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USE OF *S*-METHYLCYSTEINE AND CYSTATHIONINE BY METHIONINELESS *NEUROSPORA* MUTANTS

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ABSTRACT

WIEBERS, JOYCE L. (Purdue University, West Lafayette, Ind.), AND HAROLD R. GARNER. Use of *S*-methylcysteine and cystathionine by methionineless *Neurospora* mutants. J. Bacteriol. **88**:1798–1804. 1964.—Radioactive methionine was found in hydrolysates of various strains of *Neurospora crassa* when either *S*-methylcysteine (SMC)-$C^{14}H_3$ or SMC-S^{35} is the sole addition to minimal medium. Isotope product-precursor specific activity ratios are very similar for the two sources of label. Wild-type and methionineless mutants use sulfur from SMC in the biosynthesis of methionine, but not of cysteine, when grown in regular medium. With a medium nearly free from sulfate, SMC served as a source of sulfur for both cysteine and methionine. Suppressed methionineless mutants incorporated sulfur from SMC into cellular cysteine even in the presence of normal amounts of sulfate. SMC as a possible metabolic precursor of methionine was compared to cystathionine in an experiment with wild-type *Neurospora*. The four sources of label used were: SMC-$C^{14}H_3$, SMC-S^{35}, cystathionine-U-C^{14}, and cystathionine-S^{35}. In each flask, the organism was offered one of the labeled compounds plus an equivalent amount of the other compound without label. The amount of each compound was sufficient for either to supply its contribution to all of the cellular methionine, if it were successful in competing with endogenous sources. To avoid adaptive breakdown of substrates, the compounds were added continuously at a rate consistent with the amount of growth present. The ratio of specific activity of cellular methionine to precursor was determined for each labeled compound. The results show that SMC sulfur and methyl carbon are used equally well. Cystathionine carbon and sulfur appear to be equally utilized also. A preference for cystathionine is indicated.

Previously, we reported that the obligatory precursor role of cystathionine in methionine biosynthesis by *Neurospora* was not substantiated by isotopic tracer studies (Wiebers and Garner, 1960). Figure 1A indicates the classical pathway for methionine formation by *Neurospora* via condensation of cysteine and homoserine to form the intermediate cystathionine which is cleaved to homocysteine, and which is subsequently methylated to form methionine. If this is a major biosynthetic route, one would expect that the four-carbon chain with attached sulfur from radioactive cystathionine would be incorporated into cellular methionine by a cystathionineless mutant. A tracer experiment of this nature revealed that exogenous cystathionine serves as a major source of sulfur but not of the four-carbon skeleton of methionine. Alternatively, one may consider Fig. 1B, involving the direct formation of homocysteine from homoserine and sulfate, or Fig. 1C, first suggested by Ragland and Liverman (1956), which is a transthiomethylation of the thiomethyl group of *S*-methylcysteine (SMC) to homoserine to yield methionine. Ragland and Liverman (1956) reported the occurrence of SMC in extracts of *Neurospora* and demonstrated that SMC will serve as a sole sulfur source for certain strains. Roberts et al. (1955) reported that SMC is as effective a competitor in reducing the incorporation of S^{35} from $Na_2S^{35}O_4$ as cystathionine in *Neurospora*. Maw (1961) reported the ability of SMC to annul the inhibition of yeast growth by L-ethionine and by *S*-ethyl-L-cysteine. He suggested that SMC may abolish the ethionine effect by acting as a precursor of methionine via a pathway which excludes cysteine. The idea of a pathway from SMC to methionine not involving cysteine is also suggested by the work of Wolff, Black, and Downey (1956), who demonstrated the enzymatic synthesis of SMC in baker's yeast from methanethiol and serine.

We observed that a "cystathionineless" mutant can grow with SMC as a sole supplement to minimal medium as well as with cystathionine. Furthermore, when either methyl-labeled or sulfur-labeled SMC was the sole addition to minimal medium, radioactive cellular methionine was found in hydrolysates of wild-type and

methionineless mutants. These observations support the type of sequence in Fig. 1C and prompted the present investigation of the comparative use of SMC and cystathionine by *Neurospora*.

