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METHYLTHIOADENOSINE NUCLEOSIDE PHOSPHORYLASE DEFICIENCY IN METHYLTHIO-DEPENDENT CANCER CELLS

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SUMMARY. The cleavage of the methylthio group from methylthioadenosine is shown to involve two enzymes, a nucleosidase which catalyses the phosphorolytic cleavage of methylthioadenosine to yield adenine and 5-methylthioribose-1-phosphate and an enzyme which uses the latter compound as substrate and catalyses the release of the methylthio group as an ether-extractable product. Three malignant murine hematopoietic cell lines which require methylthio group supplementation for proliferation *in vitro* are shown to lack methylthioadenosine nucleosidase activity while retaining activity of the second enzyme. Four cell lines which are methylthio-independent *in vitro* contain activity of both enzymes. The data suggest that the requirement for exogenous methylthio groups in certain cells is caused by the block in their biosynthetic pathway imposed by methylthioadenosine nucleoside phosphorylase deficiency. Secondarily, the data suggest that all cells require methylthio or related groups for division.

A previous report described an enzyme system which catalyses the cleavage of the methylthio group from methylthioadenosine (1). This activity was shown to be present in extracts of a group of mammalian cells which grow well *in vitro* without methylthio group supplementation and to be undetectable in extracts of a group of cells which require exogenous methylthio groups for proliferation *in vitro*. It was subsequently observed that mixing extracts of the two cell types resulted in much greater activity than expected and that even very small amounts of extract from methylthio-independent cells "activated" the extracts from methylthio-dependent cells. This led to the results reported here which show that the system involves two enzymes. The first is methylthioadenosine nucleoside phosphorylase which is abundant in methylthio-independent cells and undetectable in methylthio-dependent cells. The second enzyme, given the trivial name "methylthiolase"*, catalyses the cleavage of the methylthio group from 5-methylthioribose-1-phosphate and is present in all of the cells.

METHODS

Cells and Cell Culture. The cells used and the methods of cell culture were the same as previously described (1).

* The enzyme is tentatively named 5-methylthioribose-1-phosphate methylthiolase. Although "methylthiolase" is not an ideal name, it will be used, pending more detailed information on the reaction, to imply cleavage of the CH_3-S group from the substrate.

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Chemicals and Chemical Methods. [^{35}S]methylthioadenosine and [*methyl*-^{14}C]methylthioadenosine were obtained as previously described (1). Unlabelled methylthioadenosine, xanthine oxidase from buttermilk, and other chemicals were purchased from Sigma Chemical Co. Protein was measured by the Lowry method (2), phosphate by the Fiske-Subbarow method (3), and ribose by the orcinol method (4).

Enzyme Assays.

Ether Extraction Assay for Methylthiolase Activity. Enzyme preparations were crude extracts of *in vivo* or *in vitro*-grown cells prepared as described previously (1) except that phosphate-buffered saline was replaced by HEPES-buffered saline (0.01 M K.HEPES†, pH 7.2). Reaction mixtures contained: 50 µmoles of HEPES buffer, pH 7.2; 0.15 µmole of [^{35}S] or [*methyl*-^{14}C]methylthioadenosine; 2.0 µmoles of Na.phosphate (at pH 7.2); and 2 to 5 mg of extract protein in a volume of 1.0 ml. After incubation at 32° for 90 minutes, the mixtures were extracted with diethyl ether as previously described (1).

Dowex AG-50 Assay for Nucleosidase Activity. This assay measures the conversion of labelled methylthioadenosine to a product which is not retained by AG-50-H^+ at pH 1 to 3. Reaction mixtures contained: 50 µmoles of HEPES buffer, pH 7.2; 0.15 µmole of [^{35}S] or [*methyl*-^{14}C]methylthioadenosine; 15 µmoles of Na.phosphate (at pH 7.2); and 0.1 to 0.2 mg of protein in a volume of 1.0 ml. After incubation at 32° for 20 minutes, the mixtures were acidified by adding 50 µl of 1 N HCl and passed through 0.6 x 2 cm columns of Dowex AG-50W, x8, 200-400 mesh, in the H^+ form. The passthrough and 1 ml wash were placed in scintillation fluid and radioactivity was determined. The amount of product formed was calculated from the specific activity of the substrate.

