutes at 57°C destroyed the enzymes. Attempts to increase the activity of cystathionases by altering the following conditions of growth were unsuccessful: age of mycelium (38 to 240 hours, six determinations), sulfur source (sulfate, cystine + homoserine, L-cystathionine, methionine), growth on liquid complete and growth at 35°C. It was found that particle preparations prepared by the method of Haskins et al. (43) had a maximum of 2% of the total cleavage activity of the supernatant. In two hours, 257 mg dry weight of particles produced 0.1 μM H_2S, 0.30 μM NH_3, 0.22 μM methionine (as detected by the method of McCarthy and Sullivan (44)) and no disulfides from 12 μM L-cystathionine.

2. pH optimum

The activity of cystathionase I has been measured between pH 7.4 and pH 8.6 (table 9). Cystathionase I was prepared from a me-2 strain. The enzyme was most active at pH 8.2.

3. Pyridoxal phosphate as coenzyme

The participation of pyridoxal phosphate in the enzymatic transfer of the sulfur from homocysteine to serine by rat liver extracts has been reported by Braunstein and Goriachenkova (45). Enzyme extracts from the livers of pyridoxine deficient rats showed decreased activity, while the addition of pyridoxal phosphate in vitro, or restoration of pyridoxine to the diet, restored activity to near normal levels. It was reported

Table 9

Activity of cystathionase I as a function of pH.

The enzyme was prepared from strain D 8.4-6 (me-2) by method B. Five ml of enzyme was incubated 3 hours with 0.5 mg L-cystathionine, 0.5 ml 0.3 M Tris buffer and 60% pyridoxal phosphate. Activity was determined by the Dubnoff modification of the Brand-Kassel method and assumed to be cystine. Activity is expressed as μM cysteine/10 grams wet wt., mycelium/hr. and μM H_2S/10 gm wet wt. mycelium/hr.

	Activity	
pH	H_2S	Cysteine
7.4	+*	0.12
7.7	0	0.13
8.2	0	0.20
8.6	0	0.06

* Only enough H_2S was produced for qualitative detection (less than 0.08 M total).

later that the cystathionine-synthesizing as well as the cleaving enzymes require pyridoxal phosphate (33). The activation by pyridoxal phosphate of Neurospora cystathionase I was demonstrated using a me-2 strain as an enzyme source (table 10). In two other experiments pyridoxal phosphate has increased the activity of cystathionase I 20% and 230%.

4. End products of cystathionase I activity

The isolation of cystine from rat liver extracts incubated with L-cystathionine has been reported (15). The end product of the enzymatic cleavage was assumed to be cysteine, since the sulfhydryl compounds in the extract were oxidized before the isolation of cystine.

The low activities of the Neurospora cystathionase I preparations made direct isolation and identification of the end products of Neurospora cystathionase I activity appear impractical. However, cystine has been identified as a product of cystathionase I activity by chromatography. Cystine is suspected to arise secondarily during incubation. Simultaneous determinations of disulfides and sulfhydryl compounds, using the Dubnoff modification of the Kassel-Brand method, have indicated that 40 - 100% of the sulfur-containing end product is disulfide. To determine sulfhydryl compounds, the procedure outlined for total disulfides is followed except that the Na_2SO_3 is omitted. The enzyme preparations were obtained by method A from wild type and contained cystathionases I and II. Incubations were continued 10 - 14 hours.

Cystathionase I, free of cystathionase II activity, was

Table 10

Activation of cystathionase I by pyridoxal phosphate.

The enzyme was prepared from mutant No. H98 (me-2) by method A and 5 ml incubated 12.5 hours at pH 8.2 with 0.2 mg L-cystathionine/ml. Six σ /ml pyridoxal phosphate was added as indicated. Volume, 7.5 ml. Activity was determined by the Dubnoff modification of the Kassel-Brand method and is expressed as μM cysteine/10 gm wet wt. mycelium/hr. No H_2S could be detected.

	Activity cysteine
- pyridoxal phosphate	0.06
+ pyridoxal phosphate	0.10

prepared from a me-2 strain (table 11). The methods used in this experiment are different than those regularly used and will be described. The experimental incubation was matched by a control containing enzyme, buffer and pyridoxal phosphate but no cystathionine. All operations were performed on experimental and control solutions at the same time. The reactions were stopped by adding 50% trichloroacetic acid until the concentration in the solutions reached 1%. After standing 5 hours at 6°C the precipitate was collected in a centrifuge and discarded. Volumes were reduced over P_2O_5 and NaOH pellets in vacuo. An additional precipitate was collected in the centrifuge, washed with 7% trichloracetic acid and the precipitate discarded. The washings were then combined with the supernatants and water added until the volume was 5.0 ml. An aliquot of 1 ml was saved for chromatography. The remainder of the solutions, to be used for colorimetry, was brought to about pH 7.5 to oxidize any sulfhydryl compounds and cystathionine added to the control solutions. Cystine was added to one part of the experimental solutions to determine the sensitivity of the Sullivan-Hess method. Total disulfides were determined in the usual way. The results obtained indicate that this preparation did not contain an active cystathionase II, as expected (table 11).

Volumes were further reduced in a vacuum desiccator as before to give solutions containing about 0.2 mg end products per ml. These solutions were used to detect sulfhydryl compounds. It is necessary to add pure solutions of control amino

Table 11

Cystathionase I activity in an extract of a me-2 strain (H98).

Culture grown on "sulfate free" minimal plus 60 mg DL-methionine per liter and prepared according to method C. Forty ml of the enzyme was incubated with twelve mg L-cystathionine in 6 ml 0.06 M Tris buffer and 258γ pyridoxal phosphate at pH 8.0 for 2.5 hours. Final volume, 47 ml. Activity is expressed as μM cysteine, H_2S or homocysteine/10 gm wet wt. mycelium/hr.

Activity			
H_2S	Sulfhydryl	Cysteine	Homocysteine
0	0.247	0.245	0*

* A homocysteine activity of 0.016 units would have been detected.

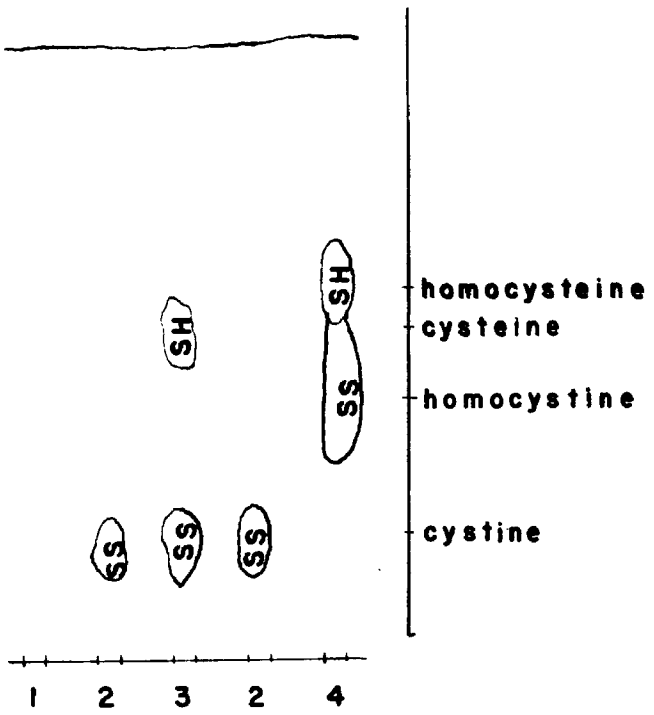
acids to the enzyme control solutions, since the R_fs are occasionally different in the presence of the substances contained in them. This was done by placing experimental and control solutions of a 3 mm diameter on Whatman #1 paper, drying and adding L-cystathionine to each control spot. After drying again homocyst(e)ine or cyst(e)ine was added as indicated in Figure 6. To detect sulfhydryl compounds the chromatograms were treated with alkaline nitroprusside (42). Cysteine was not found in experimental solutions. However cystine appeared to be present, since treatment of the same chromatogram with sodium cyanide (42) demonstrated the presence of nitroprusside-cyanide positive material in experimental solutions but not in the controls to which cystine was not added. Cyanide reduces disulfides to sulfhydryl compounds which then react with alkaline nitroprusside to give a red color. Similar chromatograms were treated with iodoplatinic acid. This reagent detects bivalent sulfur compounds (42). No spots were detected in experimental solutions which were not found in control solutions as was expected since cystine and cystathionine have the same R_f in the developing solvent.

Cystine has also been identified in the butanol acetic acid developer (figure 7). To do this the solutions were made basic with 8N NH_4OH to oxidize any sulfhydryl compounds, acidified and experimental and control solutions were applied to Whatman #1 paper as before. After treatment with the nitroprusside-cyanide reagent, experimental solutions were found to contain material with the same R_f as cystine added to

Figure 6

Chromatographic identification of cyst(e)ine as an end product of cystathionase I activity.

