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METHYLTHIOADENOSINE METABOLISM IN MALIGNANT MURINE CELLS

John I. Toohey

Division of Hematology and Oncology, Department of Medicine
University of California at Los Angeles, Los Angeles, CA 90024

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SUMMARY. An ether-extractable product formed from 5'-methylthioadenosine by extracts of malignant murine lymphocytic cells is shown to be 2-keto-4-methylthiobutyric acid. When 5'-methylthio [U-^{14}C]adenosine was used as substrate, the product was labelled, confirming earlier reports that carbons of the keto acid are derived from carbons of the ribose. When hydroxylamine was added to the reaction mixture, the ketomethylthiobutyric acid was trapped as the oxime. When glutamine was added, the main product was methionine.

In previous reports it was shown that extracts of certain malignant murine cells converted 5'-methylthioadenosine to 5-methylthioribose-1-phosphate and converted the latter compound to an ether extractable product (1, 2). The ether-extractable product contained the CH_3 group and the S atom of the substrate, it gave a negative test for ribose, and it dissociated as an acid. It was concluded that the product was an acidic derivative of the CH_3-S group and, at the time of the previous reports, it appeared likely that the CH_3-S group had been cleaved from the ribose.

Subsequently, Backlund and Smith reported a system in rat liver which converted 5'-methylthioadenosine to methionine with carbons of the ribose conserved in the amino acid (3). 5-methylthioribose-1-phosphate was shown to be an intermediate in this conversion. Shapiro and Barrett reported a similar system in *Enterobacter aerogenes* (4). They observed two products besides methionine which they concluded to be 2-keto-4-methylthiobutyric acid and 2-hydroxy-4-methylthiobutyric acid. Trackman and Abeles, using a partially-purified system from rat liver, also observed the latter two compounds (5). They showed that oxygen is consumed by the system and that formic acid is produced. Yung, Yang, and Schlenk reported a similar conversion in apple tissue in which 5-methylthioribose, formed by hydrolysis of methylthioadenosine, is converted to methionine (6).

In view of these findings, the ether-extractable product from the malignant cell system has been re-examined. This report shows that it is 2-keto-

* Abbreviations: KMTB, 2-keto-4-methylthiobutyric acid; MTA, 5'-methylthioadenosine; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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4-methylthiobutyric acid. It is trapped as the oxime when hydroxylamine is added to the reaction mixture. If glutamine is added, it is converted to methionine.

MATERIALS AND METHODS

Chemicals. 2-keto-4-methylthiobutyric acid (KMTB*), sodium salt; 2-hydroxy-4-methylthiobutyric acid, calcium salt; 5'-methylthioadenosine; and other organic chemicals were purchased from Sigma Chemical Co. [^{35}S]methionine and [U-^{14}C]ATP were purchased from Amersham. [^{35}S]methylthioadenosine and 5'-methylthio [U-^{14}C] adenosine were prepared by hydrolysis of the corresponding S-adenosylmethionine at pH 3 (7). [^{35}S]adenosylmethionine was prepared by culturing baker's yeast in the presence of [^{35}S]methionine as previously described (1). [*Adenosyl*-U-^{14}C] adenosylmethionine was prepared enzymatically with methionine adenosyl transferase from Fleischmann's "active dry" yeast purified through the acetone fractionation step as described by Stekol (8). The reaction mixture contained Tris buffer, pH 7.5, 50 mM; $MgCl_2$, 50 mM; KCl, 100 mM; GSH, pH 7.5, 10 mM; L-methionine, 20 mM; [U-^{14}C]ATP, 944,000 cpm per umole, 1 mM; and 2 units of the enzyme. After incubation at 32° for 2.5 hours, protein and K^+ ions were removed by precipitation with 450 umoles of $HClO_4$. Mg^{++} ions were removed by precipitation with 150 umoles of Na_4pyrophosphate at pH 7. Unreacted [U-^{14}C]ATP and other anions were removed by passing the solution at pH 7 through a 2 cc column of Bio-Rad AG3-X4A-chloride. The product was further purified by chromatography on Amberlite IRC-50-H^+ with 0.1 N HCl as eluant (9).

The oxime of KMTB was prepared by mixing 100 mg of Na.KMTB, 168 mg of hydroxylamine.HCl, and 200 mg of $NaHCO_3$ in 2 ml of water. The pH of the mixture was adjusted to 7.5. After 2 hours at 32°, the mixture was acidified with 0.3 ml of 12 N HCl and extracted 3 times with 2 ml volumes of diethyl ether. Removal of the ether by evaporation yielded 95 mg of white crystalline material: m.p. 133°. Chromatography of the product in butanol:acetic acid:water (see details below) gave a single spot with R_f=0.88. No spot corresponding to unreacted KMTB (R_f=0.75) was detectable.