MATERIALS AND METHODS

Organisms. Wild-type *N. crassa* G165-A was used along with a cystathionineless mutant (36104a) originally obtained from Marguerite Fling. Strain 36104a requires methionine, homocysteine, or cystathionine for growth. Strain M-4 and the suppressed strains, M-4-Su and H-98-Su, were obtained from Bernard Strauss. Strain M-4 is blocked between cysteine and cystathionine; M-4-Su is the suppressed strain. H-98-Su is the suppressed strain from H-98 A, which is blocked between cystathionine and homocysteine.

Culture methods. Cultures were maintained on agar slants (Horowitz, 1947). Fries (1948) defined medium was used for experimental work. Magnesium chloride replaced magnesium sulfate in Fries medium for "sulfur-free" studies.

Extraction of mycelia. Mycelia were extracted twice with hot 85% ethanol for determination of free amino acids. A more exhaustive extraction procedure employing a modification of the methods described by Roberts et al. (1955) was used before hydrolysis of the protein. Mycelia were harvested, washed with water, extracted with 50% ethanol overnight, filtered, extracted with 4 ml of 85% ethanol plus ether (2:2) at 40 to 50 C for 30 min, filtered, extracted with 4 ml of 5% trichloroacetic acid at 80 to 90 C for 1 hr, filtered, washed with acidified alcohol, filtered, washed with ether, and filtered.

Hydrolysis of mycelia. Subsequent to the extraction procedure outlined above, the dried mycelia were hydrolyzed in a sealed tube with 4 N HCl for 4 hr at 120 C and filtered; the hydrolysate was evaporated to dryness in a desiccator over concentrated H_2SO_4 and NaOH to remove excess HCl.

Radioactive substrates. L-Cystathionine-U-C^{14} and L-cystathionine-S^{35} were prepared as described previously (Wiebers and Garner, 1960). DL-SMC-S^{35} was obtained from Volk Radiochemical Co., Skokie, Ill. *S*-methyl-L-cysteine labeled with C^{14} in the methyl group was synthesized by reduction of L-cysteine in liquid ammonia with sodium, followed by methylation of the cysteine with $C^{14}H_3I$ (Research Specialties Co., Richmond,

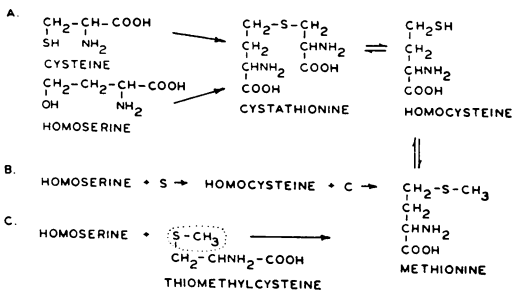

FIG. 1. *Possible biosynthetic sequences in the formation of methionine.*

Calif.) by the method of du Vigneaud, Loring, and Craft (1934). Following the synthesis, crystals of SMC-$C^{14}H_3$ were dissolved in water and band chromatographed on large sheets of Whatman no. 1 paper in butanol-acetic acid–water (2:1:1) with known SMC adjacent. The radioactive band corresponding to known SMC was located by autoradiography, cut out, and eluted with water. The chromatography procedure was repeated, and a sample of the eluted material was co-chromatographed with known SMC on a two-dimensional chromatogram (phenol–water, 80:20; butanol–acetic acid–water, 2:1:1). Autoradiography showed one major radioactive spot which corresponded identically in position and outline to known SMC. A minor radioactive spot was present which corresponded to known SMC-sulfoxide. $Na_2S^{35}O_4$ was obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn. (6.14 mc/ml).

