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Title: 5'-Methylthioadenosine and 5-Methylthioribose: Studies on their Metabolism and Function in Mammalian Cells

Abstract approved: Redacted for privacy

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Although the biological occurrence of 5'-methylthioadenosine (MTA) and 5-methylthioribose (MTR) were recognized more than a half century ago, very little light has been cast on the physiologic significance of either substance. My studies were designed to extend our knowledge of the metabolism of MTA and to determine if this pathway is important in the overall process of cell division.

MTA has been shown to exert striking inhibitory effects on cell proliferation. My efforts to study the mode of action of MTA have focused on the modulation of cAMP metabolism by the nucleoside. Evidence is provided which establishes the enzyme cAMP phosphodiesterase as the primary target site for MTA. The importance of this inhibitory activity must await further studies on the size and fluctuation of the intracellular MTA pool during the cell cycle.

One might think that since MTA inhibits enzyme reactions vital to cell growth, its rapid degradation is essential simply for maintenance of low intracellular levels. Recent studies suggest,

however, that the degradation products of MTA are required for cell growth. A nutritional requirement of methylthio groups for mammalian cells was postulated on the basis that cells which cannot degrade MTA are unable to grow unless synthetic methylthio compounds are added to the culture medium. My attempts to elucidate the critical product of MTA determined that methylthioribose could satisfy the methylthio dependence of cells lacking MTA phosphorylase activity. In addition, I also found that the thiopentose stimulated the growth rate, saturation density, and viability of other cells in culture.

The final section of my thesis describes the isolation of MTR from the serum of a variety of mammals. The results were substantiated through the use of various chemical, chromatographic, and spectroscopic methods.

In conclusion, I have demonstrated the opposing effects of MTA and MTR towards cell division. These observations suggest that the degradation of MTA and subsequent formation of MTR may represent a pivotal site of control for cell proliferation.

5'-Methylthioadenosine and 5-Methylthioribose: Studies on their Metabolism and Function in Mammalian Cells

by

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CONTRIBUTION OF AUTHORS

I am indebted to Paula A. Tower for work done on polyamine levels in S49 cells after exposure to methylthioadenosine. This work is not actually presented in this thesis but the results of her work allowed me to concentrate on different possible mode(s) of action.

I would also like to thank Donald A. Griffin for all of the mass spectroscopy work performed in this thesis.

Finally, I would like to thank Dr. John H. Fitchen for supplying human serum from healthy volunteers at the V.A. Hospital in Portland. In addition, Dr. Fitchen was helpful in discussions on the direction and significance of the work presented in the last two chapters of my thesis.

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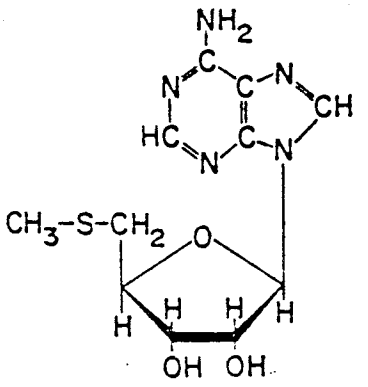
5'-Methylthioadenosine and 5-Methylthioribose: Studies on their Metabolism and Function in Mammalian Cells

CHAPTER I

Introduction

Metabolism of Methylthioadenosine

The naturally occurring nucleoside, 5'-deoxy-5'-methylthioadenosine, is ubiquitous to the living world. In mammalian cells, methylthioadenosine is synthesized primarily during the production of the polyamines spermidine and spermine from S-adenosylmethionine (28). The biosynthesis and metabolism of methylthioadenosine are detailed in Figure I.1. The low concentrations of methylthioadenosine relative to polyamine levels in normal tissue suggest that this substance is rapidly degraded. In eucaryotic organisms the major route of degradation is the enzymatic splitting of methylthioadenosine to form adenine and methylthioribose-1-phosphate (MTR-1-P) by the enzyme, methylthioadenosine phosphorylase (28). Kamatani and Carson (47) have demonstrated that the adenine moiety can be salvaged via purine nucleoside phosphorylase into purine nucleotides. The fate of MTR-1-P in mammalian cells is uncertain, but Backlund and Smith (45) have reported that the methylthio and carbohydrate portions of the molecule can be recycled into methionine.



