METHIONINE BIOSYNTHESIS FROM S-METHYLCYSTEINE BY THIOMETHYL TRANSFER IN *NEUROSPORA*[1]

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It has been demonstrated (Ragland and Liverman, 1956; Tokuno, *et al.*, 1962; Wiebers and Garner, 1964) that several methionineless mutants of *Neurospora* can utilize S-methylcysteine as a source of sulfur. It has been suggested (Wiebers and Garner, 1960; Tokuno, *et al.*, 1962) that S-methylcysteine is an intermediate in methionine biosynthesis in this organism. In order to investigate further the role of methylcysteine in methionine formation, eight methionineless mutants of *Neurospora* representing all of the known loci were tested for growth on methylcysteine as a source of sulfur (data to be published elsewhere). All but one mutant could utilize methylcysteine or methylmercaptan as a sole source of sulfur, though not as well as methionine. This finding indicated that neither methylcysteine nor methymercaptan is an intermediate in the "normal" pathway of methionine formation from sulfate, but that either compound can furnish the sulfur for methionine biosynthesis. This paper presents evidence that *Neurospora* can catalyze two reactions for formation of methionine from methylcysteine by a thiomethyl transfer.

$$\text{S-methylcysteine} + H_2O \longrightarrow \text{methylmercaptan} + \text{pyruvate} + NH_3 \quad (1)$$[2]

$$\text{methylmercaptan} + \text{O-acetylhomoserine} \longrightarrow \text{methionine} + \text{acetate} \quad (2)$$[2]

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[2]Methylmercaptan and methionine have been identified. The other products have not been identified, but are likely on the basis of analogous reactions.

These reactions provide an alternate pathway for the synthesis of methionine from methylcysteine without cysteine, cystathionine and homocysteine as intermediates (Fig. 1). Such a pathway would account for the ability of the methionineless mutants of Neurospora to utilize methylcysteine as the sole source of sulfur.

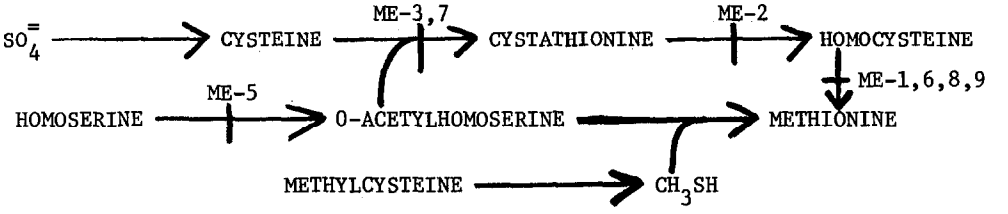

Fig. 1. Pathways of methionine formation from cysteine and methylcysteine

EXPERIMENTAL: Wild type Neurospora (FGSC #352)[3] was grown under forced aeration for one week in a 9-liter bottle containing 7 liters of liquid medium. S-methyl-L-cysteine at 6 X 10^{-4} M was used as the sole source of sulfur and $MgCl_2$ was substituted for $MgSO_4$ in the basal medium (Ryan, et al., 1943); the yield of mycelia was approximately 100 gms fresh weight.

The mycelia were ground at 0° C in a mortar with sand using an equal amount of 0.1 M potassium phosphate buffer, pH 8.5, containing 10^{-3} M mercaptoethanol and 10^{-5} M pyridoxal phosphate. After centrifugation, the residue was re-extracted in the same manner, centrifuged and the two supernatant solutions combined (crude extract). The crude extract was adjusted to pH 7, and the protein precipitating between 45 and 65% ammonium sulfate saturation was collected. This fraction was dialyzed 16 hours at 0° C against 0.01 M potassium phosphate buffer, pH 8.5, containing 10^{-3} M mercaptoethanol and 10^{-6} M pyridoxal phosphate.

The assay for methylcysteine lyase (equation 1) was carried out under an anaerobic atmosphere in a side arm test tube connected, through a cotton plug, in series with two similar tubes to trap the volatile reaction product, CH_3SH. The first trap contained 5 ml of 1 N NaOH in absolute ethanol at -78° C. The second trap contained 10^{-3} M p-chloromercuribenzoate. Gaseous nitrogen was passed slowly through alkaline pyrogallol before bubbling through the incubation mixture and the traps. After incubation, the reaction was stopped by adding 4 drops of 6 M H_3PO_4. Several drops of butanol were added to suppress foaming, and bubbling was continued for 1/2 hour. An aliquot of the trapping solutions was removed for counting by liquid scintillation. Recovery of standard labeled CH_3SH in this system was over 99%.

