METHIONINE SYNTHESIS IN NEUROSPORA. THE ISOLATION OF CYSTATHIONINE*

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Among artificially produced biochemical mutants of *Neurospora*, those which have lost the ability to synthesize methionine form the largest class. At the present writing 87 occurrences of the *methionineless* character have been observed in this laboratory following treatment of wild type spores with high frequency radiations (1) or mustard gas (2). *Methionineless* mutants differ from wild type *Neurospora* in that they fail to grow on a medium containing only sugar, inorganic salts, and biotin, but do grow if, in addition to these constituents, methionine is supplied. In many of the mutants failure of methionine synthesis results from a block in the reduction of sulfate, which, except for a trace of biotin, is the sole source of sulfur in the basal medium. These strains can utilize reduced forms of inorganic sulfur for growth, as well as methionine and other organic sulfur compounds. On the other hand, some of the mutants require organically bound sulfur for growth, an indication that in these strains the block in methionine synthesis comes at a later stage than sulfate reduction. Similar classes of methionine-requiring mutants have been reported in the mold *Ophiostoma* by Fries (3) and in *Escherichia coli* by Lampen *et al.* (4–6).

The present study is concerned with four strains which are unable to carry out certain of the terminal steps in methionine synthesis. Evidence is presented showing that the synthesis proceeds through a series of gene-controlled reactions involving cysteine, cystathionine, and homocysteine as intermediates. Of particular interest is the fact that, as a result of genetic blocking, a precursor of methionine accumulates in cultures of one of the strains. The precursor has been isolated and shown to be chemically and biologically indistinguishable from synthetic L-cystathionine.

The results of this investigation were presented at the meeting of the American Society of Biological Chemists in Chicago, May 21, 1947 (7).

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Materials and Methods

Media—The basal medium used in the experiments has the following composition, in gm. per liter: ammonium tartrate 5, ammonium nitrate 1, monobasic potassium phosphate 1, magnesium sulfate ($7H_2O$) 0.5, sodium chloride 0.1, calcium chloride 0.1, sucrose 20, biotin 5×10^{-6}, and the following trace elements (added as salts), in mg. per liter, B 0.01, Mo 0.02, Fe 0.2, Cu 0.1, Mn 0.02, Zn 2.0.

Stock cultures of the mutants were carried on agar slants of the following composition, in gm. per liter: potassium tartrate 5, sodium nitrate 4, monobasic potassium phosphate 1, magnesium sulfate ($7H_2O$) 0.5, sodium chloride 0.1, calcium chloride 0.1, glycerol 20, hydrolyzed casein 0.25, Difco yeast extract 5, Difco malt extract 5, DL-methionine 0.025, agar 15.

Growth was measured by inoculating the mold into 20 ml. of medium in 125 ml. Erlenmeyer flasks, incubating for 72 hours at 25°, drying the mycelium at 90°, and weighing to the nearest 0.5 mg.

Mutant Strains—The isolation numbers of the mutants reported in this paper are 38706, H98, 36104, and 39816. For convenience these will be referred to as *methionineless-1*, *methionineless-2*, *methionineless-3*, and *methionineless-4*, respectively, abbreviated *me-1*, *me-2*, etc. The same numbers can be used without confusion to designate the distinguishing gene of each strain. Thus, for example, strain *me-1* carries a mutation of gene *me-1*, but has all other known genes in their normal forms.

Strains *me-1*, *me-3*, and *me-4* were obtained from wild type *Neurospora crassa* after irradiation with ultraviolet light, while strain *me-2* was isolated (by Dr. Frank Hungate) from material exposed to x-rays. A genetic investigation of strains *me-2* and *me-3* has been carried out by Buss (8), who reports that both mutants differ from the wild type by different single genes. *Me-1* and *me-4* have been studied by Dr. B. Phinney in this laboratory, who finds that they are single gene mutants differing from strains *me-2* and *me-3*. Crosses between strains *me-1* and *me-4* are infertile (Phinney, personal communication), and the heterocaryon test for allelism (9) has so far given negative results; so that at present there is no genetic evidence that these two strains differ. In view of the differences in nutritional requirements to be reported below, however, there is little doubt that these strains do differ genetically, and in what follows they will be considered so to differ.

