METABOLIC RELATIONSHIP BETWEEN CYSTATHIONINE AND METHIONINE IN NEUROSPORA[1]

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The precursor relationship of cystathionine in the biosynthesis of methionine by *Neurospora crassa* was first indicated by Horowitz (1947). Isotope competition studies with *N. crassa* yielded evidence that the sulfur of cystathionine can supply a large portion of the sulfur needed for growth (Roberts *et al.*, 1955). Isolation of the cystathionine-cleaving enzymes and their respective reaction products from different *N. crassa* methionineless mutants further indicated the validity of the precursor role of cystathionine and supported the concept of transsulfuration in *N. crassa* (Fischer, 1957).

Cystathionine has been designated as a biosynthetic intermediate in methionine formation in both mammals and microorganisms. Evidence for and against its biological significance has been presented in the literature. The investigation reported here was initiated to evaluate the position of cystathionine in methionine biosynthesis by utilizing biochemical mutants of *N. crassa*, blocked in the pathway of methionine biosynthesis, in isotopic conversion studies. Primary objectives have been: (a) to determine the source of the carbon chains and sulfur of cystathionine and, (b) to find whether radioactive cystathionine contributes to the carbon chain or sulfur of methionine. The first part has been studied for several combinations of time and methionine substrate concentration.

MATERIALS AND METHODS

Organisms. Two methionine auxotrophs of *N. crassa* were selected for these studies because of their blocked position in the cysteine to methionine sequence. Strain H98A requires methionine or homocysteine for growth. It accumulates cystathionine in the mycelium and in the medium. It does not contain the enzyme which cleaves cystathionine to homocysteine (Fischer, 1957). Strain 36104a requires methionine or homocysteine or cystathionine for growth (Horowitz, 1947). The strains were originally obtained from Dr. Marguerite Fling.

Culture methods. The mutants were maintained and transferred monthly on agar slants according to the method of Horowitz (1947). Fries' defined medium (Horowitz, 1947) was used for all of the experimental work involving strains H98A and 36104a. The medium was dispensed in 20-ml quantities in 125-ml Erlenmeyer flasks. Methionine supplement was added for optimal growth, except in experiments when the effect of methionine concentration was under observation. Radioactive substrates were added to the medium as noted in specific experiments.

After sterilization, the flasks were inoculated with one drop from a very sparse conidial suspension. Growth was allowed to take place in a constant temperature room at 25 C for intervals of 2, 3, 5, and 7 days. Mycelia were harvested on a suction filter and washed.

Extraction of mycelia. Extraction of mycelia in 2 ml of 50 per cent ethanol overnight was followed by boiling water extraction for 10 min. Mycelia were extracted twice. The combined extracts were dried in a vacuum desiccator over H_2SO_4 and stored at 0 C. After extraction, the mycelial pads were dried overnight in a 55 C oven, cooled, and weighed.

Ion-exchange column chromatography. The method of Wall (1953) utilizing a Dowex 50-X12 resin column with elution of amino acids by HCl was found to separate cystathionine effectively from cystine and all other amino acids. Standard column runs on a combined sample of cystine and cystathionine gave a separation as shown in figure 1.

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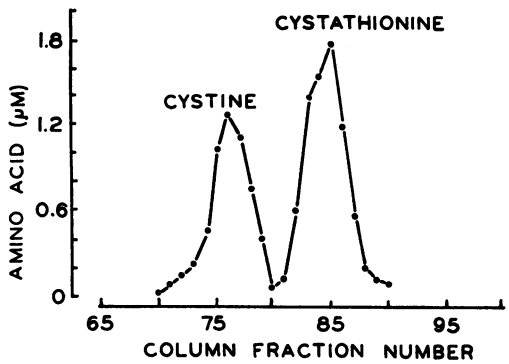

Figure 1. Cystine and cystathionine separation by ion-exchange chromatography. Identity was determined by subsequent paper chromatograms of the fractions.

Identification of cystathionine. Cystathionine isolated from radioactive extracts of strain H98A by column chromatography was identified by paper chromatography of the column fractions. A sample from each fraction was dried and spotted onto Whatman no. 1 filter paper. The paper chromatograms were run one-dimensionally with butanol-acetic acid-water (2:1:1) solvent, dried, and sprayed with ninhydrin spray reagent. Duplicate paper chromatograms were sprayed with chloroplatinic acid to confirm the sulfur content (Winegard *et al.*, 1948). Known cystathionine and the biologically produced compound show two spots in this system; the R_F value of the major spot is 0.21. The minor spot is at R_F 0.07. Further confirmation of the identity of radioactive cystathionine was done by co-chromatographing with "cold" known cystathionine. The paper chromatograms were run two-dimensionally (phenol, 80 per cent, followed by butanol-acetic acid-water, 2:1:1). Autoradiograms yielded coincident ninhydrin and radioactive spots.

