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Sulfur-containing Metabolites Secreated by an Ethionine-Resistant Mutant of *Neurospora**

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ABSTRACT: Several sulfur compounds are secreted by a mutant of *Neurospora crassa* which has lost control over methionine biosynthesis. The present paper describes the identification of these compounds as *S*-methyl-α-keto-γ-mercaptobutyric acid, *S*-methyl-α-hydroxy-γ-mercaptobutyric acid, the corresponding sulfone, *S*-methyl-β-mercaptopropionic acid, and the corresponding sulfone.

During the course of studies of the biochemical lesions involved in temperature-sensitive, irreparable mutations of *Neurospora crassa*, Metzenberg *et al.* (1964) isolated a temperature-sensitive, ethionine-resistant mutant of *Neurospora*, *r-eth*-1, which had lost control over methionine biosynthesis. In order to measure total production of methionine by *r-eth*-1, it was necessary to measure any methionine metabolites secreted into the medium as well as methionine incorporated into protein. Minimal medium in which *r-eth*-1 had grown was capable of supporting growth of a methionine-requiring strain of *Escherichia coli*. The latter mutant, which was isolated by the method of Davis (1949), was able to grow on methionine, but not on cystathionine, homocysteine, or other intermediates in the methionine biosynthetic pathway. Methionine per se, however, was shown to be absent from *r-eth*-1 culture filtrates. This paper describes the identification of the compounds derived from methionine which appeared in culture filtrates of *r-eth*-1.

Materials and Methods

Materials. The calcium salt of DL-*S*-methyl-α-hydroxy-γ-mercaptobutyric acid[1] was purchased from Mann Research Laboratories. L-[^{3}H]Methionine, specific activity 160 mc/mole, was purchased from Nuclear Chicago Corp. Snake venom (*Crotalus adamanteus*) was purchased from the Ross Allen Reptile Institute. Catalase was purchased from Sigma Chemical Co.

Fractionation of Radioactive Materials. The sample was adjusted to *p*H 8 and applied to a 1- × 20-cm column of Dowex 1 (formate). Distilled water (50 ml) was run through the column. Substances were eluted from the column by a closed-system gradient of formic acid consisting of a mixer containing 50 ml of distilled water and a reservoir containing 50 ml of 1 M formic acid. Fractions (3 ml) were collected. When the contents of the reservoir had passed into the mixer, the reservoir was replenished with 50 ml of 4 M formic acid.

Bioassay. The assay was carried out in 19- × 150-mm cuvets. Aliquots of column fractions, considered to be reasonably aseptic, were evaporated *in vacuo* in the cuvets and used without sterilization. Because the assay is quite rapid, it is necessary to avoid only gross contamination. Solutions of MKMB, MHMB, and *N*-acetylmethionine were filter sterilized. The samples were reconstituted in the salts medium of Davis and Mingioli (1950) supplemented with sodium succinate (0.005 M) to give a volume of 5.0 ml. Cells of the *E. coli* methionineless auxotroph were grown in a similar medium supplemented with methionine, harvested during the exponential growth phase, and washed with 0.5 M glucose. The cells were suspended in sufficient 0.5 M glucose so that an inoculum of 0.2 ml in the assay tubes gave an initial absorbancy of 0.010 at 650 mμ when read against distilled water. The assay tubes were read at hourly intervals until a stable terminal reading was obtained (usually 5 hours).

Synthesis of MKMB. MKMB was prepared by incubating L-methionine with snake venom L-amino acid oxidase and catalase as described by Meister (1952).

* From the Department of Physiological Chemistry, University of Wisconsin Medical School, Madison. *Received January 29, 1965.* This work was supported in part by a grant from the U.S. Public Health Service (GM-08995-03).

[1] Abbreviations and designations used in this work: *S*-methyl-α-keto-γ-mercaptobutyric acid, MKMB; *S*-methyl-α-hydroxy-γ-mercaptobutyric acid, MHMB; *S*-methyl-β-mercaptopropionic acid, MMP.

Uniformly tritiated MKMB was prepared from the radioactive amino acid in a similar manner. In this case the solution was deproteinized by vigorous agitation with 0.5 ml of chloroform followed by centrifugation at 700 × *g* for 10 minutes. The aqueous layer was acidified and washed through a 1- × 5-cm column of Dowex 50 (H^+) with distilled water to remove ammonium ions and any unreacted methionine. The radioactive eluate was neutralized and stored in the frozen state.