The volatile reaction product was identified as methylmercaptan by conversion to 1-thiomethyl, 2,4-dinitrobenzene. Dinitrochlorobenzene (0.1 ml of 0.1 M) was added to the alcohol solution in the first trap and the tube immediately closed and allowed to stand overnight (Bost, et al., 1932). A reference standard was prepared from pure $C^{14}H_3SH$ in the same way. The NaOH was removed by passing the alcoholic solution through a column (1 X 7 cm) of

[3] Culture obtained from Fungal Genetics Stock Center at Dartmouth College.

Dowex 50-X8 (200-400 mesh) in the hydrogen form (in 50% ethanol) and washing with 100 ml of 50% ethanol. The washes were dried in vacuo and dissolved in 50% ethanol. The products derived from the incubation and the reference material were chromatographed in one direction in two different solvent systems: methanol-pyridine-water (85:4:15) and butanol-acetic acid-water (12:3:5). The radioactivity was located by means of a strip scanner.

For reaction 2, the crude extract was dialyzed as above, except the pH was 7.5. $C^{14}H_3SH$ was added in 0.2 ml of ethanol at 0° C to the reaction mixture in a 15 ml centrifuge tube, and the tube was immediately closed. After incubation, the stopper was removed and the mixture was heated in boiling water for 5 minutes to remove the bulk of the unreacted CH_3SH and to denature the protein which was removed by centrifugation. The supernatant solution was evaporated to dryness in vacuo twice to complete the removal of the CH_3SH. The residue was dissolved in H_2O and the amino acids were purified by absorption on a sulfonic acid resin in the hydrogen form and elution with NH_4OH.

The labeled amino acid from the incubation was identified as methionine by co-chromatographing with methionine and methionine sulfoxide in one direction in two different solvent systems: Phenol-water (7:3) and methanol-pyridine-water. In phenol, a single radioactive peak was detected which coincided with the position of methionine and methionine sulfoxide which move together in this solvent. Two peaks of radioactivity were detected in methanol-pyridine-water; the major radioactive peak coincided with methionine and a smaller peak with methionine sulfoxide. The material associated with the methionine peak was eluted from the paper, oxidized with H_2O_2 and re-chromatographed in methanol-pyridine-water where it migrated with methionine sulfoxide. The material associated with the smaller peak, which co-chromatographed with methionine sulfoxide, was reduced with mercaptoethanol (Doney and Thompson, 1966) and chromatographed in the same solvent system where it migrated with methionine.

To determine the actual counts in methionine, labeled material from the incubation was oxidized with H_2O_2 and chromatographed with added methionine sulfoxide in two directions, using butanol-acetic acid-water in one direction and phenol-water in the other. The methionine sulfoxide spot was located by fluorescence (Morris and Thompson, 1965), eluted from the paper, and counted by liquid scintillation.

RESULTS: Preliminary experiments with a crude extract consistently demonstrated a weak methylcysteine lyase activity. Fractionation of the crude extract with ammonium sulfate showed that most of the enzymatic activity occurred in the 45-65% saturation fraction. Table I demonstrates methylcysteine lyase activity using either methyl labeled or sulfur labeled methylcysteine. The C^{14} methyl labeled methylcysteine had a slightly higher specific activity than the S^{35}-methylcysteine, and this fact probably accounts for some of the difference between the C^{14} and S^{35} radioactivity trapped. The substantially higher value for the C^{14} control (boiled) as compared to the S^{35} control has been verified in other experiments and is not due to CH_3SH.

Table I. Decomposition of C^{14}-(methyl), and S^{35}-methylcysteine by an extract of Neurospora.

TREATMENT	VOLATILE DPM TRAPPED	
	C^{14}	S^{35}
EXTRACT BOILED	12,300	530
EXTRACT UNBOILED	89,300	53,200
NO EXTRACT	250	-

Reaction mixture consisted of 2.0 ml of extract (45-65% ammonium sulfate fraction), 10^{-5}M pyridoxal phosphate and 10^{6} dpm of S-methyl-L-cysteine in a total volume of 2.2 ml. Incubation was for 2 hours at 30°C.

Proof that the volatile reaction product was methylmercaptan was obtained by reacting it with dinitrochlorobenzene and chromatographing the product in two solvents with known material (Figure 2).