Quantitative spectrophotometric determination of cystathionine. The spectrophotometric method of Moore and Stein (1954) for the quantitative estimation of amino acids was used for the determination of cystine and cystathionine isolated from standard experiments on the ion-exchange column, and for cystathionine isolated from radioactive extracts. The radioactivity of the cystathionine peaks was determined by pipetting a 0.5-ml sample from each column fraction into a glass planchet, allowing to air dry, and counting in a windowless gas flow counter. Amino acid peaks for cystathionine correspond closely with radioactive peaks (figure 2).

Quantitative paper chromatogram-densitometer determination of cystathionine. The accurate determination of specific activity of cystathionine isolated from radioactive extracts of strain H98A required that cystathionine be purified further after column chromatography. This was done by making 1.5-inch paper strip chromatograms of the column-isolated cystathionine fraction. These were run one-dimensionally in butanol-acetic acid-water (2:1:1) for 12 hr, dried, sprayed with ninhydrin spray reagent, and the color allowed to develop for 1 hr in a 60 C oven. Subsequently, the strips were placed on a Photovolt model 52-C densitometer. Optical density readings were taken for each 1 mm of the length of the entire spot. The readings were totaled for each spot and the amount of cystathionine was calculated by interpolation of the total optical density value on a standard curve for cystathionine. For determination of radioactivity, the cystathionine spot was cut out, placed in a steel planchet and counted for periods of time long enough to give counts within 5 per cent significance.

Radioactive substrates. DL-Methionine-2-C^{14} was from Tracerlab, Inc., and $Na_2S^{35}O_4$ from Oak Ridge National Laboratories.

Homoserine-U-C^{14} and serine-U-C^{14} were purified by chromatographic procedures from an extract of a garden pea plant which had been exposed to $C^{14}O_2$.

Methionine-S^{35} was prepared by a method devised after noting that Gordon *et al.* (1954) isolated large amounts of methionine from boiling water extracts of *Penicillium chrysogenum* (Wis

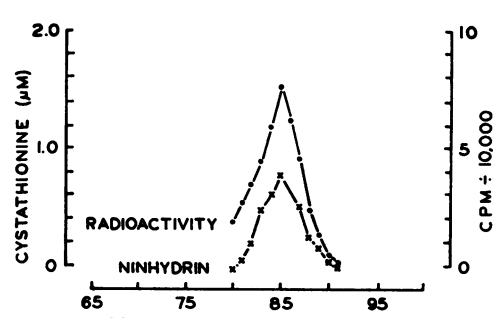

Figure 2. Correlation of radioactivity and ninhydrin determination of cystathionine in column chromatogram of extract of *Neurospora crassa* strain H98A.

49-133). In our method, *P. chrysogenum* (Q 176) was grown 5 days on semidefined medium (Gordon *et al.*, 1954) to which $Na_2S^{35}O_4$ was added. Mycelial mats were harvested, extracted for 20 min in boiling water, and the filtrate of the extract concentrated, desalted by adsorption in columns of Dowex 50-X4, 100 to 200 mesh, and the amino acids eluted with 1 N NH_4OH. Repeated band paper chromatography of the amino acid eluate resulted in methionine-S^{35} of constant high specific activity. Co-chromatograms with known DL-methionine yielded an autoradiogram with a coincident radioactive and ninhydrin spot for methionine. The purified methionine-S^{35}, diluted to approximately 7,000,000 cpm per ml, was used in tracer experiments.

Cystathionine-C^{14} and cystathionine-S^{35} for use in isotope incorporation experiments for 36104a were prepared by growing strain H98A (which accumulates cystathionine) separately in the presence of three radioactive substrates, DL-methionine-2-C^{14}, $Na_2S^{35}O_4$, and glucose-U-C^{14}. The radioactive cystathionine was isolated and purified by repeated band paper chromatography in several solvent systems.

Cleavage of radioactive cystathionine. To determine the labeling pattern in radioactive cystathionine, the method of Binkley and Boyd (1955) was employed to cleave radioactive cystathionine into the 3 and 4 carbon moieties. Half of the reaction mixture was desalted for isolation of one cleavage product, homocysteine. The other half was used to prepare a 2,4-dinitrophenylhydrazone (2,4-DNPH) (Cavallini *et al.*, 1949) for isolation of the other reaction product, pyruvate. Paper chromatograms of the desalted fraction run in butanol-acetic acid-water (2:1:1) yielded homocysteine, and ascending paper chromatography of the 2,4-DNPH in *n*-butanol saturated with an equal volume of 1.5 N NH_4OH (Walker *et al.*, 1951) purified the pyruvate derivative. The radioactivity of these two products was determined by cutting out the spots and counting in a windowless gas flow counter. Background counts were done on samples cut from duplicate chromatograms of cold reaction products.