Developer: 6 n-propanol, 2 (12N HCl), 1 H_2O, ascending. Incubation methods are described in table II. Disulfides (SS) detected with nitroprusside-cyanide, sulfhydryl compounds (SH) with nitroprusside.



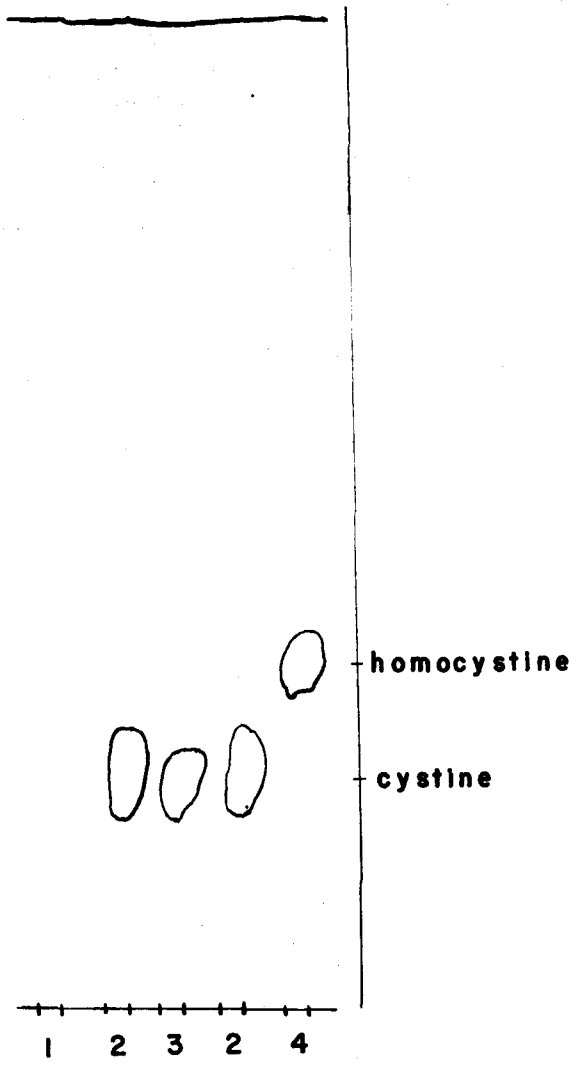
Spot Number

2 - Experimental solutions (cystathionine (csc) added at the beginning of the incubation).
1 - Control solution (matching experimental, but no csc), csc added on paper to dried spots of the control solutions.
3 - Same as 1, except cysteine and cystine also added on paper to dried spots of the control solutions.
4 - Same as 1, except homocysteine and homocystine also added on the paper to dried spots of the control solutions.

Figure 7

Chromatographic identification of cyst(e)ine as an end product of cystathionase I activity.

Developer: 2 n-butanol, 1 glacial acetic, 1 H_2O_2, ascending. Incubation methods are described in table 11. Disulfides were detected with nitroprussido-cyanide.



Spot Numbers

2 - Experimental solution (L-cystathionine (csc) added at the beginning of the incubation).
1 - Control solution (matching experimental, but no csc), csc added on paper to dried spots of the control solutions.
3 - Same as 1, except cystine also added on paper to dried spots of the control solutions.
4 - Same as 1, except homocystine also added on the paper to dried spots of the control solutions.

the control solutions.

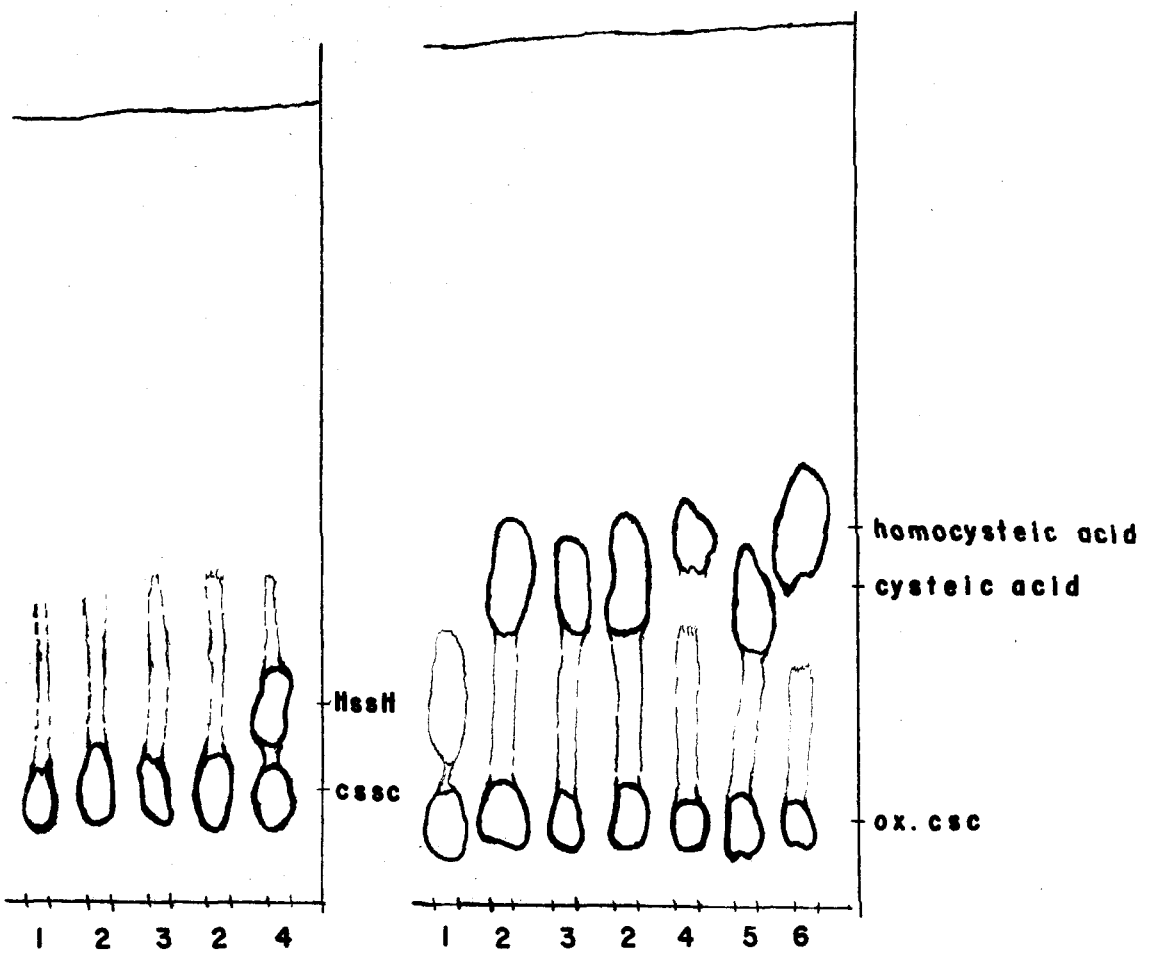
Attempts were made to oxidize the suspected cystine to cysteic acid in order to further establish its identity. Before this could be done it was necessary to remove contaminating sulfur-free amino acids by fractionating with ethanol. The solutions were neutralized with 2N NH_4OH, the volumes reduced and five to six volumes of ethanol added. The precipitate was collected in a centrifuge, suspended in water and the ethanol precipitation repeated four times. The precipitates were extracted with 0.2N HCl and the extracts used for chromatography. After oxidation with H_2O_2, new ninhydrin material with the same R_f as cysteic acid was seen in experimental solutions, but not in those controls to which no cystine or cysteic acid had been added (figure 8). The chromatogram of similar solutions which were not oxidized were used as an additional control in these experiments, since they show that the new spots were produced as a result of the oxidation. These chromatographic results indicate that cystathionase I catalyzes the production of cyst(e)ine from L-cystathionine.

The sulfur-free products of mammalian cystathionase I activity remain in doubt. Carrol et al. (46) reported production of stoichiometric amounts of ∝-ketobutyric acid (isolated as the dinitrophenylhydrazone) and cysteine as determined by the Sullivan-Hess method when rat liver extracts were incubated with L-cystathionine. This preparation also contained an active homoserine deaminase. Using preparations from which "thionase" had been reported to crystallize, Binkley reported,

Figure 8

Chromatographic identification of cysteic acid after oxidation of cystine produced by cystathionase I activity (fig. 6 and 7).

Developer: 6 n-propanol, 2(12N HCl), 1 H_2O, ascending. Experimental and control solutions were oxidized on the paper by the method of Dent (41). Color was developed with a 0.4% ninhydrin-pyridine solution.