Synthesis of S-Methyl-β-mercaptopropionic Acid and of the Sulfone Derivative. Liquid ammonia was distilled into a filter flask surrounded by dry ice and protected from atmospheric moisture by a soda lime trap until 63 ml was collected. A 20% solution (v/v) of β-mercaptopropionic acid (10 ml) in dry 1,2-dimethoxyethane was slowly added to the liquid ammonia with mechanical stirring. Small chunks of sodium were added until the blue color persisted. Methyl iodide was added dropwise until the blue color disappeared. Excess methyl iodide (1.4 ml) was added and stirring was continued for 15 minutes. The ammonia was evaporated at room temperature, and the products were dissolved in 20 ml of distilled water and washed with three 10-ml portions of diethyl ether. The water layer was cooled to 4°, acidified, and extracted with three 10-ml portions of ether. The ether layers were pooled, dried with 0.5 g of sodium sulfate, and filtered. Excess anhydrous ammonia gas was bubbled through the filtrate. The resulting white precipitate was dried *in vacuo*. A portion (200 mg) of the product was converted to the sulfone by treatment with 2 ml of performic acid for 1 hour at room temperature and dried *in vacuo*. The sirupy residue was acidified and purified by elution from a 1- × 10-cm column of Dowex 50 (H^+) with water. The product was dried *in vacuo* and dissolved in 10 ml of diethyl ether. Excess anhydrous ammonia gas was bubbled through the solution. The ether was evaporated *in vacuo*, and a carefully weighed sample of the dry residue was analyzed for nitrogen by the method of Johnson (1941). The sample was found to contain 7.77% N, compared to the calculated value of 8.29%.

Experimental and Results

Conidia from *r-eth*-1 and wild-type *Neurospora* were incubated in Fries' minimal salts (Beadle and Tatum, 1945) with substitution of $MgCl_2$ for $MgSO_4$ on a mole-for-mole basis, and addition of sucrose to give 4.3×10^{-2} M, and $Mg^{35}SO_4$ (2×10^{-4} M, 80 μc/μmole) for 4 days without shaking at 24°. At this time aliquots of the culture filtrates were subjected to paper chromatography and autoradiography. The R_F values of ^{35}S compounds are given in Table I. *R-ethl*-1 culture filtrates contained five radioactive compounds which were absent from wild-type culture filtrate. Several compounds with lower R_F values appeared in both wild-type and *r-eth*-1 filtrates.

Larger quantities of *r-eth*-1 filtrate for the identification of these compounds were obtained by incubating *r-eth*-1 conidia in 40 ml of the above-mentioned medium for 48 hours at 35° without shaking. At this temperature growth of the mutant is slow and accumulation of metabolites is favored. The culture filtrate was stored in the frozen state.

TABLE I: Paper Chromatography of Culture Filtrates.[a]

Strain	R_F Values	Compound
R-eth-1	0.89	MMP
	0.85	MHMB
	0.71	MKMB
	0.64	MMP sulfone
	0.49	MHMB sulfone
	0.30	Unidentified
	0.26	Unidentified
Wild type	0.29	Unidentified
	0.21	Unidentified

[a] Aliquots of culture filtrates containing approximately 20,000 cpm were chromatographed on Whatman No. 3MM filter paper by the ascending method with 1-butanol–acetic acid–water (12:3:5, v/v). The chromatogram was dried and exposed to X-ray film for 3 days.

A preliminary fractionation of the filtrate by gradient elution from Dowex 1 (formate) was carried out as described under Materials and Methods. The elution pattern of ^{35}S is shown in Figure 1. The fractions comprising a "peak" were pooled, and aliquots from each pool were subjected to paper chromatography. The R_F values of compounds appearing in each peak are shown in Table II. The presence of more than one radioactive compound in many fractions appears to be due to degradation during and/or after fractionation.