METHYLTHIOADENOSINE

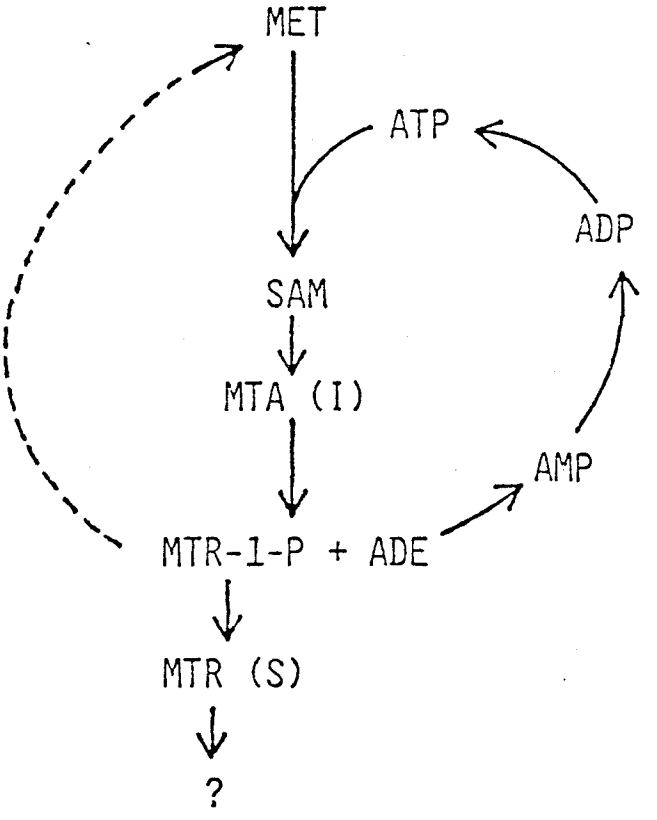


Figure I.1. Structure and metabolism of 5'-methylthioadenosine.

Biologic Effects of Methylthioadenosine

Although the biological occurrence of methylthioadenosine was recognized more than a half century ago (49), early studies cast little light on the physiological significance of this nucleoside. Recent investigations have shown that methylthioadenosine exerts some striking effects on proliferating cells. Ferro's laboratory has shown that methylthioadenosine phosphorylase activity is increased 108% during lymphocyte transformation induced by phytohemagglutinin (PHA). It was also demonstrated that methylthioadenosine could inhibit both PHA- and concanavalin A-induced lymphocyte transformation in a dose-dependent, non-toxic fashion (53). Ferro and coworkers (29) have also reported the inhibition of the growth of several murine lymphoid cell lines of both T and B cell origin by this nucleoside.

Mechanism for Inhibitory Effects

Knowledge of the primary mode of action for the biological effects induced by methylthioadenosine is of importance in ascribing a physiological role for the nucleoside in mammalian cells and because of the nucleoside's potential chemotherapeutic usefulness. Efforts to elucidate the mechanism of action have demonstrated the inhibition by methylthioadenosine of cAMP phosphodiesterase (18), S-adenosylhomocysteine hydrolase (21), and spermidine and spermine synthases (6). Although a great deal of work has concentrated on this

area, the primary target site(s) for methylthioadenosine remain uncertain.

Nutritional Requirement for the Degradation of Methylthioadenosine

Since methylthioadenosine inhibits enzyme reactions vital to cell growth, its rapid degradation was thought to be essential simply for maintenance of low intracellular levels. Recent data suggest however, that the degradation products of methylthioadenosine may be required for cell growth. Toohey (28) found that sulfhydryl-dependent hematopoietic cell lines were devoid of detectable methylthioadenosine activity. Cells of this methylthioadenosine phosphorylase-deficient phenotype excrete large quantities of methylthioadenosine into the culture medium (31). Toohey also demonstrated that the sulfhydryl-dependent cell lines, which are normally cultured in medium containing fetal calf serum (FCS) and glutathione, could flourish in a serum-free medium containing "methylthio" groups (38). These methylthio groups were provided through the exogenous addition of synthetic mixed disulfides of the type R-S-S-CH_3. It was later postulated that cells normally obtain their essential methylthio groups through the cleavage of -S-CH_3 from MTR-1-P or 2-keto-4-methiobutyrate and that only methylthioadenosine phosphorylase deficient cells require an exogenous supply 56a). The structure of the actual "methylthio" donor remains a mystery.

Summary of Objectives

The objectives of this project have been directed towards defining a physiological role for 5'-methylthioadenosine and its degradation products in mammalian cell division. To attain this goal, I have examined the biologic effects of methylthioadenosine and determined the primary mechanism for the growth inhibition produced by this substance. I also describe the charting of a previously unexplored course of metabolism for this nucleoside and expand upon its significance.