Determination of protein methionine content in Neurospora. Mycelia from a 60-hr wild-type culture were extracted as above and dried, and pooled mycelia were ground to a fine powder. Triplicate determinations for methionine on 200 mg of ground mycelia were made with the McCarthy and Paille (1959) method for determination of methionine in crude proteins.

Determination of protein methionine for specific activity values. In tracer experiments, radioactive methionine was isolated from hydrolyzed mycelia by band chromatography in butanol–acetic acid–water (2:1:1). Autoradiograms revealed the radioactive methionine band which was cut from the chromatogram, eluted with water, converted to the sulfoxide derivative with 30% H_2O_2, and rechromatographed. The radioactive methionine sulfoxide band (free from compounds which commonly contaminate the methionine band, because the R_F of methionine sulfoxide is widely

separate from that of methionine) was cut out and eluted with water. Samples of this material were counted in a windowless gas-flow counter, and analyzed for the amount of methionine sulfoxide by the method of Moore and Stein (1954). For some experiments, when enough material was available, methionine was determined by the method of Csonka and Denton (1946).

Degradation of labeled methionine. A micromodification of the method of Simmonds et al. (1943), in which methyl groups are liberated from methionine and trapped as tetramethylammonium iodide, was used to determine radioactivity in the methyl moiety of protein methionine in *Neurospora* from tracer experiments. Approximately 100 mg of cells, dried and exhaustively extracted as described above, were added to a 100-ml reaction flask with 10 ml of hydriodic acid (55%) and refluxed for 3 hr. At the end of the degradation, the traps were removed from Dry Ice, 5 ml of 5% alcoholic trimethylamine were added, and the mixture was allowed to come to room temperature slowly. Samples were transferred to planchets, dried with a gentle stream of air, and counted in a windowless gas-flow counter. Known methionine-$C^{14}H_3$ yielded 92% by this method. For specific activity values, the amount of methionine from an equivalent quantity of cells was determined by the method of McCarthy and Paille (1959).

Determination of methionine-cystine radioactivity ratios. Hydrolyzed samples of cells were band chromatographed in two solvent systems. Radioactive areas were located on the chromatogram strips by scanning with a Forro chromatogram scanner connected to an Esterline-Angus recorder through a Tracerlab ratemeter. After scanning, duplicate strips were sprayed with either ninhydrin or chloroplatinic acid. Peaks corresponding to known methionine and cystine were cut out of the recording and weighed; the ratio of the weights of the peaks represented the radioactivity ratio of the two compounds.

RESULTS

Incorporation of the sulfur and methyl moieties of SMC into protein methionine. When either SMC-$C^{14}H_3$ or SMC-S^{35} was the sole addition to minimal medium, radioactive cellular methionine was found in hydrolysates of wild-type and methionineless mutants. Proof of the conversion of at least parts of labeled SMC to protein methionine was afforded by isolation from hydrolysates of cells of a radioactive compound which had an R_F identical to that of methionine on two-dimensional chromatograms. Subsequent oxidation to the sulfoxide derivative yielded a compound with an R_F identical to methionine sulfoxide in three solvent systems. Additional confirmation was obtained by the degradation of protein methionine from cells of a cystathionineless mutant, 36104a, which received SMC-$C^{14}H_3$ as the only supplement to minimal medium. Methyl groups liberated from cellular methionine were determined. Table 1 indicates the results of this degradation. It may be observed from the product-precursor specific activity ratio that methionine methyl is labeled from SMC-$C^{14}H_3$. If cystathionine was added along with methyl-labeled SMC in the growth medium, the ratio was decreased by about half. When $MgCl_2$ was substituted for $MgSO_4$ in the minimal medium, the product-precursor specific activity ratios were diminished.