Spot Numbers

2 - Experimental solution (L-cystathionine(csc) from time zero).
1 - Control solution (matching experimental, but no csc), csc added on paper to dried spots of control solutions.
3 - Same as 1, except cystine(cssc) also added on paper.
4 - Same as 1, except homocystine (hssh) also added on paper.
5 - Same as 1, except cysteic acid also added on paper.
6 - Same as 1, except homocysteic acid also added on paper.

without supporting data, that the dinitrophenylhydrazone of ∝-ketobutyric acid was isolated when this preparation was incubated with L-cystathionine. He also states that "certain discrepancies of an analytical nature remain" (16). This is very probably in part reference to an earlier report that as "thionase" was purified, the ability to produce NH_3 from L-cystathionine was lost, while the cysteine producing activity was retained (47). In addition this preparation may or may not contain "residual" homoserine deaminase activity (16, 47). It is reported by the same author that alanine, serine, ∝-amino-butyric acid and homoserine could not be detected as products of "thionase" activity (16). Attempts to demonstrate the sulfur-free products of Neurospora cystathionase I activity have been unsuccessful.

5. Cystathionase I activity in mutants

The cystathionase I activity of certain mutants was studied to determine whether the nutritional requirement of the mutants might be related to this enzyme activity. Mitchell and Lein have reported that a single gene tryptophaneless mutant (td_1) has no detectable tryptophane desmolase activity (6). Yanofsky reported that a tryptophaneless mutant (td_2) apparently allelic to td_1, also contained no tryptophane desmolase activity (48). Mixing experiments with extracts from the mutant and wild type indicated that this lack of activity was not due to an inhibitor. Fincham has reported that a single gene mutant which requires the ∝-amino group of various amino acids is deficient in glutamic dehydrogenase activity. This

enzyme is responsible for the synthesis of the α-amino groups via glutamic acid. Mixing experiments also indicated that this lack of activity was not due to an inhibitor (49).

Cystathionase I activity has been found in most of the strains investigated. This includes wild type, two nonallelic methionine-requiring mutants and the homocysteineless mutant, H98. This result was confirmed by chromatography, as indicated (table 12). In those instances where controls of the Sullivan-Hess method were inadequate, the data in column 6 are semi-quantitative and indicate that the enzyme was active.

The data presented in table 12 show that cystathionase I activity could not be found in extracts of two cystathionineless strains (4894 and 4894-R1). However, cystathionase II activity was present, since homocyst(e)ine was produced from L-cystathionine. Column 9 gives an estimate of the maximal amount of cystathionase I activity which could have been detected. Column 11 gives the ratio of the cystathionase II activity found to this hypothetical cystathionase I activity. Since cystine was found in the enzyme control solutions, this result was not confirmed by chromatography. The cystine found may have accumulated during the growth of the mutant or have been produced endogenously (possibly by hydrolysis of peptides) during the incubation. However, the controls of the colorimetric methods are regarded as adequate to permit the conclusion that the enzyme was not active.

Experiments were done to see whether the deficiency of cystathionase I activity in me-c3 strains is due to the

Table 12

Cystathionase I and cystathionase II activities in wild type and mutant strains.

Columns 9 and 10 give an estimate of the amount of activity which could have been present and not detected. 4 - 7% pyridoxal phosphate/ml, 0.12-0.18 mg L-cystathionine/ml. Each line represents a separate preparation and experiment. Activity is expressed as μM indicated product/10 gm wet wt. mycelium/hr.

Strain No.	Genotype	Prep. Meth.	Incubation time (hrs.)	Activity				Minimum detectable activity		Activity
				Sulfhydryl	homocysteine	homocysteine	H_2S	homocysteine	homocysteine	homocysteine/cysteine
5256	wild type	B	3	0.23	0.06	0.17	0			2.8
D8.4-6	me-2	B	2	0.07	0.08*	0	0		0.03	0.4
4894	me-c3	B	4	0.15	0	0.15*	-.005	0.02		7
D8.4-6	me-2	C	3	0.27	0.24*	0	0		0.07	0.3
H98	me-2	C	2	0.247	0.245*+	0	0		0.016	0.07
4894	me-c3	C	2	0.402	0	0.402*+	0	0.01		40
4894-R1	me-c3	C	3	0.11	0	0.11	0.02			
5297	wild type	A	12	0.33	0.11+	0.22+	0			2.0
35599-R1	me-a1	A	14	0.62	0.15+	0.47+	0			3.1
29627-R1	me-b1	A	14	0.28	0.07	0.21	0			3.0
H98	me-2	A	12	0.14	0.14*+	0	0		0.05	0.4

* Cystine added to the experimental vessel.
\+ Confirmed by chromatography.

absence of or inhibition of the enzyme. The possibilities considered were: 1) lack of a cofactor, 2) presence of a dialyzable inhibitor, 3) presence of a nondialyzable inhibitor.

Cofactor deficiencies are known to reduce the activity of enzyme requiring the cofactor, whether the deficiency is induced by genetic or dietetic means. However, in these cases addition of the required cofactor in vitro commonly restores activity to near normal levels. In the experiments concerning me-c3 a known cofactor, pyridoxal phosphate, was added and cystathionase I activity could not be detected.

If an inhibitor of cystathionase I is present in me-c3 strains, it is not dialyzable, since the preparations used were regularly dialyzed. The results presented in table 13 indicate that the lack of cystathionase I activity is not due to the presence of nondialyzable inhibitors. An active cystathionase I was obtained free of cystathionase II activity and incubated as indicated with extracts obtained from the me-c3 mutant. The results show that no nondialyzable inhibitors of cystathionase I were recovered from the me-c3 mutant.

These results suggest that synthesis of cystathionase I is interfered with in me-c3 strains.

The results also indicate that the reversal of cystathionase I activity is responsible for the in vivo synthesis of cystathionine in Neurospora. However, this may not be true, since cystathionine synthesis was not observed. Preliminary experiments to detect enzymatic synthesis of cystathionine gave results which varied but suggest that such experiments are practical.

Table 13

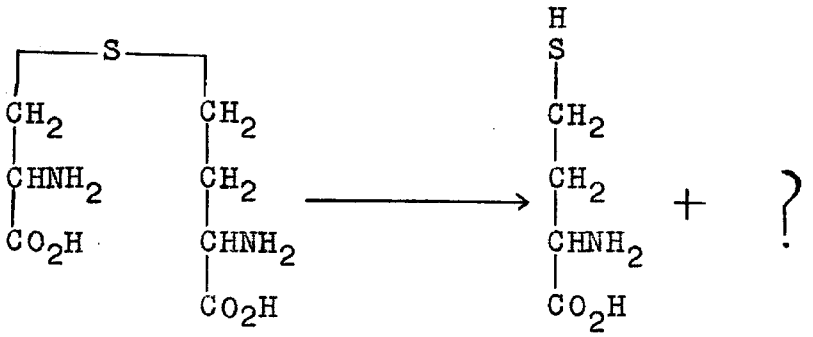
Mixing experiment of cystathionase I from a me-2 strain and cystathionase II from a me-c3 strain.

Enzymes prepared according to method C. Cultures were grown on "sulfur-free" medium with 60 mg DL-methionine per liter. Total disulfide values only were determined. Eight ml of enzyme solution, 1.5 mg L-cystathionine, 7 γ pyridoxal phosphate/ml and 1.5 ml 0.06 M Tris buffer were incubated at pH 8.05 for 2 hours. Total volume was 10 ml. Activity is expressed as μM cysteine and/or homocysteine/10 gm fresh wt. mycelium/hour.

Ml. enzyme		Activity	
me-2	me-c3	Found	Expected
8		0.25	
	8	0.40	
5	3	0.31	0.30
4	4	0.29	0.33
3	5	0.33	0.34

E. Cystathionase II

Cystathionase II catalyzes the cleavage of cystathionine to yield homocysteine.



L-cystathionine homocysteine

This enzyme is of particular interest in Neurospora since this step is involved in the transfer of the sulfur of cysteine to homocysteine (figure 1, page 3). The enzyme has been found in wild type Neurospora (table 12). In the experiments reported in this section, cystathionase II was prepared from mutants lacking cystathionase I activity (me-c3) strains), except where noted. Cystathionase II has not been previously reported as a separate enzyme, although Binkley and Hudgins state that Proteus morganii and the rat are able to enzymatically cleave L-cystathionine to produce L-homocysteine (37).

1. Methods used

The methods used to determine cystathionase I activity (pp. 26 - 32) were also used to study cystathionase II.

2. pH optimum

The activity of cystathionase II was determined between pH 6.4 and pH 9.1 and the greatest activity found at pH 7.8 as seen in table 14.

Table 14

Activity of cystathionase II as a function of pH.