Compounds—I am greatly indebted to Dr. Vincent du Vigneaud for samples of synthetic L-cystathionine, D-cystathionine, L-allocystathionine, and D-allocystathionine. I wish also to thank Dr. H. Borsook and Dr. J. Dubnoff for samples of L-homocysteine and L-homocystine.

DL-Homocysteine thiolactone hydrochloride was prepared from DL-homo-

cystine by the method of Riegel and du Vigneaud (10). The methionine, cystine, and cysteine hydrochloride were commercial preparations.

EXPERIMENTAL

Growth of Mutants on Homocysteine and Cysteine—It is known from the work of du Vigneaud *et al.* (11) that the rat can methylate homocysteine to form methionine. If a similar reaction takes place in *Neurospora*, and if the reaction is gene-controlled, then one might expect to find two classes of *methionineless* mutants with respect to homocysteine utilization, those which can use homocysteine for growth and those which cannot. A preliminary survey of a number of *methionineless* strains showed that these classes do in fact exist. Of the four strains reported here, strain *me-1* fails to grow on the basal medium supplemented with homocysteine, while the other three mutants utilize homocysteine readily (Table I). Strain *me-1* does not

TABLE I

Activity of Sulfur-Containing Amino Acids for Four Neurospora Mutants

Amino acid	Dry weight of mold after 72 hrs. at 25°			
	Strain *me-4*	Strain *me-3*	Strain *me-2*	Strain *me-1*
	mg.	mg.	mg.	mg.
None	0	0	0	0
DL-Methionine, 1 mg	22.0	54.0	66.0	57.5
L-Homocysteine, 1 mg	23.0	35.0	32.0	0
DL-Homocysteine thiolactone·HCl, 2 mg	16.5	36.5	41.0	0
L-Homocystine, 2 mg	0	0	0	0
L-Cysteine·HCl, 1 mg	25.0	0	0	0
L-Cystine, 1 mg	27.0	0	0	0

grow on homocysteine even with choline or betaine supplied additionally, suggesting that the transfer of methyl, rather than its synthesis, is blocked in this mutant.

The thiolactone of homocysteine is somewhat less active than homocysteine in promoting growth, while homocystine is inactive. Evidently the last compound is not appreciably reduced to homocysteine by the mold under the conditions of these experiments. The observed activity of homocysteine for strains *me-2* and *me-3* is approximately 0.25 that of DL-methionine, calculated on a molecular basis. This represents a minimal value, since a significant quantity of homocysteine is undoubtedly oxidized to homocystine in the course of a 3 day aerobic growth experiment.

The foregoing results indicate that the normal (wild type) form of gene *me-1* controls the methylation of homocysteine and that genes *me-2*, *me-3*, and *me-4* control earlier steps in the synthesis of methionine. The results

obtained when cystine (or cysteine) is supplied throw light on the functions of the last three genes. It is found that strain *me-4* responds to cystine and cysteine, whereas the other strains do not (Table I). It follows that the synthesis of cysteine is blocked in strain *me-4* and that cysteine can serve as a precursor of methionine. The activity of cystine (calculated as cysteine) for strain *me-4* is approximately equal to that of methionine.

The conversion of cysteine to methionine is dependent on the normal functioning of genes *me-1*, *me-2*, and *me-3* since strains carrying mutant forms of these genes fail to bring about the conversion. These conclusions are summarized in the following diagram.

$$\text{Gene } me\text{-}4 \longrightarrow \text{cysteine} \xrightarrow[\text{“ } me\text{-}3]{\text{gene } me\text{-}2} \text{homocysteine} \xrightarrow{\text{gene } me\text{-}1} \text{methionine}$$

Inspection of Table I shows that strain *me-4* grows at a slower rate than do the other mutants tested. A 3-fold increase in the concentration of cystine or methionine only slightly increases the growth rate over that shown in Table I. A depressed growth rate has been found in a number of other *cystineless* mutants, although this is not characteristic of all such strains. The phenomenon is being investigated.