Cells and Cell Extracts. Murine lymphocytic leukemia, L5178Y, was obtained from EG & G Mason, Inc., Worcester, MA, and maintained in ascites culture in DBA/2 mice. These cells contain the enzyme, methylthioadenosine phosphorylase (2) and proliferate readily *in vitro* without a requirement for methylthio disulfides (10) (unpublished observations). Cells were collected by peritoneal lavage 7 days after inoculation with 2 million cells, washed with Hepes-buffered saline, and disrupted by sonication as previously described (2). Mouse liver extracts were prepared by homogenizing livers in 0.25 M sucrose in a glass-teflon homogenizer followed by centrifugation at 100,000 *g* for 30 minutes. Protein content of cell extracts was determined by the method of Lowry (11).

Enzyme Reaction. Reaction mixtures of 1 ml volume in 12 x 75 mm plastic tubes contained: Hepes buffer, pH 7.2, 50 mM; Na.phosphate, pH 7.2, 2 mM; labelled MTA, 0.3 mM; and about 5 mg of extract protein. The mixtures were incubated at 32° for 90 minutes. For quantitative assays, the mixtures were extracted with ether as previously described (2). For qualitative analyses, the mixtures were processed as described below.

Chromatography of Ether Extracts. The reaction mixtures were transferred to centrifuge tubes with ground glass stoppers, acidified by addition of 1 ml of 1 N HCl, and extracted 3 times with 1.5 ml volumes of diethyl ether. The ether was evaporated in a stream of nitrogen gas to a volume of about 0.2 ml. Aliquots of the ether residues were streaked on 4 cm lines on Whatman #1 paper. Standard compounds were spotted at both sides of the paper. Chromatography was carried out in an ascending system in a solvent containing butanol:acetic acid:water; 72:30:18. After development for about 4 hours, the paper was dried in air. The outer edges of the paper were cut off and sprayed with 0.1% bromcresol green in

95% ethanol to detect organic acids which appeared as yellow spots on a blue background. Radiolabelled products in the center lanes were detected by cutting the paper into 4 mm bands, placing them in scintillation fluid and measuring radioactivity in a scintillation counter.

Electrophoresis of Ethanolic Extracts. To each reaction mixture was added 1.5 ml of 95% ethanol. The mixtures were centrifuged at 5000 rpm for 5 minutes. The clear supernatant solutions were removed and evaporated in a stream of N_2 to a volume of about 0.2 ml. Since salts in the samples affected mobility, the standards were incorporated into the unknowns before streaking on the paper. Aliquots were streaked along 4 cm lines on pre-wet Whatman #1 paper. Electrophoresis was carried out in a Shandon apparatus with 0.5 M acetic acid as solvent and at a voltage of 25 volts per cm of paper width for 3 hours. The paper was dried in air for at least 6 hours and sprayed with bromcresol green as described above. To detect methionine, the paper was sprayed with 0.5% ninhydrin in acetone and heated. The lanes containing unkowns were then cut into scintillation fluid and radioactivity was measured. The color reagents on the paper did not affect the counts as determined by external quench analysis and phosphorescence controls. Cyanocobalamin was used in the electrophoresis system as a marker to indicate zero mobility.

Mass Spectrometric Analysis. The enzyme reaction mixture described above was scaled up 10-fold and contained 20 mM hydroxylamine. After incubation at 32° for 2 hours, the mixture was acidified with 0.8 ml of 12 N HCl and extracted 3 times with ether. The ether extract was shaken with 3 ml of 0.06 M Hepes buffer pH 7.2. The aqueous phase was freed of ether by passing nitrogen gas over it and then passed through a 2 cc column of Bio-Rad AG50W-H^+ and washed out with water. The eluate was acidified with HCl and extracted with ether as described above. The combined ether extract was evaporated to dryness with gentle warming under a stream of N_2. The white residue was dissolved in 0.2 ml of methanol and 10 ul were applied to the direct insertion probe for mass spectrum analysis. The analysis was carried out in an AEI MS092 high resolution mass spectrometer with ionization at 70 eV.