In summary the work presented herein is divided into three studies concerning: 1) the mode of the action of methylthioadenosine, 2) the formation and significance of 5-methylthioribose in mammalian cell systems, and 3) the detection of 5-methylthioribose in serum.

CHAPTER II

Mechanism of Action of 5'-Methylthioadenosine in S49 Cells

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Summary

5'-Deoxy-5'-methylthioadenosine, a naturally occurring co-product of polyamine biosynthesis, has been shown to inhibit a variety of biological processes. To investigate the mode of action of this nucleoside and to assess the involvement of cAMP in this action, the effect of methylthioadenosine on S49 wild type and two cAMP-related mutant cells was examined. The sulfur-containing nucleoside potently inhibited the growth of the parental strain (ID_{50} = 50 μM) whereas nearly ten-fold greater resistance was demonstrated by S49 adenylate cyclase-deficient (ID_{50} = 420 μM) and S49 cAMP-dependent protein kinase-deficient mutant cells (ID_{50} = 520 μM). Methylthioadenosine was shown to competitively inhibit the S49-derived high-affinity cAMP phosphodiesterase (K_i = 62 μM) in vitro, whereas methylthioadenosine phosphorylase activity was equivalent in all three cell types. The intracellular levels of the regulatory nucleotide, cAMP, increased dramatically in the wild type (17-fold) and protein kinase-deficient (6-fold) strains in response to 100 μM concentrations of the drug. It is concluded that the growth arrest produced by 5'-methylthioadenosine in S49 cells is primarily due to the inhibition of cAMP phosphodiesterase and the subsequent increase in cAMP levels that result.

Introduction

5'-Deoxy-5'-methylthioadenosine is the co-product of the spermidine and spermine synthase reactions of mammalian cells (1). This sulfur-containing nucleoside has been shown to adversely affect several biological processes, such as lymphocyte blastogenesis (2) and the growth of murine hematopoietic cell lines (3) and virally transformed mouse fibroblasts (4). Knowledge of the primary mode of action for the growth inhibition produced by methylthioadenosine is of interest because of the potential chemotherapeutic usefulness of the drug and several of its structural analogs. It is also of importance in ascribing a biological function to the nucleoside in mammalian cells. Some methylthioadenosine-induced biochemical effects have been described in attempts to elucidate the mechanism of action of this compound. Three of these effects are notable: 1) Methylthioadenosine has been shown to potently inhibit spermidine and spermine synthetase from a variety of sources (5,6); 2) Ferro and coworkers (7) have demonstrated the "suicide like" inactivation of erythrocyte S-adenosylhomocysteine hydrolase by methylthioadenosine; and 3) Methylthioadenosine is a competitive inhibitor of the lymphocyte-derived high affinity cAMP phosphodiesterase (8). In light of these later findings, attention has focused on the modulation of cAMP metabolism by methylthioadenosine. Our investigation was aided by the availability of two mutant cell lines which were originally selected for their resistance to either cAMP itself (cAMP-dependent protein kinase-deficient) or to substances which induce the

synthesis of this growth regulatory nucleotide (adenylate cyclase-deficient) . The variant clones, S49 adenylate cyclase-deficient (9) and S49 cAMP-dependent protein kinase-deficient (10), have been useful as tools in elucidating the components of the cAMP-induced regulatory system. This system is composed of a protein kinase, which in the presence of cAMP, phosphorylates various enzyme substrates and brings about growth arrest. According to this model, if cAMP plays a central role in the biological effects of methylthioadenosine, the response of these cAMP-related mutant cells to methylthioadenosine should be altered in characteristic ways.

Specifically, in this report we compare the effects of methylthioadenosine on parental wild type, adenylate cyclase-deficient, and protein kinase-deficient S49 cells. We confirm the previous findings of the inhibition of methylthioadenosine of the cAMP phosphodiesterase, and we provide evidence suggesting that this perturbation of cyclic nucleotide metabolism by methylthioadenosine plays an important role in determining its biological effects.

Materials and Methods

Materials. 5'-Deoxy-5'-methylthioadenosine was synthesized according to the procedure of Kikugana et al. (11). [2,8-^{3}H]Adenosine 3', 5'-cyclic phosphate (27 Ci/mmole) was purchased from ICN. cAMP and 5'-nucleotidase purified from Crotalus astrox venom were purchased from Sigma. AG-1-X8 was obtained from BIO-RAD, cAMP binding protein assay kit from Amersham (UK), and all cell culture supplies from Gibco. All other reagents were obtained from Sigma.