Conversion of Cysteine to Homocysteine—As mentioned above, the genetic findings of Buss showed genes *me-2* and *me-3* to be non-allelic. It was of considerable interest to determine whether a difference in function could be demonstrated between them. Aqueous extracts of each mutant were therefore tested for their ability to support the growth of the other strain. It was found that extracts of strain *me-2*, or the medium in which it has grown, contain a factor which can be utilized for growth by strain *me-3* (but not by strain *me-2*). The finding suggested that a precursor of homocysteine accumulates in cultures of strain *me-2* as a result of the genetic blocking.

$$\text{Cysteine} \xrightarrow{\text{gene } me\text{-}3} \text{intermediate} \xrightarrow{\text{gene } me\text{-}2} \text{homocysteine}$$

The situation is identical in principle with that encountered in the accumulation of anthranilic acid by strain 10575 of *Neurospora* (12), of monomethylaminoethanol by strain 47904 (13), and of an unidentified precursor of nicotinic acid by strain 4540 (14).

The isolation of the intermediate was accomplished by the following procedure.

Isolation of Cystathionine—Strain *me-2* was inoculated into 5 gallon Pyrex carboys containing 16 liters of basal medium plus 0.4 gm. of DL-methionine. The carboys were incubated at 25° under forced aeration for a period of 7 to 10 days. At the end of this time the mycelium was collected in a basket

centrifuge and washed with water. The moist weight of the mycelium at this stage varied from 290 gm. (7 days incubation) to 360 gm. (10 days incubation) per carboy.

Bioassay with strain *me-3* having shown that the active substance is more highly concentrated in the mold than in the medium, the latter was ordinarily discarded. In one instance the medium was worked up in parallel with the mycelium from the same carboy. The yield of pure material from the mycelium was over 10 times that obtained from the medium.

The mycelium was dispersed in water in a Waring blendor and poured into 10 liters of boiling water per kilo of moist mold. After 10 minutes the suspension was filtered through cloth and the extraction repeated. The combined filtrates were brought to pH 5 with dilute HCl and placed in the cold room. The next day the precipitate was filtered off through a layer of infusorial earth and discarded. The clear filtrate was neutralized and concentrated under reduced pressure to a volume of 2.6 liters per kilo of the original mycelium. 1 volume of 95 per cent alcohol was added with stirring, and the mixture was placed in the refrigerator overnight. The precipitate was removed by filtration and discarded.

The filtrate was concentrated to 550 ml. per kilo of original mycelium and 5 volumes of alcohol were added. After standing in the cold overnight the precipitate was collected in the centrifuge and washed with successive small portions of ice-cold water until the washings were colorless. The residue was dissolved in hot water, treated with charcoal, and concentrated under reduced pressure until crystallization began. 3 volumes of alcohol were added and the mixture refrigerated overnight.

The precipitate was collected by centrifuging and was recrystallized from water. The colorless octagonal prisms were washed with cold water and alcohol and dried with ether. Yield, 360 mg. per kilo of moist mycelium. The yield from one carboy was doubled by supplementing the basal medium with 0.2 gm. of L-cystine and 0.2 gm. of DL-homoserine in addition to the usual methionine supplement. I am indebted to Dr. Marguerite Fling for the synthetic homoserine used in this experiment.

Elementary analysis of the isolated material showed the following.

$C_7H_{14}O_4N_2S$.	Calculated.	C 37.78, H 6.35, N 12.59, S 14.42
	Found.	" 37.68, " 6.46, " 12.47, " 14.80

The material gives a strong ninhydrin test. It darkens at 270° and melts with decomposition at 301°. The specific rotation, $[\alpha]_D^{25}$, of a 1 per cent solution in 1 N HCl is $+26° \pm 2°$. These values are in agreement with those obtained by du Vigneaud *et al.* (15) for synthetic L-cystathionine.

55 mg. of the isolated material were treated with benzoyl chloride by the method of Anslow *et al.* (16), yielding 50 mg. of the dibenzoyl derivative.

$C_{21}H_{22}O_6N_2S$. Calculated, N 6.51; found, N 6.36

The compound melted at 228–229°, in agreement with the reported melting point of dibenzoyl-L-cystathionine (15). This finding, taken together with the observed specific rotation of the free amino acid, defines the configuration of *Neurospora* cystathionine as of the L, or natural, configuration throughout (16).