In order to compare R_F values of known sulfur compounds with those appearing in *r-eth*-1 culture filtrate, various compounds were subjected to paper chromatography in the previously described butanol–acetic acid–water system and detected by the platinic iodide dip described by Toennies and Kolb (1951). L-Methionine, *N*-acetyl-DL-methionine, MKMB, MHMB, DL-allocystathionine, DL-homocysteine, and *S*-methylcysteine had R_F values of 0.52, 0.85, 0.71, 0.84 (0.04 and 0.11), 0.19, and 0.38, respectively.

Since MKMB has the same R_F as a radioactive compound from culture filtrates which disappears during column chromatography, carrier isolation of the putative MKMB as the 2,4-dinitrophenylhydrazone from untreated culture filtrate was undertaken. The sodium salt (0.5 mmole) of MKMB was dissolved in 1 ml of radioactive *r-eth*-1 medium. An excess of 2,4-dinitrophenylhydrazine in 2 N HCl was added. The precipitate was dissolved in 1 ml of absolute ethanol. Aliquots were counted in a Tracerlab gas-flow counter and analyzed for 2,4-dinitrophenylhydrazone by the method of Umbarger and Magasanik (1952). These analyses were repeated after each of four recrystallizations. The

TABLE II: Paper Chromatography of Peaks from Fractionation of *R-eth*-1 Culture Filtrate.[a]

Material	R_F Values
Peak A	0.24
Peak A, performic acid treated[b]	0.13, 0.36
Peak B	0.69
Peak B, performic acid treated	0.68
Peak C	0.50, 0.64
Peak C, performic acid treated	0.64
Peak D	0.27, 0.50, 0.67
Peak D, performic acid treated	0.36, 0.49, 0.66
Peak E	0.49, 0.64 (faint), 0.89
Peak E, performic acid treated	0.52, 0.64

[a] Samples containing approximately 10,000 cpm were applied to Whatman No. 3MM filter paper. The chromatogram was developed in 1-butanol–acetic acid–water (12:3:5), dried, and exposed to X-ray film for 3 days. [b] Performic acid oxidation was carried out by treating a dry sample with an excess of performic acid, prepared as described by Moore (1963), for 1 hour at room temperature.

specific activity of the derivative was the same after each recrystallization.

An aliquot of the radioactive derivative was treated with performic acid as described, dried *in vacuo*, and redissolved in absolute ethanol. Aliquots of the treated and untreated derivatives were applied to two sheets of Whatman No. 3MM filter paper. One chromatogram was run in the previously described 1-butanol–acetic acid–water solvent, and the other in a descending solvent composed of ethanol-water (83:17, v/v). The 2,4-dinitrophenylhydrazones were clearly visible as yellow spots whose shape and position corresponded exactly to the radioactive areas, as determined by autoradiography.

Final proof of the presence of MKMB in *r-eth*-1 culture filtrate was obtained by reduction of the radioactive 2,4-dinitrophenylhydrazone to radioactive methionine as follows: The remaining dinitrophenylhydrazone was dissolved in 1 ml absolute ethanol. Finely shaved tin was added, and hydrogen chloride gas was bubbled slowly through the mixture until the tin had disappeared and the solution was colorless. Water (10 ml) was added, and hydrogen sulfide gas was bubbled through the mixture for 10 minutes. The precipitate was removed by centrifugation, and the clear supernatant was dried *in vacuo*. The product was combined with 6.3 mg of L-methionine and treated with excess performic acid for 5 hours at 0°. The solvent was evaporated *in vacuo*. The residue was dissolved in 5 ml of 0.1 N acetic acid and applied to a 1- × 5-cm column of Dowex 50 (H^+) resin. The column was overlaid with 5 ml of distilled water. Methionine sulfone was eluted from the column by a closed-system gradient of hydrochloric acid consisting of a mixer containing 50 ml of