Spectrophotometric Adenine Assay for Nucleosidase Activity. The release of free adenine was measured by the method of Naher (5). Reaction mixtures contained four times the amounts of reactants used in the reaction mixtures for the AG-50 assay, i.e.; 60 µmoles of phosphate, pH 7.2; 0.60 µmole of unlabelled methylthioadenosine; and 0.4 to 0.8 mg of protein in a volume of 0.6 ml. After incubation at 32° for 20 minutes, 0.15 ml of 4.8 M $NaNO_2$ and 50 µl of 19.2 N H_2SO_4 were added. This mixture was incubated at 37° for 1 hour to deaminate adenine followed by centrifugation to remove precipitated protein. An aliquot of 0.2 ml was removed, added to 0.6 ml of water, and neutralized by adding 50 µl of 5 N NaOH. Neutralization to within pH 4 to 8 was confirmed by placing 1 µl of the mixture on pH test paper. To this solution were added: 0.1 ml of 1 M Tris buffer, pH 8.0; and 0.1 unit of xanthine oxidase bringing the volume of the assay mixture to 1.0 ml. The quantity of adenine was calculated from spectrophotometric measurements as described by Naher (5). Results are expressed as nmoles of adenine per assay mixture (one-fourth of the reaction mixture) so that the yield of product is comparable with that from the AG-50 assay. The same procedure was used for measuring hypoxanthine released from inosine except that $NaNO_2$ was replaced by water, H_2SO_4 was replaced by 4 N trichloroacetic acid, the second incubation was omitted, and the aliquot removed for assay was neutralized with 50 µl of 1 N NaOH. The xanthine oxidase preparation gave no absorbance change with adenine, thus permitting the distinction between adenine and hypoxanthine.

Adenosine Deaminase Assay. Activity was measured by following the decrease in absorbance at 265 nm by the method described by Giusti (6).

Partial Purification of Nucleosidase Enzyme. Plasmacytoma MOPC 21a or LPC-1 ascites cells (1) were washed 3 times with HEPES-buffered saline, suspended in 3 volumes of the same, disrupted by sonication (1), and centrifuged at 12,000 *g* for 20 minutes. The clear supernatant solution was made 3 x 10^{-3} M with mercap-

† Abbreviation: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

toethanol and the pH adjusted to 4.4 by adding 0.03 volumes of 1 M Na.citrate, pH 3.3. The copious white precipitate was removed by centrifugation at 12,000 *g* and 3° for 10 minutes. With the pH still at 4.4, the solution was heated to approximately 55° by swirling in a 60° water bath for 1 minute, then cooled in ice. The dense coagulum of protein was removed by centrifugation as above. The pH of the supernatant solution was adjusted to about 6.5 with 0.9 M $NaHCO_3$. The resulting preparation contained 7 to 12% of the protein of the crude extract, 100% of the nucleosidase activity, and no methylthiolase activity.

Purification of Nucleosidase Product. The following method was used for preparing ^{35}S or [^{14}C]methyl-labelled product in batches of about 0.3 µmole. Larger amounts were prepared as multiples of the following. The reaction mixture contained: 50 µmoles of HEPES buffer, pH 7.2; 1.5 µmoles of [^{35}S] or [*methyl*-^{14}C] methylthioadenosine; 20 µmoles of phosphate (pH 7.2); and 2 mg of protein as partially-purified nucleosidase in a volume of 1.5 ml. The mixture was incubated at 32° for 60 minutes. Unreacted substrate was removed by adjusting the pH to 3.5 with 1 M acetic acid and passing the solution as quickly as possible through a 0.6 x 2 cm column of Dowex AG-50, x8, 200-400 mesh, previously equilibrated with Na.phosphate at pH 3. Inorganic phosphate was precipitated by adjusting the pH to near 8 with 0.15 ml of 1 M Tris buffer, pH 8.0, and adding 1.5 ml of 1% $CaCl_2$ in 33% ethanol (7). The precipitate was removed by centrifugation followed by filtration through a 0.2 micron Millipore filter. The organic phosphate was then precipitated by adding 3 ml of absolute ethanol and 0.1 ml of 0.75 M $NaHCO_3$ (to increase the bulk of the precipitate). The mixture was cooled to -10° for 30 minutes. The precipitate was collected by centrifugation and extracted with 0.5 ml of water. The resulting aqueous solution contained about 50% of the radiolabelled product present after Dowex AG-50 treatment.