The enzyme was prepared from mutant No. 4894-R1 by method B. Five ml of enzyme was incubated 3 hours with 0.5 mg L-cystathionine, 0.5 ml 0.3M Tris buffer and 60 γ pyridoxal phosphate. Final volume, 7 ml. Total disulfides were determined by the Dubnoff modification of the Kassel-Brand method and assumed to be homocystine. Activity is expressed as μM homocysteine/10 gm wet wt. mycelium/hr. and μM H_2S/10 gm wet wt. mycelium/hr.

	Activity		
pH	H_2S	Homocysteine	Total
6.4	0	0	0
7.2	0	0.10	0.10
7.8	0.1	0.25	0.35
9.1	0	0.01	0.01

3. Pyridoxal phosphate as coenzyme

Binkley and Hudgins have reported that pyridoxal phosphate activated the production of L-homocysteine from L-cystathionine by "thionase" (37). As is seen in table 15, Neurospora cystathionase II preparations were also activated by pyridoxal phosphate.

4. End products

Binkley and Hudgins have stated in abstract that partially purified extracts of Proteus morganii, when incubated with L-cystathionine, produce L-homocysteine as a major product and NH_3 and pyruvic acid as minor products (37).

Since Neurospora cystathionase II preparations were low in activity, direct isolation and characterization of homocyst(e)ine as a product did not appear to be practical. However, homocysteine and homocystine were identified by chromatography. Data obtained using colorimetric methods indicate that the cystathionase II preparation did not contain appreciable cystathionase I activity (table 16). Aliquots of these solutions for chromatography were prepared in the same way as those described on pages 36 to 42. Homocysteine has been identified as an end product of cystathionase II activity by its R_f in one developing solvent with both the nitroprusside and iodoplatinic acid reagents (figure 9). However, cystine was present in the control solutions. Chromatography has indicated that cyst(e)ine is produced endogenously (possibly by hydrolysis of peptides) during some incubations. It is also possible that the cystine found was accumulated

Table 15

Activation of cystathionase II by pyridoxal phosphate.

The enzyme was prepared from strain 4894 (me-c3) by method B and incubated 2 hours at pH 7.2 with 0.2 mg L-cystathionine/ml. Six pyridoxal phosphate/ml was added as indicated. Activity is expressed as μM homocysteine/10 gm wet wt. mycelium/hr. and μM H_2S/10 gm. wet wt. mycelium/hr.

	Activity	
	H_2S	Homocysteine
-pyridoxal phosphate	0	0.12
+pyridoxal phosphate	±**	0.33*

* Control solutions containing cystine added at the end of the run indicated that a cysteine activity of 0.06 would have been detected.

** Qualitative test (less than .08 μM total).

Table 16

Cystathionase II activity in a preparation obtained from a me-c3 strain.

Grown on "sulfur-free" minimal plus 60 mg DL-methionine per liter and prepared according to method C. Forty ml of the enzyme was incubated with 12 mg L-cystathionine in 6 ml 0.06 M Tris buffer and 25% pyridoxal phosphate at pH 8.0 for 2.0 hours. Final volume 47 ml. Activity is expressed as μM homocysteine, cysteine or H_2S/10 gm wet wt. mycelium/hr.

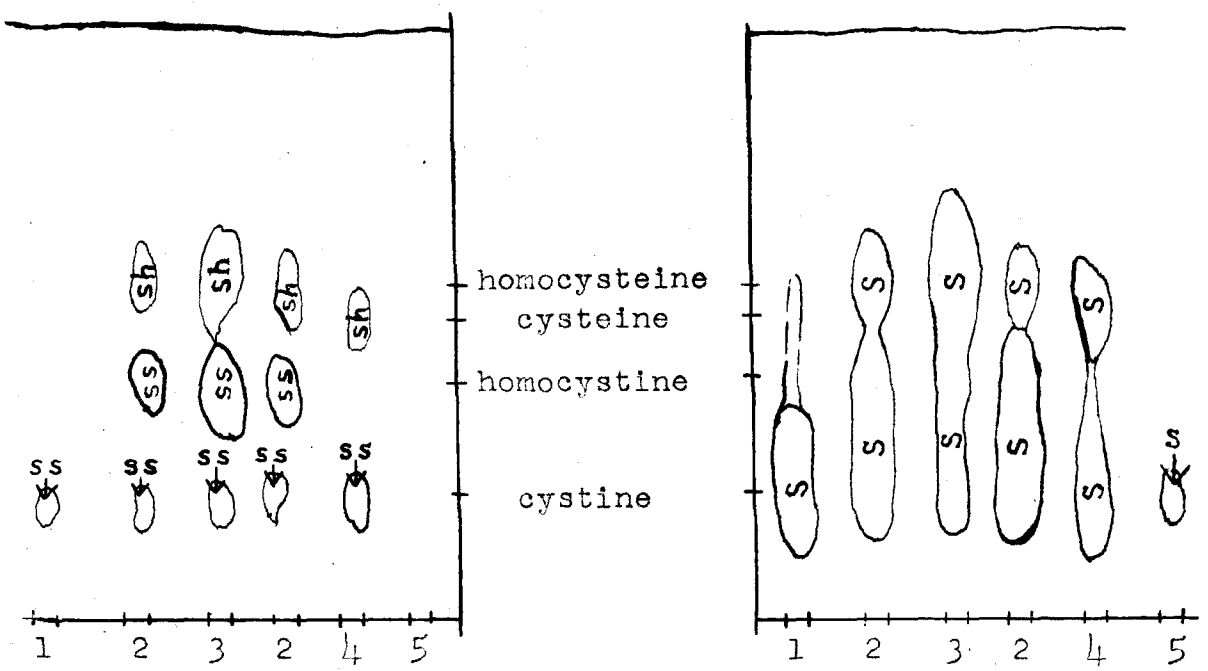
	Activity		
H_2S	Sulfhydryl	Homocysteine	Cysteine
0	0.402	0.402	0*

* A cysteine activity of 0.01 units would have been detected.

Figure 9

Chromatographic identification of homocyst(e)ine as an end product of cystathionase II activity.

Developer: 6 n-propanol, 2 (12% HCl), 1 H_2O, ascending. Incubation methods are described in table 16. Sulfhydryl (sh) compounds detected with nitroprusside, disulfides (ss) with nitroprusside-cyanide, bivalent sulfur (S) with iodoplatinic acid.



Spot Numbers

2 - Experimental solution (cystathionine (csc) added at the beginning of the incubation).
1 - Control solution (matching experimental, but no csc), csc added on paper to dried spots of the control solution.
3 - Same as 1, except homocysteine and homocystine also added on paper to dried spots of the control solution.
4 - Same as 1, except cysteine and cystine also added on paper to dried spots of control solution.
5 - Pure cystathionine.

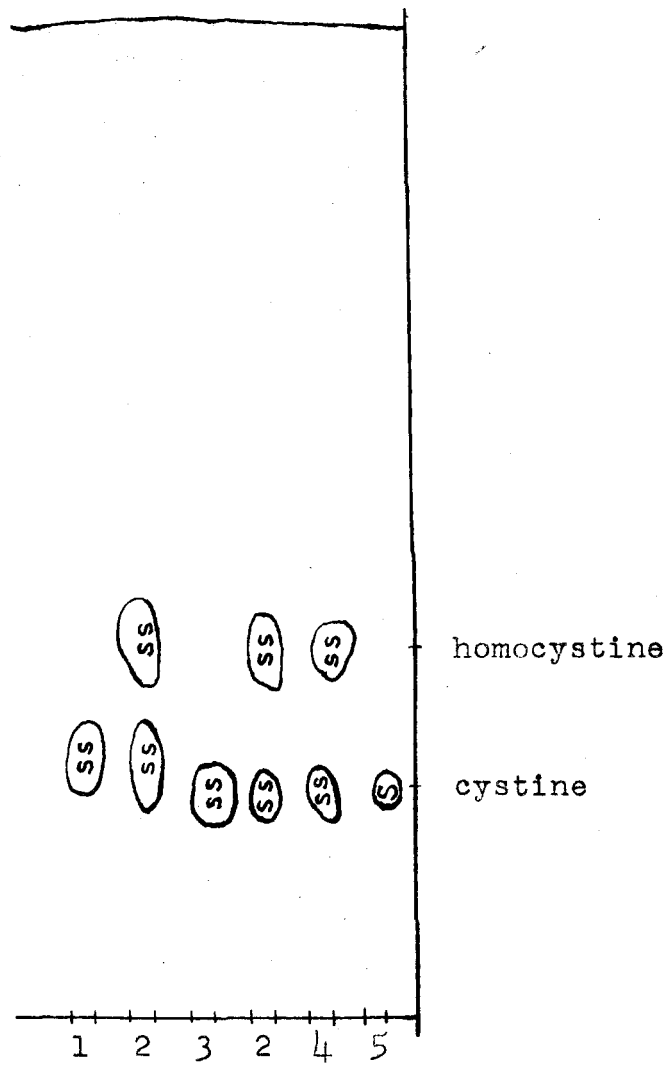
during the growth of mutant No. 4894. Homocystine has been identified by its R_f in two developing solvents, as shown with the nitroprusside-cyanide (figures 9 and 10) and iodoplatinic acid reagents (figures 9 and 10). The homocystine was also oxidized to homocysteic acid which was identified by its R_f in a single developing solvent (figure 11). From these data it is concluded that homocyst(e)ine production from L-cystathionine is catalyzed by cystathionase II.