TABLE II

Biological Activity of Natural and Synthetic L-*Cystathionine*

Cystathionine per 20 ml. medium	Growth of strain *me-3*	
	On synthetic cystathionine	On natural cystathionine
mg.	*mg.*	*mg.*
0	0.0	0.0
0.2	10.0	10.0
0.4	20.0	20.0
0.6	28.5	33.0
0.8	38.0	40.5
1.0	44.5	44.0

TABLE III

Activity of Cystathionine Isomers for Neurospora Mutants

L-Cystathionine was isolated from *Neurospora*; the other isomers were synthetic. Strain *me-4* was incubated at 25° for 96 hours, all others for 72 hours. Concentration of amino acid, 1 mg. per 20 ml.

Isomer	Dry weight of mold			
	Strain *me-4*	Strain *me-3*	Strain *me-2*	Strain *me-1*
	mg.	*mg.*	*mg.*	*mg.*
L-Cystathionine	26.0	50.5	0.0	0.0
D-Cystathionine	0.0	0.0	0.0	
L-Allocystathionine	4.0	0.5	0.0	
D-Allocystathionine	3.0	0.0	0.0	

Biological Activity—As a final confirmation of the identity of the isolated compound, its biological activity was compared with that of synthetic L-cystathionine with strain *me-3* as a test organism. The results (Table II) show that the activities of the two compounds are identical within the limits of error. The molecular activity of cystathionine for strain *me-3* is approximately 0.6 that of methionine.

Through the courtesy of Professor du Vigneaud, who supplied synthetic material, it was also possible to test the activity of the three unnatural optical isomers of cystathionine. The results are summarized in Table III.

Only L-cystathionine is active for strain *me-3*, showing that only this isomer can be cleaved by *Neurospora* to yield homocysteine. Both L- and D-allocystathionine are slightly active for strain *me-4*, indicating that these may serve to a limited extent as sources of cysteine for the organism.

DISCUSSION

S-(β-Amino-β-carboxyethyl)-homocysteine, later named cystathionine by Binkley and du Vigneaud (17), was first suggested by Brand *et al.* (18) as a possible intermediate in the biological conversion of methionine to cystine. Previously, the structure had been tentatively assigned by Küster and Irion (19) to a substance they isolated from wool, following prolonged treatment with sodium sulfide. Later the same structure was suggested by Horn and Jones (20) for an amino acid isolated from seleniferous grains in isomorphous combination with the selenium-containing analogue.

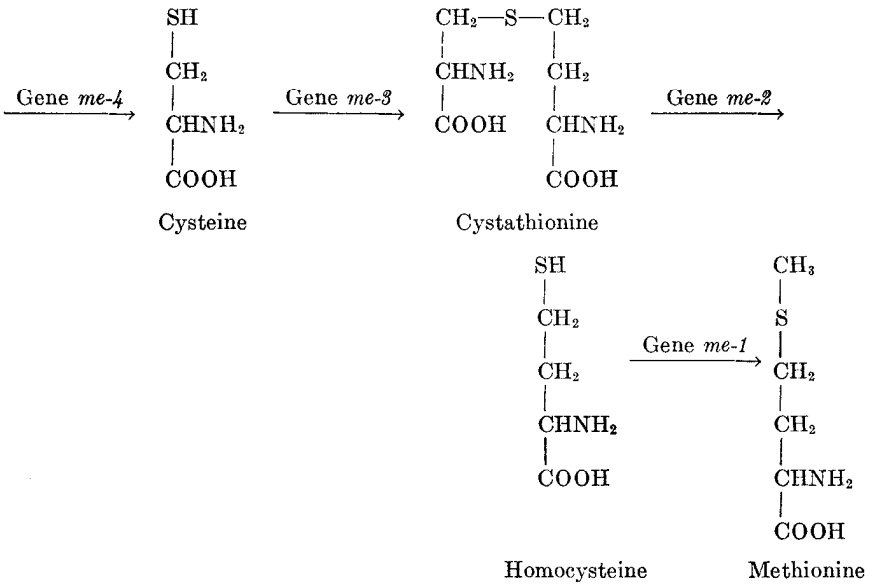
In recent years the problem has been vigorously attacked by du Vigneaud and his coworkers, who have accomplished the synthesis of optically active cystathionine as well as of active allocystathionine (15, 16). The same workers have brought forward important experimental support for the hypothesis of Brand *et al.* concerning the rôle of cystathionine in the methionine-cystine conversion (15–17, 21, 22).