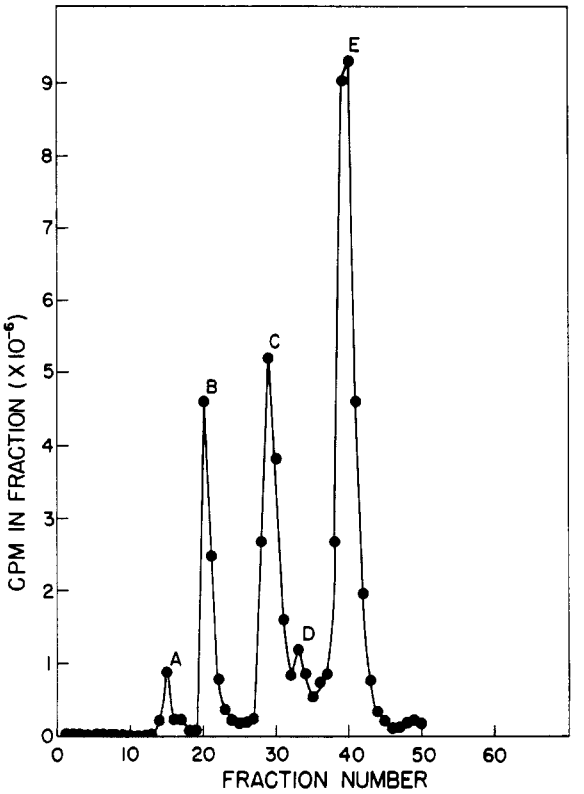

FIGURE 1: Fractionation of ^{35}S materials present in *r-eth-1* culture filtrate. An aliquot of culture filtrate containing approximately 100 μc of ^{35}S was subjected to column chromatography on Dowex 1 (formate) as described under Materials and Methods. Aliquots (5 μl) of each fraction were counted in a Tracerlab gas-flow counter.

TABLE III: Bioassay of Methionine Analogs and of Fractionated Culture Filtrate.

Compound	Sulfur in Sample (μmoles)	Methionine Determined in Bioassay (μmoles)
MKMB	0.20	0.158
MHMB	0.20	0.070
N-Acetyl-DL-methionine	0.20	0.079
Peaks from Dowex 1 (formate) column		
A	0.013	0.005
B	0.019	0.005
C	0.024	0.005
D	0.017	0.005
E	0.019	0.015
E (R_F 0.83)	0.06	0.018
E (R_F 0.49)	0.04	0.005
Untreated culture filtrate	0.05	0.020

TABLE IV: Paper Chromatography of *S*-Methyl-α-hydroxy-γ-mercaptobutyric Acid.

Compound	R_F Value in Solvent[a]				
	1[b]	2[b]	3[c]	4[c]	5[c]
MHMB, authentic	0.72	0.63	0.82, 0.55	0.21	0.52, 0.17
MHMB, putative	0.72, 0.54	0.63, 0.39	0.82, 0.55	0.21	0.52, 0.17
MHMB, authentic, performic acid treated			0.64, 0.55	0.24	0.67, 0.17
MHMB, putative, performic acid treated			0.64, 0.55	0.24	0.67, 0.17

[a] Ascending chromatography on acetic acid–washed Whatman No. 3MM paper was carried out in the following solvents: (1) pyridine–*t*-butyl alcohol–water (1:1:1); (2) 1-propanol–1 N NH_4OH (3:1); (3) 1-butanol–acetic acid–water (12:3:5); (4) *n*-amyl alcohol–5 M formic acid (1:1); (5) ethyl acetate–acetic acid–water (3:1:1). [b] Sulfur containing compounds were detected by platinic iodide. [c] Acidic compounds were detected by methyl red as described in the text.

water and a reservoir containing 50 ml of 6 N HCl. Fractions (3 ml) were collected. Aliquots of the fractions were analyzed for methionine by the ninhydrin determination of Moore and Stein (1948). The remainder of each fraction was dried *in vacuo*, dissolved in a mixture of Bray's scintillator (Bray, 1960) and water (10:1, v/v), and counted in a Packard scintillation counter. The radioactive and ninhydrin peaks were coincident.

Aliquots of untreated culture filtrates and of eluates from the column described in Figure 1 containing approximately 0.02 μmole of sulfur as judged by their radioactivity were bioassayed as described. Authentic samples of *N*-acetyl-DL-methionine, MKMB, and DL-MHMB were also assayed for comparison with L-methionine. The results are shown in Table III. It is clear that *N*-acetyl-DL-methionine, DL-MHMB, and MKMB were able to support the growth of the *E. coli* auxotroph. The low values obtained in the bioassay of DL-MHMB and *N*-acetyl-DL-methionine probably are owing to the limited ability of the bacteria to concentrate these compounds from the medium and to the racemic nature of these compounds. The less-than-expected growth obtained with MKMB, which was about 75% of that obtained from an equivalent amount of L-methionine, probably reflects the instability of this keto acid. Peak E contained the major growth-promoting activity of the fractionated medium. The following experiment was conducted in order to ascertain which of the radioactive compounds in this fraction was nutritionally active. An aliquot of this fraction was concentrated *in vacuo*, streaked onto Whatman No. 3MM paper, and subjected to chromatography and autoradiography as described. The radioactive areas were cut out and the compounds were eluted with water. Aliquots of each eluate were bioassayed as previously described. Results of the assay are shown in Table III. Only the faster-moving compound had growth-promoting activity.