Cell lines and culture. S49 mouse lymphoma cells of the wild type and mutant phenotypes, protein kinase- (24.6.1) and adenylate cyclase-deficient (94.15.1) were obtained from Dr. Phillip Coffino (Univ. of Calif., San Francisco). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter of glucose, supplemented with 10% heat-inactivated horse serum (12).

Preparation of cell extracts. All cell extracts were made from logarithmic cultures. Suspensions of cells were centrifuged at 700 x g for 5 min, and the supernatant was discarded. The cell pellet was washed in 250 mM sucrose, 100 mM Hepes (pH 7.2, 3 mM 2-mercapoethanol, and centrifuged at 4°C at 700 x g for 10 min. The cells were resuspended in 3 ml of the same buffer and gently fractured by the freeze-thaw method. After addition of dimethylsulfoxide to 5%, the suspension was homogenized in a Dounce homogenizer until breakage was 99% as indicated by the Trypan blue dye exclusion test. The crude

extract was centrifuged at 10,000 x g for 10 min. at 4°C and the supernatant fraction was collected and stored at -80°C until utilized.

Analytical procedures. cAMP present in acid soluble extracts of cells was quantitated by using the competition binding assay kit of Amersham. The nucleotides were first separated from the perchlorate salts by the Freon:alamine extraction method of Khym (13).

Enzyme Assays. cAMP phosphodiesterase activities were measured by the two-step procedure of Thompson and Appleman (14). Each reaction contained 40 mM Tris (pH 8.0), 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, [^{3}H]cAMP (300,000 cpm),and enzyme extract. Methylthioadenosine phosphorylase activity was determined by measuring the conversion of 5'-[$^{14}CH_3$]methylthioadenosine to 5-[$^{14}CH_3$]methylthioribose-1-phosphate (15).

Results

Effect of 5'-methylthioadenosine on the proliferation of parental wild type and mutant S49 cells. The effect of exogenous supplementation of methylthioadenosine on cultures of wild type and mutant S49 cells is shown in Figure II.1. Untreated cultures of each cell type proliferate with a generation time of 17-18 hrs. Parental wild type cells, which are extremely susceptible to killing by cAMP (16), are likewise growth-inhibited by methylthioadenosine. Significant growth inhibition (30%) was observed at concentrations as low as 10^{-5} M in these cultures. Exhibiting an ID_{50} of 50 μM, the S49 wild type cells proved to be the most sensitive to methylthioadenosine of the three cell lines studied. By contrast, sensitivity is greatly reduced in mutant cell lines which are either incapable of synthesizing cAMP (adenylate cyclase-deficient) or resistant to the effects of the regulatory nucleotide (protein kinase-deficient). The former demonstrate nearly ten-fold greater resistance to methylthioadenosine (ID_{50} = 420 μM) than the parental strain. Similarly, cAMP-dependent protein kinase-deficient cells also were 10-fold more resistant (ID_{50} = 510 μM) to the growth inhibitory effect of the drug. These gross differences in biological responses among these cell lines suggest a major involvement of cAMP in the inhibitory effects of methylthioadenosine.

Figure II.1 Comparison of the inhibitory effect of 5'-methylthio-adenosine on the proliferation of S49 parental wild type and mutant cells. Cells were seeded at 1 x 10^5/ml in Dulbecco's modified Eagle's Medium supplemented with 10% horse serum and variable concentrations of the nucleoside. S49 wild type (●); S49 adenylate cyclase deficient (■); and S49 protein kinase deficient, (▲). The results represent the average of three independent experiments after 72 hrs of incubation.