RESULTS

Effect of the amount of methionine supplement upon the total amount of cystathionine synthesized. Typical growth curves for strain H98A when grown in the presence of three levels of methionine supplement are presented in figure 3. Five-tenths mg methionine per flask is considered optimal, 1.0 mg, excess, and 0.1 mg as starvation conditions. To learn if this mutant actually accumulates cystathionine and to determine whether the amount of exogenous methionine available in the medium affects the quantity of cystathionine synthesized, the amount of cystathionine formed by strain H98A during a 7-day growth period for three levels of methionine supplement was determined. Figure 4 indicates that the amount of cystathionine increases with the weight of the mycelial mat. If the amount of cystathionine is calculated per 1 mg dry weight of mycelia, it can be observed in figure 5 that more cystathionine accumulates when the mutant is starved for methionine. This agrees with the findings of Fischer (1957).

Labeling of cystathionine. Isotopic conversion

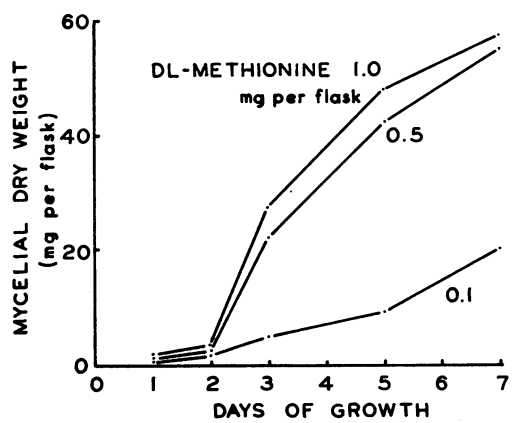

Figure 3. Growth curves of *Neurospora crassa* strain H98A for three levels of exogenous DL-methionine. Each point represents the average of two mycelial pads, each harvested from 20 ml of medium in a 125-ml Erlenmeyer flask.

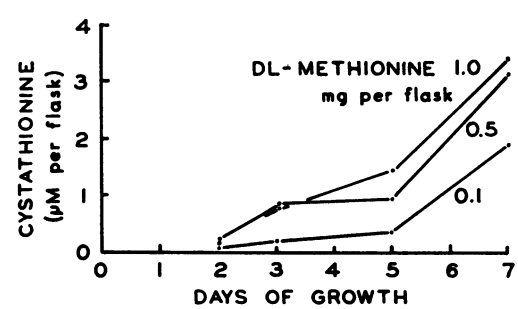

Figure 4. Cystathionine formed by *Neurospora crassa* strain H98A for three levels of exogenous DL-methionine.

studies were employed to determine sources of the carbon and sulfur of cystathionine. Radioactive cystathionine of high specific activity can be isolated from the mutant if strain H98A is grown in the presence of any of the following tracer compounds: DL-methionine-2-C^{14}, homoserine-U-C^{14}, serine-U-C^{14}, methionine-S^{35}, or $Na_2S^{35}O_4$.

Kinetic comparison of the specific activity of radioactive cystathionine derived from three different tracer compounds in extracts of strain H98A. An attempt was made to correlate the incorporation of methionine-2-C^{14}, homoserine-U-C^{14}, and $Na_2S^{35}O_4$ into cystathionine with the metabolic events that occur over a 7-day growth period of the mutant and to determine the effect of methionine substrate concentration on their incorporation. The mutant was grown in the presence of the appropriate radioactive compound, the radioactive cystathionine was isolated, and its specific activity determined. Specific activity values for cystathionine were obtained from 36 individual samples, each one representing a specific combination of the three parameters, namely, radioactive source, time, and methionine substrate concentration. Figures 6, 7, and 8 present the results. The data of figure 6 may be interpreted as follows. When homoserine-U-C^{14} is the source of label, the specific activity of cystathionine for the three levels of methionine is very similar, being somewhat higher in the case of 0.1 mg of DL-methionine per flask. A smaller

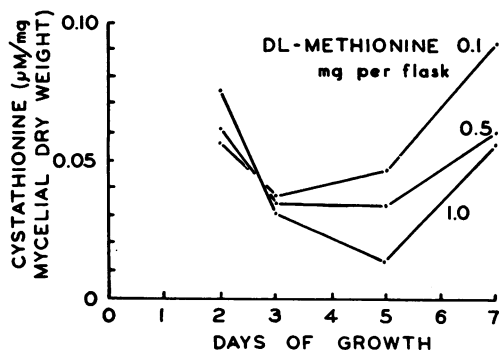

Figure 5. Cystathionine formed per milligram dry weight of mycelium by *Neurospora crassa* strain H98A for three levels of exogenous DL-methionine.