The radioactive and nutritionally active compound with an R_F of 0.83 in the butanol–acetic acid–water system was identified as MHMB on the basis of the following evidence: Mixed samples containing 0.2 μmole of authentic DL-MHMB and 8000 cpm of radioactive putative MHMB eluted from a paper chromatogram were applied to each of two sheets of Whatman No. 3MM filter paper. The chromatograms were developed in two different solvent systems, dried, dipped in the platinic iodide reagent, and subjected to autoradiography as described. Table IV shows that the R_F values of the radioactive substance coincides with those of authentic MHMB in both cases. Samples containing 0.6 μmole of authentic MHMB and 8000 cpm of putative MHMB were applied before and after performic acid treatment to three sheets of Whatman No. 3MM filter paper which had been washed previously with 0.2 N acetic acid. Chromatograms were run in three different ascending solvents, each containing 0.008% (w/v) methyl red, recommended by Lawson and Hartley (1958). The chromatograms were dried, briefly exposed to ammonia vapor, and returned to the air. The cherry-red spots which soon appeared against a yellowish-pink background were marked with pencil, and the chromatograms were subjected to autoradiography. The R_F values for acidic and radioactive compounds are reported in Table IV. It is clear that performic acid oxidation of the radioactive material yields two products which are chromatographically identical with those from MHMB.

The substance from culture filtrates with an R_F of 0.49 in the butanol–acetic acid–water solvent is probably the sulfone of MHMB. It is acidic to methyl red, but is not detected by the platinic iodide reagent, which is specific for sulfur compounds in the −2 valence state. This compound is formed spontaneously and irreversibly from MHMB, and is slowly converted to putative MMP sulfone after treatment with performic acid.

The disappearance of some compounds and the increase in others following performic acid treatment suggested that some of the compounds might be formed by

the breakdown of others. In order to investigate this possibility an aliquot of untreated [^{35}S]*r-eth*-1 culture filtrate was subjected to paper chromatography in butanol–acetic acid–water and autoradiography as described. Radioactive areas were cut out and the substances were eluted from them with water. Aliquots of the eluate were applied to Whatman No. 3MM paper with and without performic acid treatment, and the chromatogram was rechromatographed in the same solvent and subjected to autoradiography.

The results, given in Table V, show that MKMB, under the conditions of chromatography and elution, is partially converted to a mixture of putative MMP, putative MMP sulfone, and two unidentified substances of low R_F. Performic acid oxidation quantitatively converts both MKMB and putative MMP to putative MMP sulfone and the two unidentified substances. MHMB is partially converted to the sulfone during chromatography and elution. Performic acid treatment converts MHMB to a mixture of MHMB sulfone and putative MMP sulfone.

TABLE V: Rechromatography of Substances Eluted from Paper Chromatograms.

Original R_F	R_F After Rechromatography	R_F after Performic Acid Oxidation and Rechromatography
0.90	0.90, 0.63	0.63
0.83	0.83, 0.49	0.63, 0.49
0.71	0.91, 0.71, 0.62 0.33, 0.27, 0.21	0.64, 0.28, 0.22
0.65	0.65, 0.38, 0.27	0.65, 0.29, 0.21
0.49	0.49	0.65, 0.49

In order to confirm that the several compounds have an identical breakdown product, authentic [^{3}H]MKMB was prepared as described under Materials and Methods, mixed with ^{35}S-labeled compounds eluted from paper chromatograms of *r-eth*-1 culture filtrate, and treated with performic acid. The products in each case were eluted from a Dowex 1 (formate) column as described under Materials and Methods, and the number of ^{3}H and ^{35}S counts in each fraction was obtained with a Packard scintillation counter.