Sulfate inhibition of SMC-S^{35} and SMC-$C^{14}H_3$

TABLE 1. *Degradation of labeled protein methionine from cystathionineless mutant 36104*

Supplement	Medium	Total count/min in protein fraction of cells	Count per min per mg of cells	Count/min recovered in CH_3 after degradation*	Specific activity of methionine (count per min per μmole)	Ratio of specific activity of methionine/SMC
SMC-$C^{14}H_3$ (2 mg)	$MgSO_4$	13,728	264	7,789	7,489	0.32
SMC-$C^{14}H_3$ (2 mg)	$MgCl_2$	10,379	107	4,318	2,225	0.09
SMC-$C^{14}H_3$ (2 mg) (+ 2 mg of "cold" cystathionine)	$MgSO_4$	13,680	210	4,174	3,162	0.14
SMC-$C^{14}H_3$ (2 mg) (+ 2 mg of "cold" cystathionine)	$MgCl_2$	13,804	119	2,389	1,029	0.04

* No measurable radioactivity in the 4-carbon moiety of methionine (CH_2—CH_2—$CHNH_2$—C=O) after degradation.

utilization. Strauss, Tsuda, and Tokuno (1961) reported that sulfate or cysteine inhibits the growth response of certain methionine-requiring mutants to SMC. Furthermore, their studies with suppressed methionineless mutants suggested that these strains probably utilize an alternate pathway for methionine synthesis directly involving SMC. We were able to corroborate the observations of Strauss et al. (1961) by the following tracer experiment which demonstrated that sulfate inhibits the utilization of SMC. A cystathionineless mutant, M-4, its corresponding suppressed mutant, M-4-Su, and wild type were grown with SMC-$C^{14}H_3$ or SMC-S^{35} as the sole supplement in Fries medium (which contains 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter). They were also grown in Fries medium in which an equivalent amount of $MgCl_2$ replaced $MgSO_4$. Cells grown under these conditions were hydrolyzed and chromatographed; the labeled methionine was isolated, and its specific activity was determined. The product-precursor specific activity ratios derived from this (Table 2) show that the ratios were increased when the mutant or wild-type was grown in "low sulfur" medium with SMC-S^{35}. This difference was not apparent with the suppressed mutants in which the ratio was essentially the same in both media. Utilization of the methyl group of SMC by the mutant, the wild type, or the suppressed strains, as expressed by the SMC-$C^{14}H_3$ product-precursor specific activity ratios, did not exhibit the inhibitory effect of sulfate. The ratios were similar regardless of the amount of sulfate in the medium. This would indicate that the sulfur and methyl group were used separately. The presence of added cystathionine in the two media does not appreciably affect the ratios from SMC-$C^{14}H_3$.

TABLE 2. *Effect of sulfate on the use of SMC-S^{35} and SMC-C^{14} by methionineless mutants, suppressed mutants, and wild-type Neurospora*

Strain*	Isotope	Medium	Ratio of specific activity of methionine/SMC
M-4	SMC-S^{35}	$MgSO_4$	0.23
M-4	SMC-S^{35}	$MgCl_2$	1.00
M-4-Su.	SMC-S^{35}	$MgSO_4$	0.50
M-4-Su.	SMC-S^{35}	$MgCl_2$	0.45
H-98-Su.	SMC-S^{35}	$MgSO_4$	0.40
H-98-Su.	SMC-S^{35}	$MgCl_2$	0.15
W.T.	SMC-S^{35}	$MgSO_4$	0.24
W.T.	SMC-S^{35}	$MgCl_2$	0.64
M-4	SMC-$C^{14}H_3$	$MgSO_4$	0.06
M-4	SMC-$C^{14}H_3$	$MgCl_2$	0.03
M-4	SMC-$C^{14}H_3$ (+ 2 mg of cystathionine)	$MgSO_4$	0.06
M-4	SMC-$C^{14}H_3$ (+ 2 mg of cystathionine)	$MgCl_2$	0.07
M-4-Su.	SMC-$C^{14}H_3$	$MgSO_4$	0.08
M-4-Su.	SMC-$C^{14}H_3$	$MgCl_2$	0.08
M-4-Su.	SMC-$C^{14}H_3$ (+ 2 mg of cystathionine)	$MgSO_4$	0.04
M-4-Su.	SMC-$C^{14}H_3$ (+ 2 mg of cystathionine)	$MgCl_2$	0.07
W.T.	SMC-$C^{14}H_3$	$MgSO_4$	0.04
W.T.	SMC-$C^{14}H_3$	$MgCl_2$	0.10

* Su. = suppressed; W.T. = wild-type.