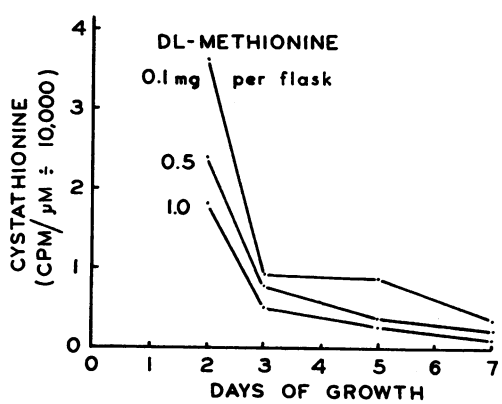

Figure 6. Specific activity of cystathionine isolated from extracts of *Neurospora crassa* strain H98A grown in the presence of L-homoserine-U-C^{14} for three levels of exogenous DL-methionine.

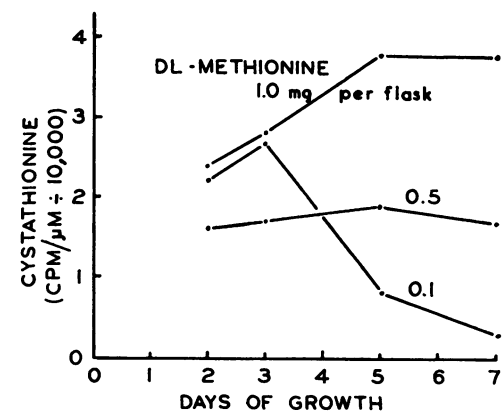

Figure 7. Specific activity of cystathionine isolated from extracts of *Neurospora crassa* strain H98A grown in the presence of three levels of DL-methionine-2-C^{14}.

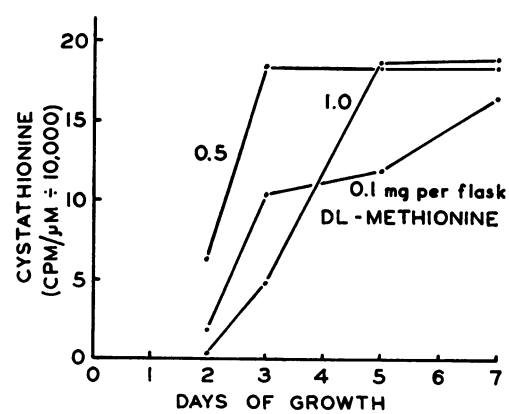

Figure 8. Specific activity of cystathionine isolated from extracts of *Neurospora crassa* strain H98A grown in the presence of $Na_2S^{35}O_4$ for three levels of exogenous DL-methionine.

amount of growth with 0.1 mg of DL-methionine per flask gives a higher specific activity in cystathionine which would be expected if the use of labeled homoserine is accompanied by proportionately less production of endogenous homoserine than in the presence of larger amounts of growth with more DL-methionine. Homoserine does act as a source of carbon for cystathionine as expected (Teas *et al.*, 1948). The specific activity of the cystathionine is dependent upon the amount of growth which is in turn determined by the amount of methionine supplement, and by the time factor, the two things interacting to determine the relative amounts of exogenous and endogenous homoserine which have been available up to the time the cells were harvested. The drop in specific activity with time is probably explained by the free homoserine being used up by the third day of growth. This was expected because only 0.167 mg of L-homoserine-U-C^{14} per flask was used. This level avoided the possibility of inhibition by homoserine.

In the case of methionine-2-C^{14} labeling (figure 7), the specific activity values for cystathionine reflect the initial amount of C^{14} and indicate a direct relation between the amount of exogenous methionine and the amount used to make cystathionine. With 0.5 mg per flask, the specific activity remains fairly constant with time. With 1.0 mg methionine per flask, the specific activity of cystathionine rises slowly with time, showing that when an excess of exogenous methionine is present, some of the labeled carbon chain remains available for cystathionine synthesis and that under these conditions the exogenous methionine is used somewhat in preference to the endogenous homoserine. With 0.5 mg DL-methionine per flask it would appear that endogenous synthesis and use of exogenous methionine remain in balance to keep a constant specific activity. With 0.1 mg DL-methionine, the endogenous synthesis exceeds the exogenous material and the specific activity drops.

In the case of $Na_2S^{35}O_4$ incorporation into cystathionine (figure 8), little S^{35} is used at first when 0.5 mg DL-methionine is present, but eventually a maximal equilibration with the $S^{35}O_4^-$ of the medium is reached. When 1.0 mg of DL-methionine is present, the equilibration occurs later. This indicates that methionine is a preferred source of sulfur, possibly because in methionine it is already reduced. With 0.1 mg DL-methionine, the slower growth presumably took longer to use the exogenous material, so the specific activity remained low for a longer time.