RESULTS

Figure 1 shows the results of chromatography of ether extracts and electrophoresis of ethanolic extracts of enzyme reaction mixtures with labelled MTA as substrate under 3 conditions: 1) basal conditions with no additions other than those described in Methods, 2) with 5 mM hydroxylamine, and 3) with 10 mM glutamine. The products are labelled *a*, *b*, and *c* for ease of discussion. Under basal conditions, the ether extract shows a major product, *a*, which migrates with an R_f of 0.75 and which coincides with KMTB. The corresponding ethanolic extract shows product *a*, again moving with KMTB, and another major product, *b*, which migrates toward the cathode with the same mobility as methionine. Addition of 10 mM glutamine to the reaction mixture causes a marked decrease in the amount of product *a* and an increase in the amount of product *b*. Addition of 5 mM hydroxylamine causes a 50% decrease in the amount of product *a*, almost complete absence of product *b*, and a marked increase in the amount of product *c*. When the concentration of hydroxylamine was increased to 20 mM, the amounts of products *a* and *b* were essentially nil and product *c* was the only significant product (data not shown).

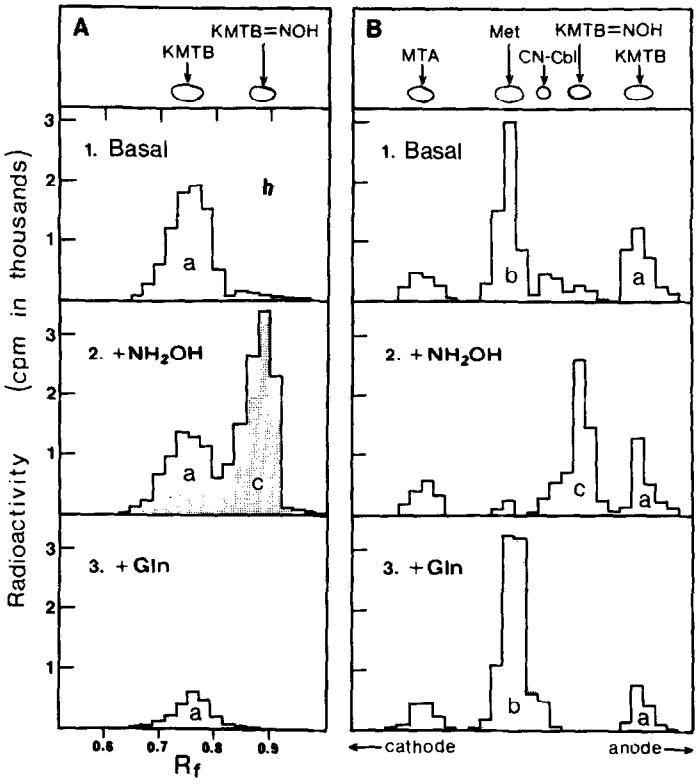

Figure 1. A. Chromatography of Ether Extracts of Enzyme Reaction Mixtures
B. Electrophoresis of Ethanol Extracts of Enzyme Reaction Mixtures

|^{35}S|MTA, 300,000 cpm per umole (***solid lines***) or |*adenosine*-U-^{14}C|MTA, 944,000 cpm per umole (***stipled areas***) were used in the enzyme system described in Methods under 3 conditions: 1) with no addition, 2) plus 5 mM hydroxylamine, and 3) plus 10 mM glutamine. In part B3, with |*adenosine*-U-^{14}C|MTA as substrate, a double peak corresponding to MTA and adenine and extending off the graph to the left is omitted. The positions of the standard compounds are shown at the top of the figure. Abbreviations: KMTB, 2-keto-4-methylthiobutyric acid; KMTB=NOH, the oxime of KMTB; MTA, methylthioadenosine; Met, methionine; CN-Cbl, cyanocobalamin; Gln, glutamine.

Product *c* moves in the chromatographic and electrophoretic systems with the same mobility as the oxime of KMTB. The mass spectra of the two compounds are shown in Figure 2. The spectra produced by the two compounds are identical and are consistent with the structure, CH_3-S-CH_2-CH_2-C(=NOH)-COOH.

When 5'-methylthio[U-^{14}C]adenosine is used in the enzyme system, it gives the same radiolabelled products as does [^{35}S]MTA (demonstrated in Fig. 2, parts A2 and B3), confirming reports in other systems that carbons of the KMTB are derived from carbons of the ribose.

I have repeated several of the experiments described above using extracts of mouse liver. The results (not shown) are qualitatively similar to those shown for the malignant cell extracts. No product corresponding to 2-hydroxy-4-methylthiobutyric acid was observed in either of these systems. When the two systems