RESULTS

Properties of the Nucleosidase from Plasmacytoma Cells. When methylthioadenosine is subjected to the assays described above, the features of a nucleosidase reaction are demonstrated by the separation of the methyl group and the S atom from the positively-charged base and by the appearance of free adenine (Fig. 1). The reaction is time-dependent (Fig. 1A), enzyme-dependent (Fig. 1B), and substrate-dependent (Fig. 1 C). The substrate-dependence curve shows that, at low substrate concentrations (enzyme excess), up to 80% of the substrate is converted to product in 20 minutes (Fig. 1C). The reaction shows an absolute dependence on phosphate when the enzyme is dialysed to reduce the endogenous phosphate content (Fig. 1D). Phosphate can be replaced by arsenate (Fig. 1D).

The enzyme activity is completely destroyed by heating at 90° for 5 minutes. However, it is stable to brief heating at 55° at pH 4.4 and the enzyme can be purified 10-fold by this procedure without loss of activity (Methods). The partially-purified enzyme can be stored for several days at 0° or for weeks at -10° with only slight loss of activity.

Extracts of methylthio-independent cells contain nucleosidase activity for inosine as well as for methylthioadenosine (Table 1, col. 3). The rate of cleavage of inosine is several times that for methylthioadenosine. Adenosine is also cleaved (at about the same rate as methylthioadenosine); however, the extracts contain adenosine deaminase activity and it appears that adenosine is

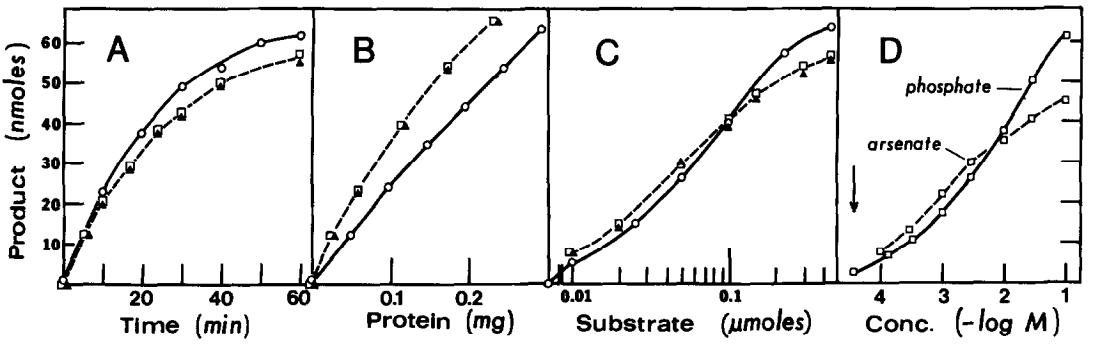

Figure 1. Characteristics of the Nucleosidase Reaction.

[^{35}S]methylthioadenosine, 441,000 cpm per μmole (□) and [*methyl*-^{14}C]methylthioadenosine, 281,000 cpm per μmole (▲) were used in the AG-50 assay and unlabelled methylthioadenosine (○) was used in the adenine assay with partially-purified nucleosidase from MOPC 21a ascites cells. Different enzyme preparations were used for the two sets of assays.

A. Time Dependence. A single reaction mixture of 10x usual volume was used for each substrate and appropriate aliquots were removed for assay at various times. The protein content of each assay mixture was 0.15 mg for AG-50 assays and 0.07 mg for adenine assays.

B. Enzyme Dependence. The amount of cell extract was varied and the amount of substrate was twice the usual or 0.30 μmole per assay.

C. Substrate Dependence. Substrate concentration was varied as shown on the abscissa in units of μmoles per assay. The protein content of each assay mixture was 0.17 mg for AG-50 assays and 0.10 mg for adenine assays.