Specific activity of reference compounds in hydrolyzates of strain H98A. After alcohol extraction, mycelia from the above experiments were hydrolyzed in 4.0 N HCl in sealed tubes for 4 hr in the autoclave. Specific radioactive compounds were isolated by means of two-dimensional paper chromatography and their specific activities determined. In the case of the homoserine-U-C^{14} labeling experiment, threonine served as a reference compound. Figure 9 presents the specific activity of cellular threonine for three levels of exogenous DL-methionine supplement. If this is compared with figure 6 it can be observed that the specific activity of the cellular threonine is very similar to that of cystathionine, indicating that the exogenous homoserine is used equally well for synthesis of the two compounds. Figure 10 represents a comparison between the specific activity of cystathionine derived from the methionine-2-C^{14} source and the specific activity of methionine sulfoxide representative of methionine in the cells from the same experiment. The specific activity of both the cystathionine and methionine sulfoxide remain constant when 0.5 mg methionine is the supplement, but when 1.0 mg is present, there is a drop in specific activity of methionine sulfoxide but a rise in specific activity of cystathionine with time.

When the ratio of the specific activity of cystathionine to the specific activity of methionine sulfoxide is calculated and plotted against time, curves are obtained as seen in figure 11. The ratio is fairly constant with 0.5 mg methionine supplement but rises with 1.0 mg.

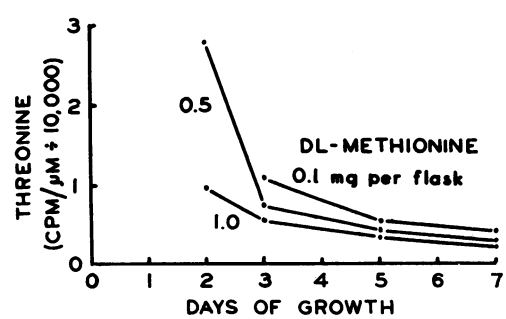

Figure 9. Specific activity of threonine isolated from hydrolyzates of *Neurospora crassa* strain H98A grown in the presence of L-homoserine-U-C^{14} for three levels of exogenous methionine.

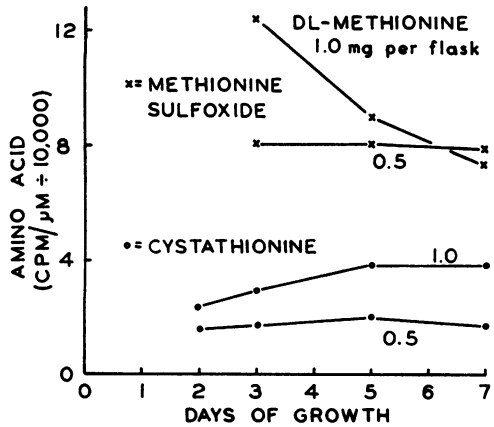

Figure 10. Specific activity of methionine sulfoxide isolated from hydrolyzates and cystathionine isolated from extracts of *Neurospora crassa* strain H98A grown in the presence of DL-methionine-2-C^{14} for two levels of exogenous methionine.

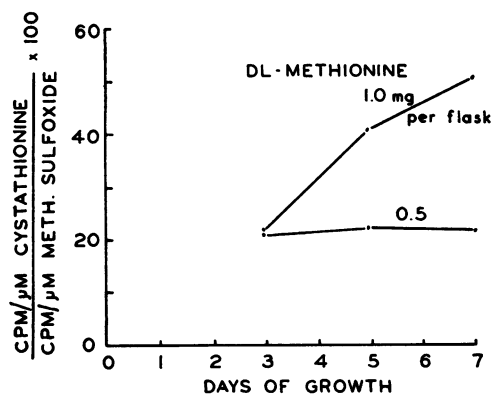

Figure 11. Comparison of the ratio of specific activity of cystathionine to specific activity of methionine sulfoxide for two levels of exogenous methionine.

Incorporation of radioactive cystathionine into methionine. Cystathionine-C^{14} and cystathionine-S^{35} (biological preparations from strain H98A grown in the presence of either $Na_2S^{35}O_4$, DL-methionine-2-C^{14}, or glucose-U-C^{14}) were fed to strain 36104a, a methionineless mutant which can use cystathionine as the sole supplement. Samples of the cystathionine-C^{14} preparations were cleaved to determine the labeling pattern that DL-methionine-2-C^{14} and glucose-U-C^{14} contribute to cystathionine. The cleavage products homocysteine and pyruvate, representing the four and three carbon moieties of cystathionine, respectively, were examined for radioactivity. Table 1 indicates that both the three and four carbon chains of cystathionine are labeled when glucose-U-C^{14} is the source of label, but only the four carbon chain is labeled when DL-methionine-2-C^{14} is the source. The three preparations of radioactive cystathionine, labeled either in the four carbon chain, both carbon chains, or the sulfur, were used singly as supplements for strain 36104a. After growth for 6 days, the mycelia were harvested, extracted, and hydrolyzed. The radioactivity present in media before growth, media after growth, extract and hydrolyzate is indicated in table 2. There was no radioactivity in traps attached to the flasks. The hydrolyzates were examined for radioactive methionine on paper chromatograms. The radioactivity in methionine and methionine sulfoxide was added and the total is indicated in table 2 as hydrolyzate methionine. The data show that of