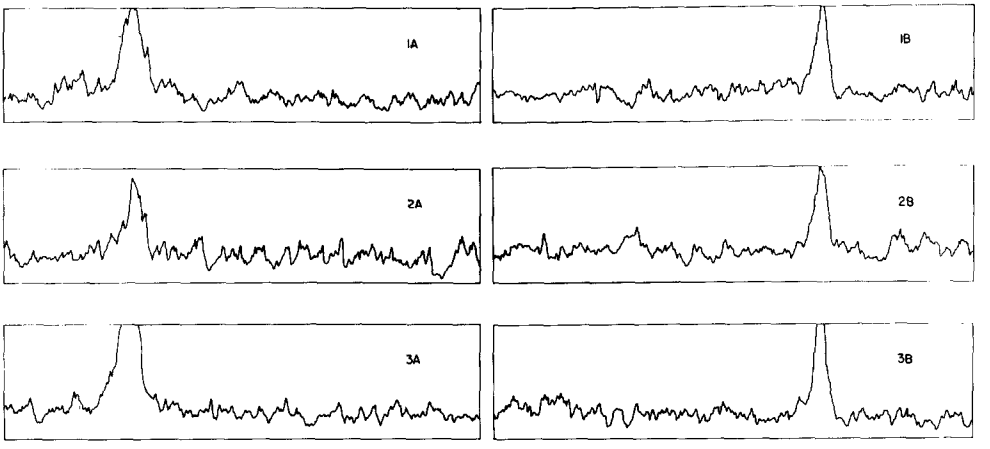

Fig. 2. Identification of methylmercaptan from labeled methylcysteine by radioactivity on chromatograms. The origin is at the right end of each box. Solvent for A series was methanol:pyridine:water. Solvent for B series was butanol:acetic acid:water. 1, 2 and 3 refer to the reaction product of volatile S^{35}-incubation product, volatile C^{14}-incubation product or $C^{14}H_3SH$ with dinitrochlorobenzene, respectively.

Pyridoxal phosphate was included in the reaction mixture because it would be expected to be a cofactor; however, preliminary experiments with dialyzed extracts failed to demonstrate any pyridoxal phosphate requirement.

Table II shows that an extract from Neurospora was able to form methio-

nine by reaction 2. Although O-acetylhomoserine was very active, DL-homoserine was inert. The labeled product of the reaction was proven to be methionine as described in the experimental section.

Table II. Formation of methionine from CH_3SH by an extract of Neurospora.

TREATMENT	DPM IN METHIONINE SULFOXIDE	
	BOILED	UNBOILED
CH_3SH	180	1,650
CH_3SH + HOMOSERINE	700	2,760
CH_3SH + O-ACETYLHOMOSERINE	360	226,300

Reaction mixture consisted of 5.0 ml of extract, 10^6 dpm of $C^{14}H_3SH$ and 25 µmoles of DL-homoserine or O-acetyl-DL-homoserine, as indicated, in a total volume of 5.3 ml. Incubation was for 2 hours at 30°C.

DISCUSSION: The net result of reactions 1 and 2, demonstrated above, is the synthesis of methionine from methylcysteine in Neurospora without going through cysteine, cystathionine or homocysteine (Figure 1). These reactions are consistent with the observation that methylcysteine or CH_3SH as the sole source of sulfur support the growth of methionineless mutants blocked at 7 of the 8 known loci involved between cysteine and methionine. The mutant (me-5), unable to utilize these sulfur compounds, is blocked in the formation of O-acetylhomoserine (Nagai and Flavin, 1966), which is in agreement with the participation of this compound as a substrate in reaction 2.

The leakiness of many methionineless mutants (Tokuno, et al., 1962; data to be published elsewhere) could be the result of the conversion of methylcysteine to methionine, but this possibility must await the demonstration of the pathway of methylcysteine biosynthesis. The biosynthesis of methionine from methylcysteine bypassed all but one of the known genetic blocks between cysteine and methionine. This fact, plus the poorer growth on methylcysteine than on methionine, leads to the conclusion that the methylcysteine pathway is an alternate pathway of secondary importance when sulfate is the sulfur source.

The decomposition of methylcysteine to CH_3SH by γ-cystathionase from Neurospora has been reported recently (Flavin and Slaughter, 1967a), although definite identification of the product was not indicated. Since γ-cystathionase is found in wild type Neurospora and since it has no obvious function in "normal" methionine biosynthesis, it may well be present in all methionineless mutants. The direct synthesis of methionine from CH_3SH and O-succinylhomoserine by extracts of Salmonella (Flavin and Slaughter, 1967b) has also been reported. This latter reaction would appear to be similar to reaction 2 reported in this communication, except that in Neurospora, O-acetylhomoserine provides the four-carbon skeleton.

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