Attempts to determine the sulfur-free end products of cystathionase II activity were unsuccessful. Alanine could not be found by chromatography. It was found, however, that enzyme extracts of wild type prepared by method B and containing cystathionases I and II produce pyruvic acid in the presence of L-cystathionine. The dinitrophenylhydrazones were separated chromatographically and the material with the R_f of the pyruvic acid derivative was found to represent 70% of the optical density found before chromatography (29). In addition, as the preparations were purified, keto acid production increased, when compared with the production of sulfhydryl compounds. The same observation was made when the NH_3 producing activity was followed. However, the keto acids producing activity only reached about 50% of the homocysteine plus cysteine activities. Since pyruvic acid is a 3-carbon compound, it is assumed to arise as a result of cystathionase II activity. The finding that cystathionase II activity was low might suggest that pyruvic acid and NH_3 are products of a second enzymatic reaction, since the large amount of protein present might

Figure 10

Chromatographic identification of homocyst(e)ine as an end product of cystathionase II activity.

Developer: 2 n-butanol, 1 glacial acetic 1 H_2O, ascending. Incubation methods are described in table 16. Disulfides (ss) detected with nitroprusside cyanide, bivalent sulfur (S) with iodoplatinic acid.

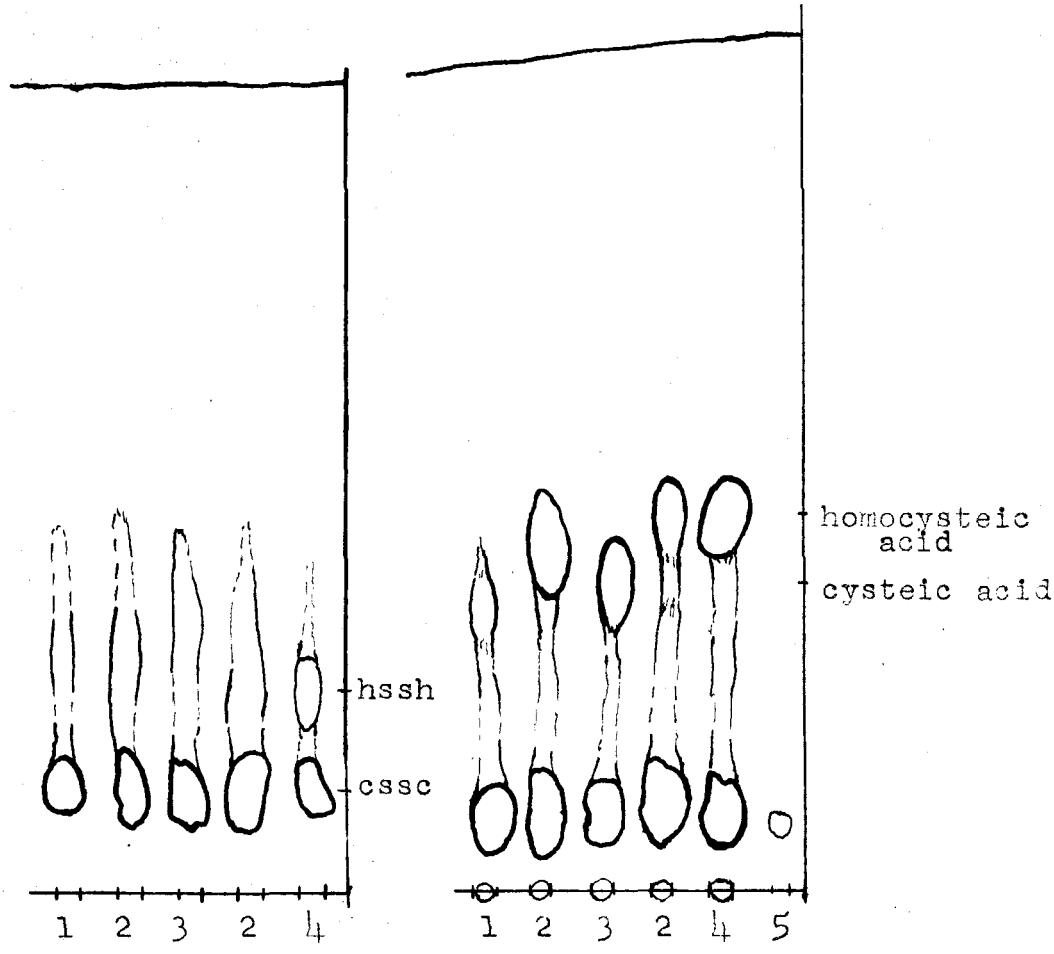


2 - Experimental solution (cystathionine (csc) added at the beginning of the incubation).
1 - Control solution (matching experimental, but no csc), csc added on paper to dried spots of the control solution.
3 - Same as 1, except cystine also added on the paper.
4 - Same as 1, except homocystine also added on the paper.
5 - Pure cystathionine.

Figure 11

Chromatographic identification of homocysteic acid after oxidation of homocyst(e)ine produced by cystathionase II activity (figures 9 and 10).

Developer: 6 n-propanol, 2 (12N HCl), 1 H_2O, ascending. Experimental and control solutions were oxidized on paper with the method of Dent (41). Color was developed with a 0.4% ninhydrin-pyridine solution.



Spot Numbers

2 - Experimental solution (cystathionine (csc) from time zero).
1 - Control solution (matching experimental, but no csc), csc added on the paper to dried spots of control solution.
3 - Same as 1, except cystine (cssc) also added on the paper.
4 - Same as 1, except homocystine (hssh) also added on the paper.
5 - Pure cystathionine.

include such an enzyme. However, the observed pyruvic acid production is very probably closely related to cystathionase II activity and provides a useful clue for future work.

5. Cystathionase II production in mutants

Data presented in table 12 show that cystathionase II activity was recovered in wild type Neurospora, as well as in methionineless and cystathionineless mutants. These results show that the enzyme was recovered by the methods used.

Cystathionase II activity could not be detected in two me-2 strains, H98 and D 8.4-6, although an active cystathionase I was present. These results have been confirmed by chromatography (table 12). Column 10 gives an estimate of the maximal cystathionase II activity which could have been detected. Column 11 gives the ratio of this hypothetical cystathionase II activity to the cystathionase I activity actually found. These results concerning the cystathionases in me-2 strains do not appear to be dependent upon the age of the cultures, since mycelial extracts prepared from 3.5 to 6.5 day-old cultures gave the same results.

Experiments were done to see whether the deficiency of cystathionase II activity in me-2 mutant strains is due to the absence or inhibition of the enzyme. Similar experiments have been described in relation to cystathionase I activity.

The addition of a known cofactor, pyridoxal phosphate, did not restore activity, indicating that a cofactor deficiency is not involved.

Dialysis did not restore activity. The preparations were regularly dialyzed before use. In addition to the

experiments presented in table 12, preparations from a me-2 strain have been dialyzed for 8 - 18 hours and no activity detected.

Data presented in table 13 show that no nondialyzable inhibitors of cystathionase II activity were recovered from the me-2 strain, since cystathionase II was as active in the presence of as in the absence of extracts from the me-2 mutant. Similar results have been obtained in each of three tests using mixtures of a me-2 strain and wild type prepared by method A and incubated 14 hours.

These results suggest that me-2 strains are deficient in the ability to synthesize cystathionase II.

F. Cystathionase I and Cystathionase II as Separate Enzymes

Since extracts of wild type Neurospora catalyzed the cleavage of L-cystathionine to produce cyst(e)ine and homocyst(e)ine, it is important to know whether one or two enzymes are involved. The fact that these activities varied independently in different strains (table 12) indicates that two enzymes are involved. However, the views of Binkley on "thionase" specificity (p. 15, p. 23) would lead to the conclusion that the different cystathionase activities found in mutant and wild type strains represented different specificities of a single enzyme. Binkley also states that the "thionase" from Proteus morganii differs from the "thionase" of mammals in that the former enzyme has a preference for attachment to a 3-carbon α-amino acid with a bivalent sulfur on the β-carbon and the latter enzyme has a preference for a 4-carbon α-amino

acid with a bivalent sulfur on the γ-carbon. This is assumed to be true since extracts from Proteus morganii produce more homocysteine than cysteine from L-cystathionine and extracts from rat livers produce more cysteine than homocysteine from the L-cystathionine (37).