TABLE 1

Radioactivity in three and four carbon moieties of cystathionine synthesized by Neurospora crassa strain H98A

Source of Label	Cpm in Pyruvate	Cpm in Homocysteine
Methionine-2-C^{14}	0	72
Sucrose-U-C^{14}	56	60

TABLE 2

Distribution of radioactivity from cystathionine after growth of Neurospora crassa strain 36104 for 6 days

	Source of Label in Cystathionine		
	Methionine-2-C^{14}	Glucose-U-C^{14}	$Na_2S^{35}O_4$
	Total counts of cystathionine in medium at start		
	70,160	42,980	4,177,480
	Distribution after growth:		
	%	%	%
Medium	86	78	80
Extract	5.6	14.0	6.5
Hydrolyzate of cells	5.7	11.6	18.0
Hydrolyzate methionine	0.12	0.38	11.7
Recovery	97.5	104.4	104.6

the total amount of radioactivity incorporated into cells from C^{14} labeled cystathionine, only 2 to 3 per cent is present as methionine. The corresponding figure for S^{35} in methionine from S^{35} labeled cystathionine is 65 per cent.

DISCUSSION

Previous studies on the methionineless mutants of Neurospora have proposed that methionine synthesis proceeds over the pathway diagrammed in figure 12A (Horowitz, 1947; Fling and Horowitz, 1951). Fischer (1957) reported that the pathway is reversible. Certain of the data in our study lend support to this concept of methionine formation. Other experimental results in the study, however, suggest rather strongly that other pathways may be operative.

The fact that the methionineless mutant (H98A) accumulates the greatest amount of cystathionine per mg of cells throughout the growth period when the exogenous methionine present represents starvation conditions (figure 5) supports the idea that cystathionine is a valid intermediate in methionine formation. It corresponds with observations of others (Houlahan and Mitchell, 1948; Fischer, 1957) that mutants tend to accumulate more of the compound just prior to that point where they are blocked in a synthetic pathway when starvation amounts of their required supplement are present.

The information that highly labeled cystathionine results from the utilization of serine-U-C^{14} and homoserine-U-C^{14} by strain H98A could indicate the necessary position of cystathionine in the cysteine to methionine sequence since serine is considered to be a precursor of cysteine, and homoserine, a precursor of methionine.

In isotope competition studies (Roberts *et al.*, 1955) with *N. crassa*, cystathionine had the competitive behavior which one would expect of an intermediate, i. e., S^{32}-cystathionine proved to be an effective competitor in reducing the incorporation of S^{35} from $S^{35}O_4^-$ and S^{35}-methionine in *N. crassa*. Our data reveal that when cystathionine-S^{35} is used as the sole supplement for the methionineless mutant 36104a (which requires cystathionine for growth), approximately 65 per cent of the total amount of radioactivity incorporated into the cells from cystathionine-S^{35} is present as methionine. This verifies that transsulfuration occurs in *N. crassa* but does not confirm the obligate precursor role of cystathionine in methionine biosynthesis since the carbon chains of cystathionine have not been shown to be incorporated into cellular methionine.

Evidence against the biological significance of cystathionine in methionine biosynthesis has been presented in isotope competition studies with *E. coli* strain B and mutant strains of *E. coli* (Roberts *et al.*, 1955) wherein no competitive effects of cystathionine when tested with C^{14}- or S^{35}-labeled compounds were found. They concluded that cystathionine and allocystathionine are neither intermediates in the carbon transformations nor are capable of furnishing sulfur to *E. coli*. They suggested that it would be desirable to complete this negative picture by using labeled cystathionine to show that the cell is permeable to this compound and that its sulfur or carbon is not incorporated.