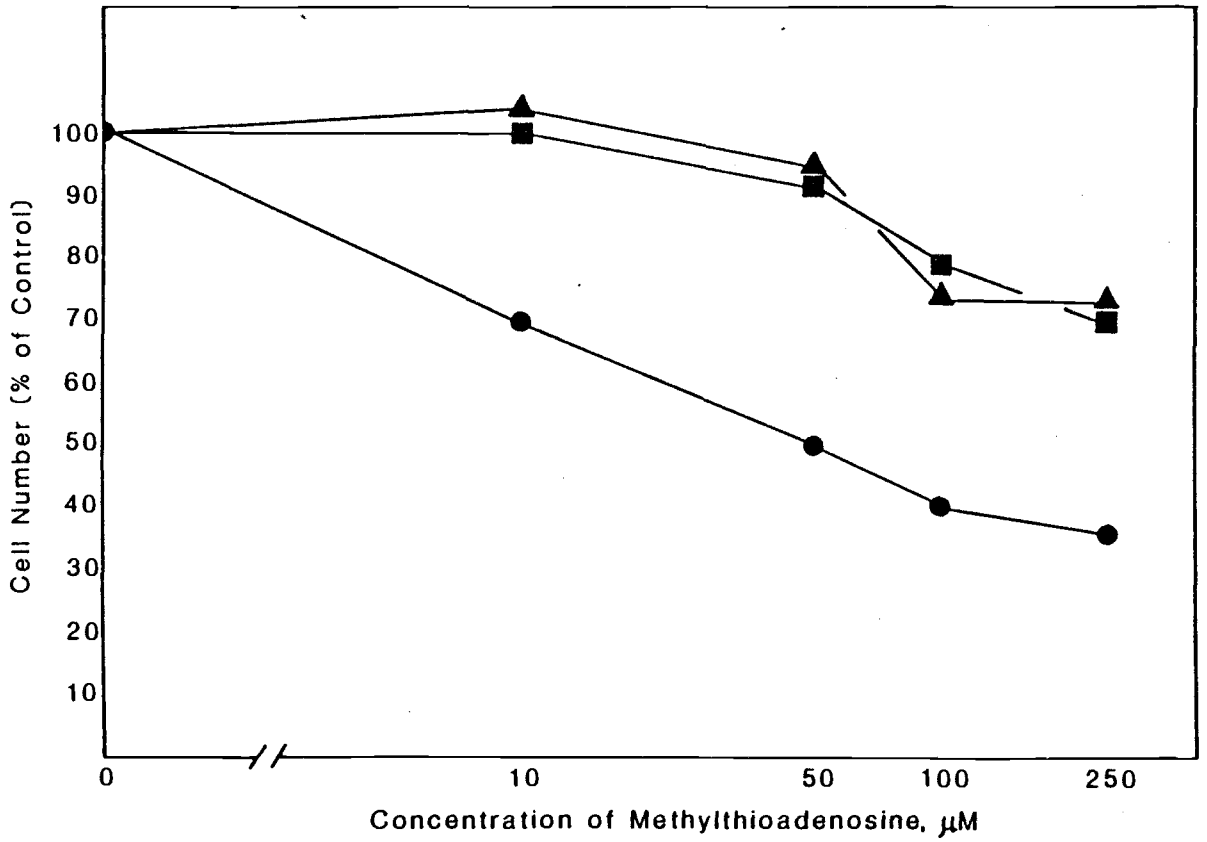
Cell Number (% of Control)
100
90
80
70
60
50
40
30
20
10
0
10
50
100
250
Concentration of Methylthioadenosine, μM

Figure II.1

Methylthioadenosine phosphorylase activities of S49 wild type and mutant cells. Differences in the sensitivity to methylthioadenosine between the wild type and mutant cells could have been due to an enhanced ability of the mutant cells to degrade the nucleoside. To examine this possibility methylthioadenosine phosphorylase activities from logarithmic cultures of the three cell types were measured. The S49 wild type, S49 adenylate cyclase-deficient, and S49 protein kinase-deficient cells contain equivalent methylthioadenosine phosphorylase activities; 36.5, 36.1, and 33.6 pmol/min/mg protein, respectively. The data indicate that the differences in sensitivity to methylthioadenosine noted for the three strains is not due to differences in their ability to degrade the nucleoside by this pathway.

Inhibition of the high affinity cAMP-phosphodiesterase by 5'-methylthioadenosine. Previous kinetic analysis of S49 cell homogenates for cAMP phosphodiesterase activity established the presence of two enzyme forms: a high affinity enzyme exhibiting a K_m value of 0.52 μM, and a second low affinity enzyme exhibiting a K_m of 16.9 μM (17). Zimmerman et al. (18,19) have reported that methylthioadenosine and several of its structural analogs inhibited the high affinity phosphodiesterase of murine cytolytic lymphocytes. To evaluate this possible site of action of methylthioadenosine in the S49 cell strains, we tested the inhibitory potency of the nucleoside on the high affinity phosphodiesterase activity from homogenates of S49 wild

type cells (Figure II.2). Our data confirm the previous estimate of the Km value (0.50 μM) as well as the potent competitive inhibition exerted by methylthioadenosine on the enzyme (K_i = 62 μM).