Binkley's views also lead to the conclusion that cysteine desulfhydrase activity and, perhaps, homocysteine desulfhydrase activity in addition to cystathionine cleavage activities are due to a single enzyme -- "thionase." The reported failure to fractionate cysteine desulfhydrase and cystathionase I after 40-fold purification of rat liver extracts is support for the argument (47). A fractionation experiment using Neurospora extracts is presented in table 17. The data show that the desulfhydrase activities can be separated from the cystathionase activities by fractionation of crude extracts with $(NH_4)_2SO_4$. This indicates that any cystathionase activity of the desulfhydrases is slight. This separation has been repeated twice. (In addition, roughly 60% of the cystathionase I activity was lost during the fractionation and about 90% of the cystathionase II activity was recovered, indicating again that two cystathionase activities involve separate enzymes.) A re-isolate (D 8.4-1) has been found which has no detectable homocysteine desulfhydrase activity, but contains cystathionase I and cystathionase II activity (tables 7 and 20), indicating that cystathionases I and II have, at best, a low desulfhydrase activity toward homocysteine.

These results indicate that cystathionase I and cystathionase II are separate enzymes.

Table 17

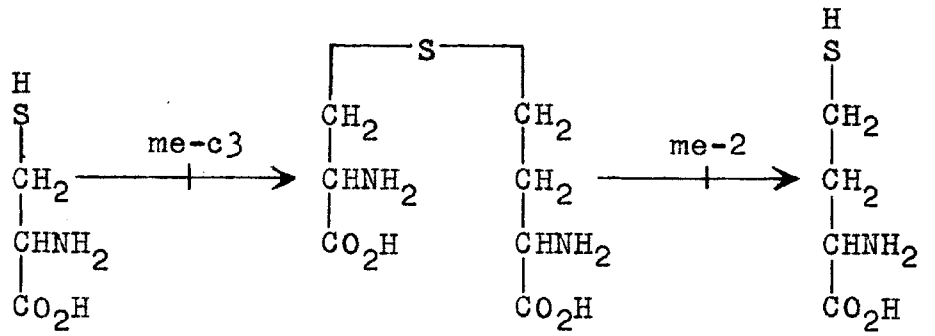
Cystathionase and desulfhydrase activities in crude and fractionated extracts of 4.5 day 5256A preparations.

Grown with aeration on minimal plus 40 mg DL-methionine and 1 mg pyridoxine hydrochloride per liter. 1.5 mg substrates, 0.04 M Tris buffer, 6γ /ml pyridoxal phosphate, pH 8.1, 2.5 hrs. Activities are expressed as μM end product/10 gm wet wt. mycelium/hr.

Fraction	Substrate	Activity		
		H_2S	cysteine	homocysteine
crude extract	L-cystathionine	±	0.20	0.19
0 - 50% $(NH_4)_2SO_4$	L-cystathionine	0.02	0.05	0.17
	L-cysteine	0.097		
	DL-homocysteine	0.170		
50 - 60% $(NH_4)_2SO_4$	L-cystathionine	0	0	0
	L-cysteine	0.04		
	DL-homocysteine	0.04		

G. A Suppressor

While studying the behavior of a me-c3 strain, Giles obtained an albino-2 (symbol, al_2) linked suppressor (symbol, su-1) of me-c3 which also suppressed me-2 (23). Dr. Giles has generously supplied this suppressor in order that its effect



cysteine cystathionine homocysteine

on the cystathionase-synthesizing activities might be studied.

The suppression of me-2 and me-c3 was confirmed (p. 9). The methods to carry out crosses have been described (50). Giles reported that strains of genotypes su-1 and su-1 me-c3 grew less on minimal than wild type and that methionine in the medium restored the growth to that of wild type (23). These results have been confirmed and the partial requirement for methionine has been shown in an additional genotype, su-1 me-2 (table 18).

Strain 9666, a cystathionineless mutant nonallelic to me-2 or me-c3, has been shown to be in linkage group D, at least 26 units from the centromere (9). As shown in table 19, su-1 does not suppress mutant No. 9666, since no asci containing 3 spore pairs which grew on minimal were found. Tests of random ascospores also demonstrated the failure of su-1 to suppress mutant No. 9666, since 47% required cystathionine

Table 18

The partial requirement of su-1 me-2 strains for methionine.

Mg. dry weight, "sulfur free" minimal plus the indicated supplements (6.6 μM).

Strain number	Genotype	Sulfur source	Mg. dry weight 41 hour	72 hour	96 hour	135 hour
D 15.1-7	me-2 su-1	Na_2SO_4	2.0	16.0	40.5	72.5
		DL-methionine	19.0	61.5	78.5	85.0
D 8.1-7	me-2 su-1	Na_2SO_4	0.5	27.0	65.0	77.5
		DL-methionine	17.0	57.5	68.0	65.5
D a-3	me-2 su-1	Na_2SO_4	2.0	33.5	68.0	111.5
		DL-methionine	28.0	63.5	78.0	88.5
H98-B203-CL3-12.7	me-2 su-1	Na_2SO_4	0.5	28.0	45.5	48.5
		DL-methionine	24.5	57.0	70.0	70.5
4894-CL6-9.1	su-1	Na_2SO_4	3.5	22.5	65.0	111.5
		DL-methionine	16.5	48.0	66.5	91.0
D 5.1-7	(su-1)*	Na_2SO_4	6.0	44.0	90.5	113.0
		DL-methionine	29.0	76.5	81.0	91.0
H98 A	me-2	DL-methionine	23.0	54.5	63.5	70.0
5297a	wild type	Na_2SO_4	24.5	86.5	99.0	79.5
		DL-methionine	30.0	61.0	76.5	85.0

* Not confirmed, however it arose in a 2:2 ascus from cross D (al_2su-1 x me-2).

Table 19

Failure of su-1 to suppress a cystathionineless mutant. Orientation of the first and second division spindles is ignored.

4894-CL6-9.1a x 9666A

al_2 = albino$_2$
m = cystathionineless

Types of Asci Spore pair				Total Asci
1	2	3	4	
+ m	+ m	al_2 +	al_2 +	3
+ +	+ +	al_2 m	al_2 m	2
+ m	+ +	al_2 m	al_2 +	3*
+ m	al_2 m	+ +	al_2 +	3
+ m	al_2 +	+ m	al_2 +	3
+ m	al_2 +	al_2 m	+ +	3*
+ +	al_2 m	+ +	al_2 m	5
			Total Asci	22

Random Ascospores + m	+ +	al_2 m	al_2 +	Total Ascospores Germ.
15	21	14	12	62

Conclusion: Mutant No. 9666 is not suppressed by su-1.

* The order of ascospores was such in one ascus that it appeared that two spores had been transposed during the dissection.

for growth when 25% would be expected if su-1 suppressed mutant No. 9666. In addition, 14 or about half of the 26 albino strains required cystathionine for growth.

1. Enzyme studies

The cystathionase I and cystathionase II activities of strains of genotype su-1, su-1 me-2 and su-1 me-c3 were investigated to determine whether the suppressor gene affected the cystathionase-synthesizing activities in these strains. The results are presented in table 20. It is seen that each of these strains contain both cystathionase I and cystathionase II, although the activities seem to be lower than in wild type.

It is of interest that no cystathionase II activity could be detected in extracts of su-1 me-2 strains prepared by method A. However, when the same strains were extracted by method B, cystathionase II activity was found. It has been observed that method B resulted in a decrease of total cystathionine cleavage activities when wild type strains were studied. When strains carrying the suppressor were examined for cystathionase activities, the results obtained show that the suppressor gene restores the original synthetic pathway to me-2 strains, since cystathionase II activity was found in su-1 me-2 strains, but not in me-2 strains. The return of cystathionase I activity to a me-c3 strain carrying the suppressor is supporting evidence for the conclusion that the reversal of this activity is responsible for the *in vivo* synthesis of cystathionine.

Table 20

Cystathionase I and cystathionase II activities of strains carrying the suppressor.

5 - 7% pyridoxal phosphate/ml, 0.15 - 0.18 mg L-cystathionine/ml. Each line represents a separate preparation and experiment. Activity is expressed as μM indicated product/10 gm wet wt./hr.

Strain Number	Genotype	Prep. Meth.	Time of Incubation (hrs.)	Activity			
				cysteine homocysteine	cysteine	homocysteine	H_2S
D8.4-1	su-1	B	2	0.05	0.03	0.02	0.005
D8.1-7	su-1 me-2	A	12	0.04	0.04	0	-0.02
D8.1-7	"	A	12	0.03	0.026	0	0
D8.1-7	"	B	9	0.072	0.033	0.039	0
D8.1-7	"	B	6	0.103	0.103	0.085*+	0
D8.1-7	"	B	4	0.045	0	0.040	0
4894-CL1 5a	su-1 me-c3	B	5	0.034	0.012 *+	0.022	0

+ Confirmed by chromatography.