In earlier studies in our laboratory, cystathionine-C^{14} (from a sucrose-U-C^{14} source) was used as a supplement for growth of *N. crassa* strain 36104a. Methionine was isolated from the cells and found not to be radioactive (Garner and Teas, 1954). This finding suggested either that: (a) the cystathionine was not radioactive in the four carbon part or, that (b) cystathionine is not a precursor of methionine for *N. crassa*. Permeability considerations were eliminated since strain 36104a will grow if given cystathionine, homocysteine, or methionine. The cleavage data presented in this paper show that radioactive cystathionine prepared by growing cells in the presence of glucose-U-C^{14} is labeled in both the four and three carbon chains, hence, the first postulation is not valid. When cystathionine-C^{14} labeled either in the four carbon chain or both carbon chains was used as the sole supplement for strain 36104a, only 2 or 3 per cent of the total amount of radioactivity incorporated into cells was present as methionine. This result will not support the schema proposed in figure 12*A* because this pathway proposed the cleavage of the cystathionine molecule to yield homocysteine which is methylated to become methionine, and would require that the four carbon chain of cystathionine be incorporated totally into methionine.

The possibility exists that strain H98A can metabolize exogenous methionine and utilize some of the breakdown fragments to build up an accumulation of cystathionine as a side product of the catabolism. DL-Methionine-2-C^{14} and methionine-S^{35}, when fed to strain H98A, contribute

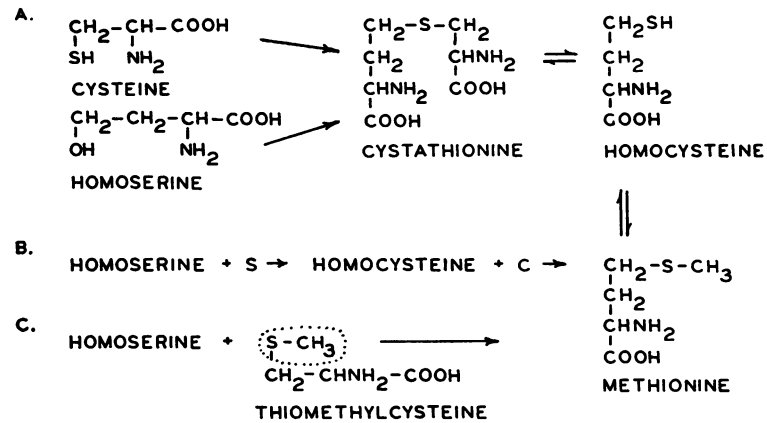

Figure 12. Possible biosynthetic sequences in the formation of methionine

label to cystathionine. Cystathionine is presumably labeled from some fragment set free when methionine-2-C^{14} is catabolized by the mutant. This fragment may include the sulfur of methionine, since methionine-S^{35} contributes label to cystathionine. (The conversion of methionine-S^{35} to cystathionine-S^{35} in intact rats was demonstrated by Tabachnick and Tarver, 1955.) The fragment may be homocysteine, resulting from the demethylation of methionine, which then condenses with serine to yield cystathionine. An enzyme which synthesizes cystathionine from homocysteine and serine with the simultaneous deamination of serine has been isolated from rat liver by Selim and Greenberg (1959). However, cystathionine formed from exogenous methionine has only about one-fourth of the specific activity of the reference compound, methionine sulfoxide, when strain H98A is grown in the presence of methionine-2-C^{14} (figure 10). This shows that endogenous sources of the four carbon chain of cystathionine do not go through methionine. It cannot be postulated that the mechanism of cystathionine formation from methionine in Neurospora involves a catabolism of methionine similar to that in animals in which homoserine is the first degradative product (Matsuo and Greenberg, 1955). If this did happen, homoserine from exogenous labeled methionine would then be expected to contribute carbon to threonine, and this has not been observed.

The significance of the kinetic study of the specific activity of cystathionine is that there are apparently two sources of the four carbon chain of cystathionine. One appears to be an endogenous source via homoserine, and the other, the exogenous source from methionine supplement. Confirmation that the four carbon chain is indeed derived from methionine, was achieved by the cleavage of radioactive cystathionine, which in turn was derived from growth of the mutant in the presence of DL-methionine-2-C^{14}, yielding a four carbon fragment, homocysteine, which was radioactive, and a three carbon fragment, pyruvate, which was not radioactive. The incorporation of C^{14} from homoserine, wherein specific activities are related to the amount of homoserine and inversely related to the amount of growth, indicates that homoserine acts as a source of the four carbon chain. Exogenous homoserine also acts in the predicted fashion as a precursor of threonine, isoleucine, and methionine. The similarity between the specific activity curves for cystathionine and cellular threonine (figures 6 and 9) from this experiment indicates that homoserine is apparently equally available for formation of the two compounds.