TABLE 3. *Effect of sulfate on protein methionine-cysteine radioactivity ratio*

Strain*	Isotope	Medium	Methionine/cysteine
M-4	SMC-S^{35}	$MgSO_4$	—†
M-4	SMC-S^{35}	$MgCl_2$	2.8
M-4-Su.	SMC-S^{35}	$MgSO_4$	4.7
M-4-Su.	SMC-S^{35}	$MgCl_2$	2.7
H-98-Su.	SMC-S^{35}	$MgSO_4$	7.5
H-98-Su.	SMC-S^{35}	$MgCl_2$	3.0
W.T.	SMC-S^{35}	$MgSO_4$	—†
W.T.	SMC-S^{35}	$MgCl_2$	2.8

* Su. = suppressed; W.T. = wild-type.
† No measurable radioactivity in cysteine.

SMC-S^{35} conversion to cysteine in "low sulfur" medium. Band chromatograms of hydrolysates of cells of wild-type, mutant, and suppressed mutants, which had been grown with SMC-S^{35} as the sole supplement in medium with and without $MgSO_4$, were examined for the distribution of radioactivity in methionine and cysteine. Radioactivity was found in cysteine as well as methionine in some strains (Table 3). There was no measurable radioactivity in cysteine from SMC-S^{35} in hydrolysates of wild-type or the cystathionineless mutant (M-4). However, in "low-sulfur" medium, SMC served as a source of sulfur for cysteine and a methionine-cysteine ratio may be expressed. The suppressed mutants (M-4-Su and H-98-Su), however, utilized SMC

TABLE 4. *$Na_2S^{35}O_4$ isotopic competition with S-methylcysteine and cystathionine*

Supplement	Medium	Ratio of methionine/cysteine
$Na_2S^{35}O_4$	$MgCl_2$	1.6
$Na_2S^{35}O_4$	$MgSO_4$ (0.25 g/liter)	1.8
$Na_2S^{35}O_4$	$MgSO_4$ (0.50 g/liter)	1.7
$Na_2S^{35}O_4$ + 1 μmole of SMC	$MgCl_2$	3.2
$Na_2S^{35}O_4$ + 1 μmole of SMC	$MgSO_4$ (0.25 g/liter)	2.0
$Na_2S^{35}O_4$ + 1 μmole of SMC	$MgSO_4$ (0.50 g/liter)	1.8
$Na_2S^{35}O_4$ + 1 μmole of cystathionine	$MgCl_2$	1.6
$Na_2S^{35}O_4$ + 1 μmole of cystathionine	$MgSO_4$ (0.25 g/liter)	1.9
$Na_2S^{35}O_4$ + 1 μmole of cystathionine	$MgSO_4$ (0.50 g/liter)	1.8

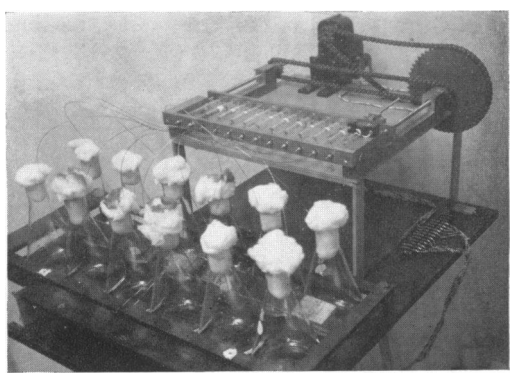

FIG. 2. *Programmed device for continuous addition of sterile substrate to growing Neurospora at a rate consistent with the amount of growth present at a given time.*

for both methionine and cysteine regardless of sulfate content in the medium.