D. Phosphate Dependence. Reaction mixtures contained varied amounts of phosphate or arsenate as shown, 0.22 μmole of [^{35}S]methylthioadenosine, and 0.23 mg of protein. The enzyme preparation had been dialysed against HEPES buffer to decrease the phosphate content. The endogenous phosphate concentration contributed by the extract, 3.8 x 10^{-5} M, is indicated by the arrow.

deaminated before phosphorolysis since the base is recovered as hypoxanthine (data not shown). Adenosine deaminase activity is readily demonstrated in extracts of all of the cells studied, both methylthio-dependent and methylthio-independent, at a rate of about 30 nmoles per mg of protein per minute. The deaminase has no effect on methylthioadenosine, a finding which is in agreement with the results of other studies on enzymes from mammalian sources (8, 9).

Addition of inosine at concentrations up to 3 mM has no effect on the rate of phosphorolysis of methylthioadenosine (concentration = 0.15 mM) as measured by the AG-50 assay. However, the reaction is strikingly inhibited by the addition of adenine. The concentration of adenine which decreases the rate by 50% varies directly with the concentration of phosphate; it is 0.36 mM at 50 mM phosphate, 0.23 mM at 20 mM phosphate, 0.17 mM at 10 mM phosphate, 0.080 mM at 2 mM phosphate, 0.041 mM at 0.5 mM phosphate, and 0.025 mM at 0.2 mM phosphate.

Table 1. Nucleosidase and Methylthiolase Activities in Extracts of Various Cells.

Cells were grown *in vitro* and crude extracts were prepared as described in Methods. Nucleosidase activity was measured by the hypoxanthine assay for col. 3 and by the AG-50 assay for col. 4. Methylthiolase activity was measured by the ether extraction assay. For the assays in col.6, 0.2 mg of protein was added as partially-purified nucleosidase from LPC-1 ascites cells. For the assays in columns 7 and 8, phosphate was omitted and the substrate was ^{35}S-labelled nucleosidase product purified as described in Methods, 0.15 µmole per assay, 371,000 cpm per µmole. For the assays in col. 8, the product was exposed to 0.1 N HCl at 100° for 10 minutes and neutralized before addition to the assay mixtures. Activities are expressed as rates in units of nmoles of product per mg of protein per 20 minutes (for nucleosidase assays) or per 90 minutes (for methylthiolase assays).

Cell Type Extracted		Nucleosidase Activity		Methylthiolase Activity			
				Standard assay	Nucleosidase added	Substrate = nucleosidase product	
Methylthio requirement	Cell Line	Substrate = inosine	Substrate = [^{35}S]methylthioadenosine			untreated	hydrolysed
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Methylthio-dependent	L1210	3200	0.0	0.0	16	16	0.0
	P388	6500	0.0	0.0	13	12	0.0
	MOPC 21aSH	4500	0.0	0.0	15	14	0.0
Methylthio-independent	LPC-1	340	120	17	18	15	0.0
	MOPC 21a	430	120	20	23	18	0.0
	AKS-9	440	29	21	25	21	0.0
	Friend	460	24	20	20	17	0.0

Properties of the Nucleosidase Products. The basic product from the phosphorolysis of methylthioadenosine does not serve as a substrate for xanthine oxidase unless it is first deaminated, indicating that it is adenine. The glycosidic product can be substantially purified by the procedure described in Methods. Group analyses of purified samples of the product from [^{35}S] and [*methyl*-^{14}C] methylthioadenosine gave the following results: ^{35}S, 0.113 µmole; ribose, 0.105 µmole; phosphate, 0.111 µmole; and ^{14}C, 0.118 µmole; ribose, 0.107 µmole; phosphate, 0.115 µmole. These analyses are consistent with the structure, methylthioribose-1-phosphate. The phosphorylated product is very unstable. The freshly purified product and about 50% of the product in reaction mixtures is retained by anion exchange resins such as Dowex AG-1-acetate but the negative charge is lost after brief exposure to 0.1 N HCl at 100° or to acid or alkaline phosphatase. The lability is also reflected in the fact that the phosphate is measured in the Fiske-Subbarow assay (3) without previous hydrolysis, as is the phosphate of ribose-1-phosphate (7).