The results of the present study indicate that the most probable pathway of conversion of cysteine S to methionine S in *Neurospora* is by way of the gene-controlled steps shown in Diagram 1. Mutation of the indicated genes, singly, to inactive forms would result in strains with the nutritional requirements described in this paper. This pathway is exactly the reverse, with respect to the S-containing intermediates, of that which has been proposed for the conversion of methionine S to cysteine S in the mammal. In view of the data of Lampen *et al.* (6) it is likely that a similar sequence of reactions operates in *Escherichia coli;* in this organism, however, a mutant corresponding to strain *me-3* has not yet been found.

Binkley and du Vigneaud (17) found that cysteine is formed from homocysteine and serine in the liver, presumably through the intermediate formation of cystathionine, while Stetten (23) has shown that N^{15}-labeled serine gives rise to isotopic cystine in the rat. The question therefore arises whether serine is produced in the homocysteine-yielding step in *Neurospora.* Present evidence indicates that serine is not produced in this step by direct hydrolysis of L-cystathionine into 1 molecule of serine and 1 molecule of homocysteine. If this were the case, it would be impossible to obtain a serine-requiring mutant which is not at the same time *methionine-less*, since a block in serine synthesis would necessarily involve either the cleavage of cystathionine or a step in the synthesis of cystathionine. A monogenic, *serineless* mutant of *Neurospora* has, however, been described by

Hungate (24). This mutant does not require exogenous methionine for growth, although a slight stimulatory effect of methionine, of doubtful significance, was found. It is probable, therefore, that serine and homocysteine are not products of the same reaction. Consistent with this conclusion is the result of a single experiment by the author in which the *serineless* mutant was found not to respond to L-cystathionine. The evidence does not exclude the possibility of serine production indirectly from cystathionine by phosphorolytic cleavage, yielding phosphoserine, from which serine might be subsequently liberated. Such a phosphorolysis

DIAGRAM 1



would be analogous with that which, according to the experiments of Binkley (22), is undergone by L-cystathionine in the cysteine-yielding reaction in liver preparations.

Strain *me-4*, as well as all other known *cystineless* strains of *Neurospora*, grows in the absence of added cystine if methionine is supplied. This means that in the mold, as in animals, methionine can be utilized for cystine formation. Similarly, cystathionine and homocysteine can also be converted to cystine by *Neurospora*. By analogy with the rat, it seems probable that the conversion of methionine S to cysteine S is simply the reverse of the synthesis of methionine from cysteine, shown in Diagram 1. However, no direct evidence has yet been obtained on this point. It is

conceivable that the formation of cystine from methionine in the mold proceeds over a route not involving homocysteine or cystathionine. A test of this hypothesis should be possible by the use of double mutants.

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SUMMARY

Four radiation-induced, single gene mutants of *Neurospora* which are unable to synthesize methionine from the constituents of the basal medium are described. The synthesis is blocked at a different stage in each mutant. Strain *me-4* can utilize cystine, cystathionine, and homocysteine for growth, in addition to methionine. Strain *me-3* can utilize cystathionine, homocysteine, and methionine, but not cystine. Strain *me-2* grows on homocysteine or methionine, but not on cystine or cystathionine. Strain *me-1* utilizes only methionine. A substance is produced by strain *me-2* which is active for strains *me-3* and *me-4* but not for strains *me-2* and *me-1*. The substance has been isolated and identified as L-cystathionine on the basis of the following criteria: elementary analysis, optical rotation, decomposition temperature, melting point of the dibenzoyl derivative, and biological activity. The results indicate that L-cystathionine is a normal intermediate in the synthesis of methionine by *Neurospora* and that cysteine S is converted to methionine S over the following pathway.

Cysteine → cystathionine → homocysteine → methionine

Each step is under genic control. Of the four optical isomers of cystathionine, only L-cystathionine is cleaved to homocysteine.

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