Labeling from DL-methionine-2-C^{14} shows a relationship between the methionine concentration and synthesis of cystathionine. The increasing ratio of the specific activity of cystathionine to the specific activity of methionine sulfoxide (figure 11) with 1.0 mg methionine per flask suggests that some of the excess methionine is converted to an intermediate in cystathionine biosynthesis which is not available for reconversion to methionine. Hence, the cystathionine can be continually made from a labeled source while the cellular methionine is increasingly supplied

by unlabeled endogenous sources. It is more difficult to interpret the constancy of specific activity with 0.5 mg of supplement when, as shown in figure 8, new S^{35} is definitely being introduced into cystathionine. To reconcile these data, one would need to postulate two intermediates, one without sulfur and capable of being converted into either methionine or cystathionine, and another, formed in much larger amounts when excess methionine is present, capable of being converted only to cystathionine. The second would possibly contain sulfur and thus account for the delay in incorporation of S^{35} into cystathionine (figure 8) when 1.0 mg of methionine is fed.

If cystathionine is not an obligate precursor to methionine biosynthesis in *N. crassa*, the question arises as to what other pathways for methionine formation are feasible. Homoserine is known to be a precursor for methionine. Homoserine-U-C^{14} gives rise to cellular methionine-C^{14} in strain H98A. Homoserine would require the loss of the hydroxyl group and addition of a thiomethyl group to become methionine. Serine has been shown to be a precursor of cysteine (Roberts *et al.*, 1955). It may be possible that its homolog, homoserine, could give rise to homocysteine in an analogous fashion. In this type of schema, outlined in figure 12*B*, homoserine could become homocysteine which is methylated to become methionine. The schema would eliminate a need for cystathionine. Another possibility is the schema presented in figure 12*C*, in which *S*-methyl-cysteine may function as a thiomethyl donor to a four carbon compound, possibly homoserine, to yield methionine as first suggested by Ragland and Liverman (1956). Preliminary experiments have demonstrated that strain 36104a will utilize *S*-methyl-cysteine as a sole supplement as well as it utilizes cystathionine in a medium containing inorganic sulfur. Perhaps strain 36104 cleaves *S*-methyl-cysteine to yield a thiomethyl group which is transferred to homoserine in a transthiomethylation process. Ragland and Liverman (1956), reported the occurrence of *S*-methyl-cysteine in extracts of strain H98 and wild type, hence, this type of sequence has nearly the same weight of evidence supporting it as has the sequence involving cystathionine.

It should be remembered that the phenomenon of "leakage" described by Bonner (1951) may also be involved. Strauss and Minagawa (1959) have described the formation of methionine by a methionine-requiring mutant of *N. crassa*. This mutant (584), which has nutritional characteristics similar to strain H98A, incorporated S^{35} from sulfate into methionine representing net synthesis of methionine. It was proposed that methionine synthesis in strain 584 proceeds by the normal pathway but that not all of the normal proteins can be synthesized when methionine is limiting, which accounts for the long lag before the mutant begins to grow. Although strain H98A is not usually considered to be a "leaky" mutant, it is possible that it may be synthesizing methionine slowly in a normal fashion. This mutant is customarily given exogenous methionine to support growth. This situation may result in the use of alternate pathways of methionine synthesis and metabolism for the mutant. A further complication is the fact that the utilization of exogenous and endogenous methionine may be different, i. e., exogenous methionine is a carbon source for isoleucine, but endogenously produced methionine has not been shown to be used in this way.

It is our belief that the obligatory precursor role of cystathionine in methionine biosynthesis has not been completely substantiated. Other intermediates and alternative pathways appear probable and await proof by enzymatic and tracer experiments.

SUMMARY

Radioactive cystathionine can be isolated from extracts of a mutant (strain H98A) of *Neurospora crassa* when grown in the presence of any of the following tracer compounds: DL-methionine-2-C^{14}, methionine-S^{35}, $Na_2S^{35}O_4$, serine-U-C^{14}, and homoserine-U-C^{14}.

A kinetic study of the specific activity of cystathionine isolated from cells of *N. crassa* strain H98A, which were supplied separately with three sources of tracer label, homoserine-U-C^{14}, $Na_2S^{35}O_4$, and DL-methionine-2-C^{14}, plus three levels of exogenous methionine supplement, indicates that there are two sources of the four carbon chain of cystathionine. One appears to be the endogenous source via homoserine, and the other, simultaneously but not equally, the exogenous source from the methionine supplement.

An actual accumulation of cystathionine by this mutant over a 7-day growth period was confirmed. The greatest accumulation of cysta-

thionine per mg of cells occurs when starvation amounts of DL-methionine are supplied.

Optimal concentrations of exogenous methionine-2-C^{14} result in a ratio of specific activity of cystathionine to specific activity of cellular methionine which remains fairly constant over time. Excess concentrations of exogenous methionine result in a ratio of specific activity of cystathionine to methionine which rises with time.

Values for the incorporation of cystathionine-S^{35} and cystathionine-C^{14} into cellular methionine by a cystathionineless mutant (strain 36104a) of *N. crassa* indicate that exogenous cystathionine acts as an important source of sulfur but not of the four-carbon skeleton of methionine.

Transsulfuration in *N. crassa* is confirmed.

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