Distribution of Methylthiolase and Nucleosidase in Cells. Nucleosidase Product as Substrate for Methylthiolase. The methylthiolase assay measures the release of the methylthio group of appropriate substrates as an ether-extractable product (1). As previously reported (1), crude extracts of methylthio-independent

cells, LPC-1, MOPC 21a, AKS-9, and Friend cell line, catalyse the cleavage of the methylthio group of methylthioadenosine while extracts of methylthio-dependent cells, L1210, P388, and MOPC 21aSH, do not (Table 1, col. 5). The distribution of methylthioadenosine nucleosidase activity in extracts of these cells was found to correspond exactly with the distribution of methylthiolase activity (Table 1, col. 4). Although extracts of methylthio-dependent cells demonstrate no methylthiolase activity under standard assay conditions, these extracts show abundant activity when small amounts of partially-purified nucleosidase from LPC-1 ascites cells is added to the system (Table 1, col. 6). Similarly, if purified nucleosidase product is used as substrate, extracts of methylthio-dependent cells convert it efficiently into ether-extractable product (Table 1, col. 7). If the labelled nucleosidase product is exposed to 0.1 N HCl at 100° or to acid or alkaline phosphatase, then it is no longer converted to ether-extractable product by any cell extract (Table 1, col. 8).

The above results indicate that the phosphorylated product of the nucleosidase reaction serves as the substrate for the methylthiolase reaction. This is further substantiated by the effect of phosphate and arsenate on the combined system (nucleosidase plus methylthiolase) present in crude extracts of MOPC 21a ascites cells (Fig. 2). Crude extracts contain adequate phosphate to sustain maximal rates of methylthiolase activity in this system. However, the phosphate can be removed from the extracts by dialysis to a level where the system is stimulated by addition of phosphate (Fig. 2B, upper curve). The residual activity in such as system is further reduced by adding arsenate (Fig. 2A). In a system inhibited by 1 mM arsenate, addition of phosphate restores the activity (Fig. 2B, lower curve). Concentrations of phosphate in excess of 2 mM inhibit the methylthiolase reaction (Fig. 2B).

Although extracts of methylthio-dependent cells contain no detectable nucleosidase activity for methylthioadenosine, they contain high levels of activity for inosine (Table 1, col. 3). Inosine nucleosidase activity in extracts of these cells is 10 to 20-fold higher than in extracts of methylthio-independent cells.

DISCUSSION

The system for cleaving the methylthio group from methylthioadenosine is shown by the above results to contain two enzymes. The first enzyme is a nucleosidase which catalyses the phosphorolytic cleavage of methylthioadenosine to yield adenine and methylthioribose-1-phosphate. The reaction can be followed by measuring the liberation of free adenine or by measuring the separation of the labelled methyl group or S atom from the positive charge of the substrate using Dowex AG-50. The nucleosidase is quite stable. It can be purified 10-fold and freed of the second enzyme (methylthiolase) by the simple procedure of

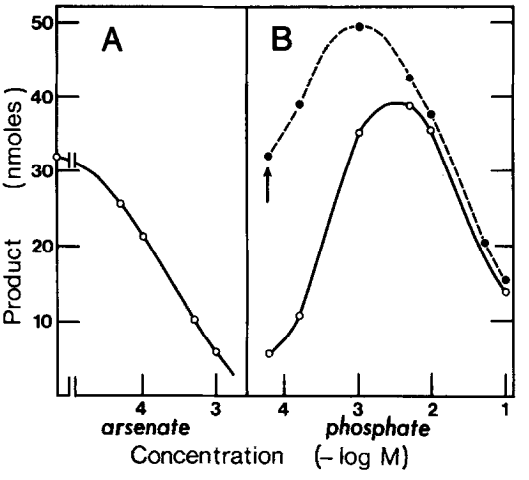

Figure 2. Effect of Arsenate and Phosphate on Methylthiolase Activity.

A crude extract of MOPC 21a ascites cells was dialysed for 6 hours against HEPES buffer, 0.05 M, pH 7.2, containing Dowex AG-1-formate in suspension, to decrease the phosphate content of the extract from 3.2 µmoles per ml to 0.21 µmole per ml. The extract was used in the ether extraction assay with 0.22 µmole of [^{35}S]methylthioadenosine as substrate (441,000 cpm per µmole). Each assay mixture contained 5.4 mg of dialysed protein which contributed a phosphate concentration of 6.3×10^{-5} M, indicated by the arrow.