Isotopic competition between sulfate and cystathionine or SMC. Amounts of cystathionine or SMC, which would be expected to contribute all of the sulfur needed for synthesis of 1 μmole of methionine at 65 hr of growth of wild-type, were added to $Na_2S^{35}O_4$ in Fries medium with three levels of sulfate in the medium. Cells were harvested at 65 hr, extracted, and hydrolyzed. Radioactive methionine and cysteine were isolated from the hydrolysates by repeated chromatography, located by autoradiography, and then eluted and counted as described in Materials and Methods. The protein methionine-cysteine radioactivity ratio was determined for each growth condition (Table 4). The only significant increase in the methionine-cysteine ratio was found when SMC was used in "low sulfur" medium. Cystathionine did not exhibit a similar phenomenon. It appears that neither SMC nor cystathionine is an effective competitor with sulfate in the medium for contribution to methionine sulfur, unless sulfate is limiting in which case SMC, but not cystathionine, can contribute to some extent.

Utilization of SMC-$C^{14}H_3$, SMC-S^{35}, cystathionine-U-C^{14}, and cystathionine-S^{35} by wild-type under "steady state" conditions. This experiment was designed to avoid adaptive breakdown of substrates which may occur when excessive amounts of substrate are present in the medium at the time flasks are inoculated. It was thought that if the radioactive substrates could be added continuously at a rate consistent with the amount of growth present at a given time, a more accurate estimation of the actual utilization of the substrates would be possible. Thus, a device (Fig. 2) which allows the continuous sterile addition of substrate to flasks of growing *Neurospora* was used (details of the construction and use of this device will be published). Wild-type was selected, because amounts of SMC and cystathionine could be offered at a low level that would be sufficient to supply all the precursors to cellular methionine, if they could compete with endogenous sources. The amount of substrate to be added was controlled by a programmed tape (Fig. 3), the program of which was predetermined based on the typical growth curve of wild-type. In this manner, SMC-$C^{14}H_3$, SMC-S^{35}, cystathionine-U-C^{14}, and cystathionine-S^{35} were offered to wild type. The experiment was designed so that the organism was offered one of the labeled compounds alone and, in another flask, the labeled compound plus an equivalent amount of the other compound without label. The cells were harvested at 65 hr, extracted, and hydrolyzed. Radioactive methionine from cellular protein was isolated, its specific activity was determined, and the product-precursor specific activity ratios were calculated. It may be observed from the ratios (Table 5) that the SMC sulfur and methyl moieties were used equally well, suggesting that in this situa-

tion the intact thiomethyl group may be converted to methionine. The ratios for cystathionine sulfur were approximately ten times those for SMC, indicating a preference for cystathionine under these conditions.

DISCUSSION

In the study of metabolic pathways by use of isotopic tracer techniques and mutant organisms, the purpose of determining product-precursor specific activity ratios is to quantitatively evaluate the significance of the postulated intermediates in the metabolic route. An analysis of the data in this investigation leads us to believe that neither SMC nor cystathionine are intermediates of major importance in methionine biosynthesis in *Neurospora* growing under the specified conditions. A product-precursor specific activity ratio of 1.0 was achieved only under altered growth conditions—i.e., when "sulfur-free" medium was used and SMC was able to contribute significantly. When conditions were set up so that radioactive SMC or cystathionine were added in small increments at a rate consistent with the growth rate of wild-type *Neurospora*, the product-precursor specific activity ratios were of such low magnitude that it appears doubtful that either cystathionine or SMC are obligate precursors for methionine. These results emphasize the importance of studying precursor relationships under "steady state" conditions wherein (i) endogenous pools are limited as to ability to regulate the use of the proposed intermediate, and (ii) the formation of adaptive enzymes for degradation of the intermediate is curtailed.