In a preliminary control experiment, [^{3}H]MKMB prepared as described and [^{35}S]MKMB eluted from a paper chromatogram of culture filtrate were mixed, dried *in vacuo*, and treated with performic acid. The products were fractionated on a Dowex 1 (formate) column as described under Materials and Methods. Most of the ^{3}H and ^{35}S counts appeared in fractions 22–25 as a sharp peak which showed satisfactory coincidence of the two isotopes. The preliminary fractionation of crude *r-eth*-1 culture filtrate on an identical column resulted in the appearance in this peak of a single substance, apparently MMP sulfone. Small but significant portions of radioactive materials appeared in fractions 13–15 and in fractions 31–34. The preliminary fractionation of whole culture filtrate (summarized in Table II) showed that substances having low R_F values in the butanol–acetic acid–water system appeared in these peaks. Table V shows that "pure" MKMB after elution from a chromatogram and rechromatography in the same solvent yields minor but significant amounts of substances with identical R_F values. On this basis it is argued that the unknown radioactive substances in these fractions are also derived from MKMB.

In the second experiment, putative MMP which had been eluted from a paper chromatogram of untreated culture filtrate was mixed with [^{3}H]MKMB, and the mixture was treated with performic acid and chromatographed as described. Most of the ^{3}H and ^{35}S counts again appeared in tubes 22–25.

In the third experiment, [^{3}H]MKMB and putative MHMB sulfone eluted from a paper chromatogram of culture filtrate were mixed, treated with performic acid, and chromatographed on a column of Dowex 1 (formate) as described. The pattern of ^{3}H counts was the same as in the first two experiments. Most of the ^{35}S counts appeared in fractions 27–29. The preliminary fractionation of crude *r-eth*-1 culture filtrate on an identical column resulted in the appearance in this peak of a single substance, apparently MHMB sulfone. A minor but distinct peak of ^{35}S material also appeared in fractions 22–25, showing that some MMP sulfone had been formed. These experiments show that most of the MKMB and putative MMP, as well as a significant amount of MHMB sulfone, are converted to a common product, putative MMP sulfone, by treatment with performic acid.

In order to compare the properties of authentic and putative MMP and MMP sulfone, the compounds were synthesized as described under Materials and Methods. Synthetic MMP (200 μg) and 10,000 cpm of putative MMP eluted from a paper chromatogram of culture filtrate were mixed, and one-half the mixture was treated with performic acid as described. These two mixtures, as well as a mixture of synthetic and putative MMP sulfone, were subjected to paper chromatography in the following three solvents: (1) 1-butanol–acetic acid–water (12:3:5), (2) *n*-amyl alcohol–5 M formic acid (1:1), (3) ethyl acetate–acetic acid–water (3:1:1). The R_F values of MMP in the above-mentioned solvents were 0.85, 0.32, and 0.68, respectively; those of MMP sulfone were 0.65, 0.37, and 0.62. The shape and position of radioactive substances on the chromatograms were determined by autoradiography. The radioactive and acidic substances showed no tendency to separate in any of the solvent systems employed.

Discussion

MKMB is the probable source of the methionine-

related compounds appearing in *r-eth*-1 culture filtrates. It is converted nonenzymatically to MMP and MMP sulfone. As a substrate for lactic dehydrogenase (Meister, 1952) it may be converted enzymatically to MHMB.

However, methionine, rather than MKMB, is thought to be the primary compound accumulated by *r-eth*-1. This mutant has lost control over its methionine production and produces methionine under conditions which completely repress synthesis of the amino acid by wild type, as will be discussed in a subsequent publication. MKMB is not an intermediate in any known anabolic pathway. Methionine is a good substrate for L-amino acid oxidase, and *Neurospora* is an excellent source of this enzyme (Thayer and Horowitz, 1951). Hence it seems probable that the keto acid is derived from methionine.

A number of examples have been reported of overproduction of metabolites by mutants which have lost feedback control (e.g., Moyed, 1961) or repression control (e.g., Ames and Hartman, 1963). The results obtained with *r-eth*-1 emphasize the fact that a simple bioassay of culture filtrates for the primary product accumulated may not reflect the magnitude of the derangement in metabolism.

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