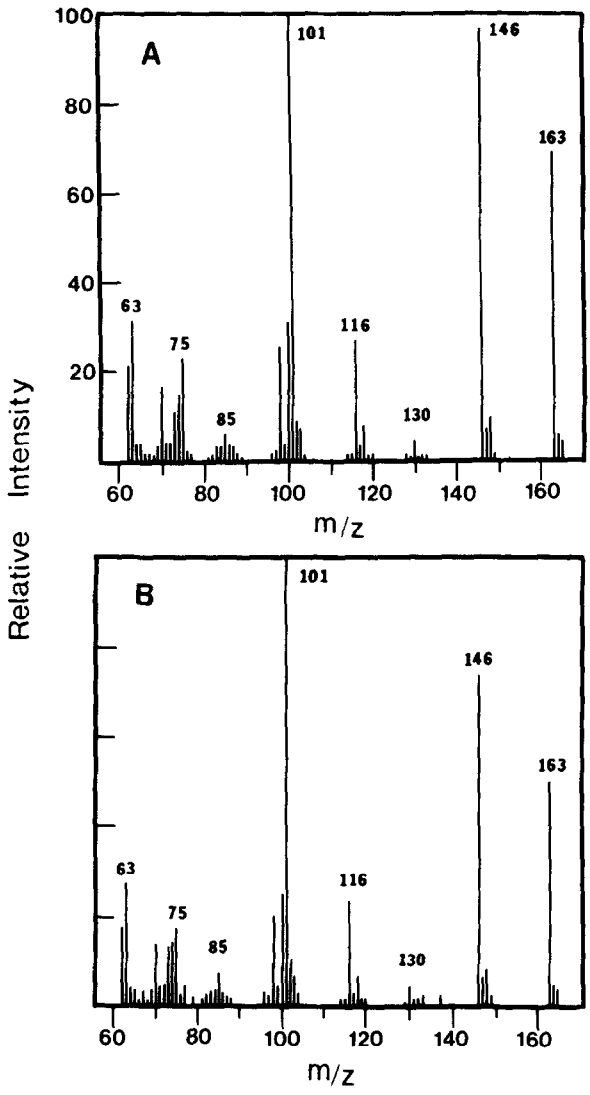

Figure 2. A. Mass spectrum of synthetic KMTB-oxime.

B. Mass spectrum of the product from the enzyme reaction with MTA as substrate in the presence of 20 mM hydroxylamine (purified as described in Methods).

were compared in the quantitative ether extraction assay, the specific activity in L5178Y extracts was almost 10-fold greater than that in mouse liver extracts (19 and 2.2 nmoles of product per mg protein per 90 minutes, respectively). This difference might be attributable in part to more rapid conversion of KMTB to methionine in the liver extracts. However, when 10 mM hydroxylamine was added to trap KMTB and prevent its conversion to methionine, the L5178Y extracts still showed 4-fold higher specific activity than the liver extracts (36 and 9.6 nmoles of product per mg of protein per 90 minutes, respectively).

DISCUSSION

The results described above show that the ether-extractable product formed from MTA by extracts of certain malignant murine cells is KMTB. The results of experiments with ^{35}S, *methyl*-^{14}C, and *adenosyl*-U-^{14}C-labelled substrates indicate that the CH_3-S group and carbons of the ribose are all conserved in the product. Therefore, it is necessary to correct an earlier interpretation which inferred that the CH_3-S group is cleaved from the ribose and to withdraw the term "methylthiolase" (1,2). The previously-used term "methylthiolase activity" should now be construed as "activity in forming KMTB".

The system which catalyses the conversion of MTA to KMTB, with 5-methylthioribose-1-P as an intermediate, has a wide distribution in nature. It has been demonstrated in a bacterium (4), a yeast (12), liver (3,5), and apple tissue (6) as well as in the malignant mammalian cells used in this study. The specific activity of the system is quite high and the pathway appears to be a major one. It has been difficult to achieve purification of the enzymes in the system and, therefore, to determine the cofactor requirements. Trackman and Abeles have reported fractionation of the system from rat liver into at least two parts (5). The first enzyme in the system, MTA phosphorylase, is stable and well-characterized. It is abundant in all of the normal tissues examined and in some malignant cells (reviewed in 13 and 14) but it is completely absent in other malignant cells (2,15,16).

The product, KMTB, can be metabolized in at least two ways. It can be converted to methionine by transamination. This pathway can be regarded as a means of salvaging the sulfur of the methionine used for ethylene production in plants or for polyamine formation in dividing cells. Secondly, the KMTB can be degraded, the principal products being CO_2, sulfate, and methyl mercaptan (17,18). The production of methyl mercaptan from KMTB by liver homogenates has been demonstrated by Steele and Benevenga (17,18) and in earlier studies Canellakis and Tarver described the formation of methyl mercaptan from methionine by liver mitochondria (19). I have confirmed the formation of CH_3-SH from KMTB by homogenates of the malignant cells used in this study (unpublished observations). CH_3-SH appears to be an essential growth factor for many cells *in vitro* (10) and it is possible that the pathway described above is the source of this factor in dividing cells.

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