The fact that either SMC or cystathionine can allow "cystathionineless" mutants to grow, and the fact that both compounds, when radioactive, can give rise to radioactive methionine suggest that at least parts of these amino acids can be converted to methionine. It is conceivable that when either SMC or cystathionine is cleaved, its sulfur-containing product may be in a valence state that is required for attachment to a carbon chain; consequently, they may enter into the biosynthesis of methionine indirectly. Flavin (1962) reported that wild-type *Neurospora* contains a cleavage enzyme which catalyzes a heterogeneous decomposition of cystathionine, yielding homocysteine, α-ketobutyrate, NH_3, and elemental $S^=$. An analogous decomposition of SMC can be envisaged if it is first demethylated to cysteine, and subsequently degraded by cysteine desulfhydrase. Cysteine desulfhydrase and cystathionase are considered identical in the rat liver system (Mondovi, Scioscia-Santoro, and Cavallini, 1963). Nomura, Nishizuka, and Hayaishi (1963) isolated *S*-alkylcysteinase from SMC-adapted cells of *Pseudomonas cruciviae*. This enzyme catalyzes the stoichiometric conversion of SMC to methyl mercaptan, pyruvic acid, and NH_3. Both methionine and cysteine are inert as substrates, indicating that the enzyme is different from cysteine desulfhydrase and methioninase.

Our data also indicate that SMC and cystathionine are not very effective competitors with sulfate for methionine biosynthesis. Consideration

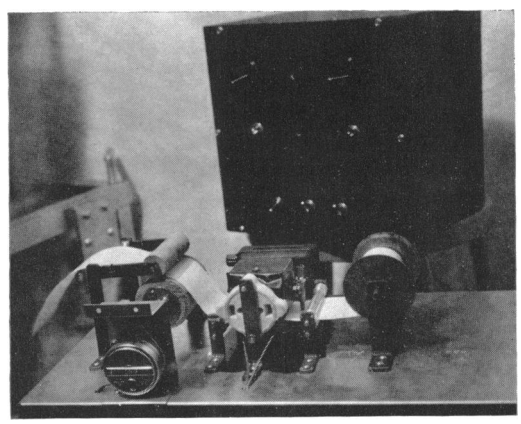

FIG. 3. *Programmed tape regulates the amount of substrate to be added to flasks. Holes punched in tape pass over a photoelectric cell, which triggers the syringe-driver.*

TABLE 5. *Comparison of cystathionine and S-methylcysteine as precursors to methionine under steady-state conditions*

Substrate*	Ratio of specific activity of methionine/precursor
SMC-$C^{14}H_3$	0.009
SMC-$C^{14}H_3$ + cystathionine	0.008
SMC-S^{35}	0.013
SMC-S^{35} + cystathionine	0.011
Cystathionine-U-C^{14}	0.14
Cystathionine-U-C^{14} + SMC	0.08
Cystathionine-S^{35}	0.19
Cystathionine-S^{35} + SMC	0.13

* All amounts were 3 μmoles.

of the probability of the direct formation of homocysteine from a four-carbon donor and sulfate (Fig. 1B) seems to be in order. We recently (Wiebers and Garner, 1963) demonstrated the isolation of an enzyme from *N. crassa* which is capable of forming homocysteine from homoserine and H_2S (and cysteine from serine and H_2S). This finding suggests that the homocysteine that is utilized for methionine formation arises in this anabolic fashion, and is distinct from the homocysteine derived from the degradative cleavage of cystathionine or methionine. Subsequent methylation of the homocysteine does not appear to present a problem, as it has been shown that resting cells and cell-free extracts of *N. crassa* can synthesize methionine from homocysteine and formate or other C_1 donors (Dalal, Rege, and Sreenivasan, 1961). We are currently investigating the validity of this hypothesis of methionine biosynthesis. We propose that SMC and cystathionine contribute to sulfur metabolism in general as alternative sources of reduced sulfur, or function as regulatory compounds.

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