* Cystine added to the experimental vessel.

H. Discussion

For the purpose of discussion, each gene is assumed to have a primary product which controls a chemical process of the cell. This is also expressed as the one gene - one function, or one gene - one enzyme hypothesis (5). The hypothesis has been supported by the observation that single gene nutritional mutants commonly have a single nutritional requirement (5) and by experiments indicating the isolation methods used to obtain these mutants do not unduly favor the recovery of such unifunctional mutants (51). In addition, it has been found in a number of cases, when the enzyme which synthesizes the required metabolite is obtained from wild type, that this enzyme activity is missing in the mutant. Two such cases, the tryptophane desmolase and glutamic dehydrogenase mutants of Neurospora, have been discussed (6, 49, 50). A number of experiments which are perhaps similar have recently been reported from E. coli; however, the data are incomplete (52 - 56). Wagner has reported that a single gene pantothenate-requiring mutant of Neurospora has, under certain in vivo and in vitro conditions, as active a pantothenate-synthesizing system as wild type Neurospora. These data are interpreted to indicate that the mutant has an enzyme sensitive to a compound normally present in Neurospora, or that the mutation has resulted in the production of an inhibitor which interferes with a normal enzyme (57). In these cases, a quantitative alteration of enzyme activity is suggested.

Evidence that an altered gene produces an altered protein has recently been presented, since the production of a

thermo-labile tyrosinase in Neurospora has been shown to be controlled by a single gene (58). In a similar report, Pauling has presented evidence that individuals with the sickle cell disease produce an altered hemoglobin molecule (59).

The data obtained concerning cystathionase I activity in me-c3 strains and cystathionase II activity in me-2 strains suggest quantitative alteration of enzyme activity as a result of gene mutation. Mixing experiments, dialysis and cofactor addition have indicated that this lack of activity is due to the enzyme molecule and not to inhibitors. The in vivo significance of cystathionase II is clear, since its activity is responsible for the conversion of cystathionine to homocysteine and is directly correlated with the nutritional requirement of me-2 strains. The data obtained with cystathionase I suggests that the reversal of this enzyme activity is responsible for the in vivo synthesis of L-cystathionine. However, this may not be true, since cystathionine synthesis was not observed. A limited number of attempts to find such a system have given results which vary but suggest that such experiments are practical, particularly if the me-2 mutant strain is sued as an enzyme source.

It appears that the suppressor gene not only causes me-2 and me-3 strains to grow on minimal, but also produces a partial requirement for methionine, since suppressor-carrying strains are stimulated by methionine, irrespective of the presence of wild or mutant alleles of me-2 and me-c3.

Suppressor-carrying strains are frequently reported to grow less on minimal than does wild type. In some instances it has been shown that the nutritional requirement of the mutant which is suppressed increases the growth of these strains. Giles has reported that a suppressor of an inositol-less mutant reduces the growth of wild type on minimal and that the addition of inositol partially overcomes this effect. This same effect is reported to be present in suppressed inositol-less strains although more pronounced (60). Lein and Lein have reported that three suppressed acetateless strains grow less on minimal than does wild type and that this effect is partially overcome by acetate (61). The suppressor of a tryptophaneless mutant obtained by Yanofsky was reported to reduce the growth of wild type and suppressed tryptophaneless strains. Tryptophane increased the growth rate of suppressed tryptophaneless strains, but not wild type strains carrying the suppressor (48). In this case it was reported that the tryptophane-synthesizing enzyme, tryptophane desmolase, is absent in the tryptophaneless strain, but present in the suppressed tryptophaneless strain to the extent of 5% of the amount found in wild type. The wild type strain carrying the suppressor was reported to have 33% of the enzyme of wild type. Strains carrying either of the two non-allelic suppressors of the sulfa-requiring mutant were reported by Emerson to have a non-wild type growth response on minimal, irrespective of the presence of the wild or mutant alleles for sulfa requirement (62). The suppressor of pyrimidineless or ornithineless mutants found by Mitchell

et al. appears to be an exception to the rule. No difference between wild type strains, suppressed ornithineless strains, or wild type strains carrying the suppressor was observed (63).

One explanation of the slow growth on minimal of strains carrying a suppressor could be that the suppressor opens an alternative pathway of synthesis of the required metabolite (61). In suppressed mutants it might be expected that the new pathway limits growth, since such strains are usually stimulated by the nutritional requirement of the mutant which is suppressed. In the case of su-1, as well as in other cases where the compound stimulates the growth of wild type strains carrying the suppressor, the alternative route might be expected to operate to the exclusion of the original route. For example, some precursor might be diverted to the new route and no longer be available to the original route.

If gene su-1 opens an alternate route of methionine synthesis, it would be expected that cystathionase II activity would not be found in strains of genotype su-1 me-2. The fact that such activity was found in this strain indicates that the original synthetic pathway is reopened in me-2 strains. The return of cystathionase I activity to a suppressed me-c3 strain is regarded as supporting evidence for the conclusion that the *in vivo* reversal of this enzyme activity is responsible for cystathionine synthesis. It is important that appropriate experiments with a cystathionine-synthesizing system be done before these experiments are related to a discussion of the ability of the suppressor gene to suppress

mutants blocked both before and after cystathionine. It should be noted however, that there are data reported which indicate that a suppressor gene may function by causing inactive alleles to become active (64). Yanofsky found that a suppressed td_2 mutant contained tryptophane desmolase activity and that the same suppressor did not cause the return of tryptophane desmolase to, or suppress, the apparently allelic td_1 mutant (48).

IV. CYSTATHIONINE CONTENT OF MUTANTS

In the course of the investigations which have been described, a semi-quantitative method of cystathionine determination was devised. This method was applied to the question of whether the pathway of methionine synthesis from cystathionine to methionine is reversible, i. e., whether Neurospora can synthesize cystathionine from methionine without going through cystine as an intermediate.

A. Preparation of Extracts

The cystathionine content of deproteinized Neurospora extracts was determined by visual comparison of the area and intensity of ninhydrin spots produced by known and unknown cystathionine solutions after the chromatographic separation of oxidized cystathionine from other naturally occurring amino acids.

Mycelia, which were grown in 125 ml Erhlenmeyer flasks in quadruplicate, were extracted as in procedure A, except that half the volume of water was used and the material was placed in a boiling water bath for ten minutes before centrifuging. An aliquot of the solution was brought to 5% trichloroacetic acid concentration with 50% trichloroacetic acid and the precipitate was collected by centrifugation and discarded. Two mycelia from each set were separately dried and weighed.

B. Chromatographic Methods

Cystathionine concentration was determined in two steps. The extracts and standard cystathionine solutions of range 0.25 to 1.0 mg/ml were first applied to Whatman #1 paper in

spots of constant diameter (2mm or 3 mm). Half of the spots were oxidized with H_2O_2 by the method of Dent (41) and an ascending chromatogram was run in the propanol developer (table 9). The chromatograms were dried at room temperature, color was developed by dipping the chromatogram in a 0.4% ninhydrin pyridine solution, and a first approximation of the cystathionine concentration of the unknown solution obtained. Secondly, the solution was concentrated over P_2O_5 or diluted with H_2O to yield a solution of about 0.3 mg/ml, the range which gave the best sensitivity. The chromatographic procedures were repeated except that the range of the standard solutions was narrowed to 0.20 to 0.50 mg/ml. Six determinations were made on each unknown solution.

C. Results

To determine whether Neurospora can synthesize cystathionine from methionine without going through cystine as an intermediate, the cystathionine content of wild type, me-2 and me-c3 strains was investigated as influenced by the omission of sulfate from medium containing methionine. The me-2 strain did not accumulate cystathionine in the absence of sulfate (table 21). The low level of cystathionine which was found very probably represents contamination of the medium with sulfur compounds. When the sulfate was present, the me-2 strain accumulated about one mg of cystathionine per flask. This result indicates that me-2 strains produce cystathionine from sulfate, as expected. On the other hand, me-c3 strains appear to contain about the same amount of extractable

Table 21

Cystathionine accumulation by different strains of Neurospora*

Growth Conditions			Strain Number and Genotype					
			me-2 D8.4-6		Wild type 5256A		me-c3 4894-R1	
Age in days	Na_2SO_4 mg.	Meth. mg.	Total mg.	Per cent	Total mg.	Per cent	Total mg.	Per cent
3	0	1.2	0.20	0.13	0.17	0.13	0.20	0.15
"	5.6	1.2	2.1	1.5	0.18	0.14	0.31	0.26
"	"	0.6	2.1	1.8	0.10	0.078	0.15	0.14
"	"	0.3	2.0	2.2	0.06	0.051	0.15	0.19
"	"	0.15	1.8	2.4	0.02	0.021		
6	0	1.2	0.07	0.043	0.13	0.10	0.08	0.053
"	5.6	1.2	1.6	0.94	0.20	0.14	0.12	0.074
"	"	0.6	1.6	0.86			0.06	0.043
"	"	0.3	2.0	1.5	0.16	0.12	0.06	0.056
"	"	0.15	1.7	1.4			0.06	0.061

* In 18 determinations of the concentration of 4 cystathionine solutions of unknown concentration the actual mean was 0.341 mg/ml and was estimated to be 0.347 ± 0.044 mg/ml.

cystathionine in the presence of methionine whether sulfate is present or not. These results suggest that me-c3 strains can synthesize cystathionine from DL-methionine without the intervention of sulfate or cysteine.