A. Na.arsenate (pH 7.2) was added at varied concentrations to the above system.

B. Varied amounts of K.phosphate (pH 7.2) were added to the system alone (●), or in the presence of 1×10^{-3} arsenate (○).

acidification to pH 4.4 and heating. An enzyme with nucleosidase activity for methylthioadenosine and properties quite similar to the above was obtained from rat prostate tissue and reported by Pegg and Williams-Ashman in 1969 (9). The inhibition of the reaction by adenine and its relationship to phosphate concentration indicate that the reaction is readily reversible and that the equilibrium is very sensitive to the relative concentrations of reactants and products, a property similar to that described by Kalckar for purine nucleoside phosphorylase (10).

The nucleosidase appears to be specific for methylthioadenosine. Methylthioinosine has not been tested as a substrate but it does not appear to be an intermediate in the phosphorolysis of methylthioadenosine because the extracts have no deaminase activity for methylthioadenosine and because the base released from methylthioadenosine is recovered as adenine (not hypoxanthine). Adenosine does not appear to serve as a substrate since the enzyme preparations do not liberate adenine from it. The preparations catalyse the phosphorolysis of inosine but it is not established whether there is a single enzyme which uses both

methylthioadenosine and inosine as substrates or whether there are two specific enzymes present. The presence of two specific enzymes is indicated by two findings; firstly, that addition of inosine, even at relatively high concentrations, does not decrease the rate of phosphorolysis of methylthioadenosine, and secondly, that methylthio-dependent cells retain inosine nucleosidase activity while lacking activity for methylthioadenosine.

The second enzyme (methylthiolase) catalyses the formation of an ether-extractable product which contains the methyl group and the S atom but no ribose and no adenine (1). The enzyme is very unstable and has been studied only in crude extracts of cells. However, it is obtained free of nucleosidase activity in extracts of methylthio-dependent cells and this permits analysis of the second reaction in some detail. Thus it can be shown that the second enzyme acts on the product of the nucleosidase reaction by combining extracts of methylthio-dependent cells either with methylthioadenosine plus partially-purified nucleosidase or with purified nucleosidase product. It is shown that the methylthiolase is specific for the phosphorylated product by three lines of evidence; by its failure to act on hydrolysed nucleosidase product (Table 1), by the inhibition of the reaction by arsenate in the combined enzyme system (Fig. 2), and by the previous demonstration that synthetic methylthioribose does not serve as a substrate (1). The effect of arsenate is interpreted as substitution for phosphate at the enzyme site followed by rapid hydrolysis of the arsenylated sugar product (11). Because the methylthiolase reaction is relatively slow and requires about ten times as much crude extract protein per assay as the nucleosidase reaction, it is likely that the characteristics of the combined system previously reported (ref. 1, Fig. 1) represent the properties of the methylthiolase enzyme. The inhibitory effect of excess phosphate on methylthiolase activity is unexplained.

Of particular interest is the finding that all of the methylthio-dependent cells lack detectable nucleosidase activity for methylthioadenosine. This deficiency blocks the formation of methylthioribose-1-phosphate and prevents methylthio group cleavage despite the fact that these cells contain abundant methylthiolase enzyme. Since methylthio-independent cells contain the intact pathway and methylthio-dependent cells are defective, it appears that this metabolic deficiency may account for the requirement for exogenous methylthio groups in the latter cells. These data are also consistent with the theory that all cells require methylthio groups for division.

A second pronounced difference between the two groups of cells is the 10 to 20-fold greater activity of inosine nucleosidase in extracts of methylthio-dependent cells than in extracts of methylthio-independent cells. The increased

activity in the first group may reflect a feedback control system stimulated by the accumulation of methylthioadenosine or it may represent a more complex disturbance of nucleoside metabolism in these cells.

It is concluded that the following pathway occurs in certain cells:

$$\text{methylthioadenosine} + \text{phosphate} \xrightarrow{\text{nucleosidase}} \text{methylthioribose-1-phosphate} + \text{adenine} \quad (1)$$

$$\text{methylthioribose-1-phosphate} \xrightarrow{\text{methylthiolase}} \text{"}CH_3\text{-S"} + [\text{ribose-1-phosphate}] \quad (2)$$

The identity of the last product has not been established. A defect in reaction (1) appears to be the cause of the requirement for exogenous methylthio groups in certain malignant cells. The exact nature of the "CH_3-S" product and the reason for its apparent essentiallity in dividing cells remain unknown.

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