When methionine is decreased and the sulfate kept constant, the percent dry weight of cystathionine in me-2 increases. Wild type shows the opposite effect in three-day cultures. This effect has been confirmed by bioassay of partially purified cystathionine from aerated cultures. The percent dry weight of cystathionine extracted decreases with decreasing levels of methionine. The possibility that homocyst(e)ine accumulated in me-2 grown on higher levels of methionine could not be confirmed by chromatography.

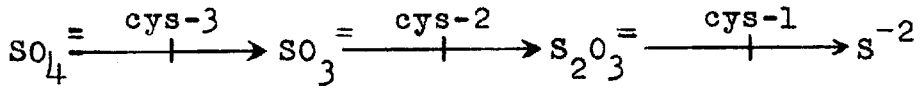
D. Discussion

It appears that Neurospora can produce cysteine from methionine without the intervention of sulfate or homocysteine, since the double mutant strains, cysteineless - methionineless (22) and cysteineless-homocysteineless (65), grow on methionine alone. The data from these experiments indicate that this reversal may involve cystathionine. The increased percentage of soluble cystathionine in the me-2 strain when the DL-methionine is decreased and sulfate kept constant is probably caused by the me-2 gene, since wild type shows the opposite effect.

Biochemical mutants have been observed in several instances to show increased accumulation when their nutritional requirement is limited (66, 67). At present, discussion of the cause of this would seem highly speculative.

V. CYSTEINE SYNTHESIS

The cysteineless mutants may be divided into three groups on the basis of the level of sulfur reduction to which they respond as follows:



Level of Reduction	S^{+6}	S^{+4}	S^{+2*}	S^{-2}

Cys-1 mutants include at least two genetic classes (68) and grow on cyst(e)ine but not on thiosulfate. When this work was begun, it was not specifically known whether these mutants were deficient in the synthesis of the carbon-nitrogen part of cystine or in sulfur metabolism. Shen had previously tested a number of possible carbon-nitrogen cystine precursors without success (69). This approach was not continued. Instead, attempts were made to determine whether these mutants were deficient in the ability to reduce sulfur compounds. Shen had obtained inconclusive results in experiments with H_2S since it is toxic, unstable and rapidly oxidized in the medium (22). It was decided to test elemental sulfur before testing H_2S in growth experiments, since it was known that fungal spores produce H_2S from this compound (70). Conidia or mycelia of Neurospora also produce H_2S from elemental sulfur by a heat labile mechanism.

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* Average

A. Growth on Elemental Sulfur

Wild type Neurospora and eight cys-1 mutants, as well as cys-2 and cys-3 mutants grow on elemental sulfur as a sole sulfur source. No cys-1 mutants were found which did not respond to elemental sulfur. Data obtained are presented in table 22. When the mycelia were harvested, attempts were made to remove the remaining sulfur with the pads. Subsequently the weight of sulfur added to each flask was subtracted from the dry weight found, to obtain the weights reported. Each value is the average of two determinations. Flasks of sulfate-free minimal, containing about 50 mg of sulfur without an innoculum, were regularly free of contamination. One cys-1 mutant was grown on autoclaved elemental sulfur as a sole sulfur source. The growth of the mutants on elemental sulfur was not the result of contamination. All mutants listed in table 22 were also grown on washed roll sulfur. Methionine-less mutants did not grow on elemental sulfur.

B. Growth on Hydrogen Sulfide

The growth of cysteineless mutants on elemental sulfur suggests that it may be used after reduction to H_2S. Experiments done with Dr. Marguerite Fling indicate that this is the case. To minimize the problems of toxicity and instability, H_2S was supplied in small doses during growth immediately after being collected from a commercial tank in sterile H_2O, the amount determined by iodometric titration. The results of two such experiments are presented in table 23. Although growth on H_2S was slow, the dry weights were about the same

Table 22

The growth of cysteineless mutants on elemental sulfur.

Mutant No. class hours of growth	Mg sulfur compound added to "sulfur-free" minimal			Mg. dry wt. of pad
	$Na_2S_2O_3$**	S	Cysteine·HCl	
5256 wild type 228		7.2		45*
85518 cys-3 84		10		42*
			1.0	50
80702 cys-3 72	1.0			48.5
		10		33.0*
6125 cys-1 132	1.0			0
		10		40.5*
			1.0	37.0
34555 cys-1 132	1.0			0
		10		44.0*
			1.0	44.0
36103 cys-1 132	1.0			0
		10		64.0*
			1.0	63.5
36106 cys-1 132	1.0			0
		10		41.0*
			1.0	54.0
39816 cys-1 132	1.0			0
		10		26.0*
			1.0	29.0
47409 cys-1 132	1.0			0
		10		40.5*
			1.0	41.5
65111 cys-1 132	1.0			0
		10		39.5*
			1.0	40.0
71310 cys-1 132	1.0			0
		10		46.5*
			1.0	73.0

* The dry wt. of sulfur added was subtracted from the dry wt. observed to give the reported values.

** Sterilized by filtration through a sintered glass filter and added to cool flasks of sterile minimal.

Table 23

Growth of cysteine mutants on H_2S

Length of Expt.	Substrate		Dry weight in mg cys-1	cys-2	cys-3	Wild type
89 hrs.	0		0.0	0.5	1.5	3.0
	2.8 ♂	H_2S	0.5	5.0	4.5	4.5
	12.0 ♂	cystine	0.5	4.0	3.0	4.5
	10.1 ♂	H_2S	3.5	7.5	11.0	8.5
	36.0 ♂	cystine	5.0	9.5	10.5	11.5
	14.1 ♂	H_2S	5.0	10.0	12.5	9.5
	60.0 ♂	cystine	9.0	11.0	15.5	12.5
	17.0 ♂	H_2S	6.0	10.5	15.5	10.5
	84.0 ♂	cystine	8.5	14.5	17.0	16.0
8 days	2.8 ♂	H_2S	1.0			
	12.0 ♂	cystine	1.0			
	6.5 ♂	H_2S	3.0			
	36.0 ♂	cystine	10.0			
	14.9 ♂	H_2S	9.5			
	84.0 ♂	cystine	15.0			
	27.7 ♂	H_2S	14.0			
	132.0 ♂	cystine	16.0			

Both H_2S and cystine were added sterilly in small amounts once and twice daily during the course of the experiment. The amounts given above represent the total amount added during the growth period.

as those found with cystine. Cys-1 mutants do not distinguish cystine from cysteine in growth experiments.

Glutathione does not serve, or serves very poorly, as a sulfur source for cys-1 mutants (table 24), although cys-2 mutants are able to utilize glutathione to a limited extent. Glutathione as supplied in this experiment was not toxic to cys-1 mutants, suggesting that most of the glutathione is oxidized before being used.

C. Discussion

All known classes of mutants involved in the reduction of sulfate are able to use inorganic sulfur compounds as a sole sulfur source. Eight mutants blocked between thiosulfate and cystine grow on elemental sulfur as a sole sulfur source. This suggests that cysteineless mutants are deficient in their ability to reduce sulfur compounds at the thiosulfate level. Elemental sulfur is probably used after reduction to H_2S, since H_2S supports the growth of cys-1 mutants.

Table 24

Glutathione as a sulfur source for cysteineless mutants.

Compound added	Mg. of S in compound added	Mg dry wt. in 72 hrs.				
		5256 Wild type	85518 cys-3	80702 cys-2	39816 cys-1	36106 cys-1
None	0	2.5	±*	0	0	0
Na_2SO_4	0.16	25.0	±*	0	0	0
Cysteine·HCl	0.15	25.0	24.5	24.0	23.0	19.5
Glutathione**	0.15	31.5	30.5	13.0	±*	2.1

* Pad too small to weigh conveniently.

** Sterilized by filtration through a sintered glass filter and added to cool flasks of sterile minimal.

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