Basal cAMP phosphodiesterase activities in S49 wild type and mutant cells. Since methylthioadenosine is a potent inhibitor of cAMP phosphodiesterase, the differences in the sensitivity to this substance between the S49 wild type and mutant cells could possibly reflect extreme variability in the levels of this enzyme among the three cell types. Activities of the high affinity form measured in cell homogenates (Table II.1) revealed only slight variation of these activities among the S49 cell strains (Range: 1.19-1.67 pmol/min/mg protein). These data suggest that the increased resistance of the S49 protein kinase-deficient and S49 adenylate cyclase-deficient cell lines to the inhibitory effects of methylthioadenosine is not due to an enhanced capacity to degrade cAMP.

The cAMP phosphodiesterase activities from the three cell types were also examined for their sensitivity to methylthioadenosine (Table II.1). Under our standard assay conditions (0.25 μM cAMP and 250 μM methylthioadenosine), the activity of the wild type, protein kinase-deficient, and adenylate cyclase-deficient S49 cells were inhibited by; 60.0%, 51.4%, and 63.5%, respectively. These data indicate that the high affinity form of the phosphodiesterase in these three cell types are very similar with respect to their activity and their sensitivity to methylthioadenosine.

Figure II.2 Inhibition of high affinity, cAMP phosphodiesterase activity of S49 wild type by 5'-methylthioadenosine. Initial velocities were pmol of ^{3}H adenosine formed per mg of protein per min. Each point represents the average of three determinations. No inhibitor, (0); 62.5 μM, (●); 125 μM (■); and 250 μM, (▲).

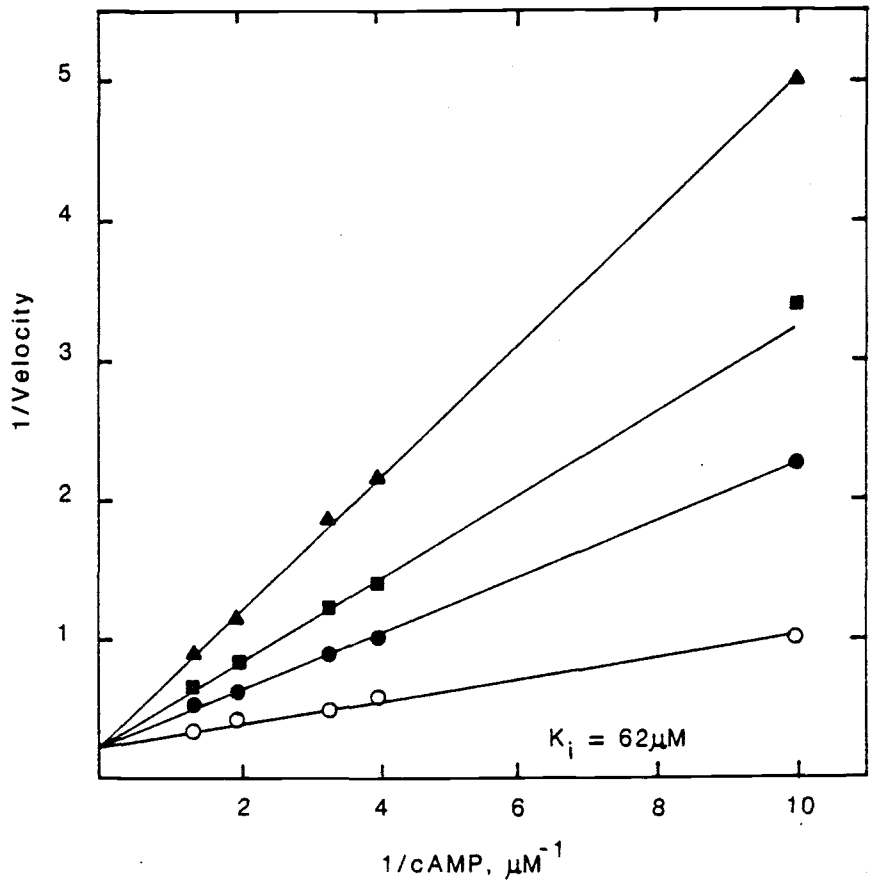

Figure II.2

Table II.1. Comparison of cAMP phosphodiesterase activities of S49 wild type and mutant cells.

Cell Type	High Affinity cAMP Phosphodiesterase Specific Activity (pmol/min/mg)[a]	% Inhibited by Methylthioadenosine[b]
S49 wild type	1.67	60.0
S49 adenylate cyclase deficient	1.53	63.5
S49 protein kinase deficient	1.19	51.4

[a]Specific activities were determined at 0.25 M substrate.

[b]Assay conditions were 40 mM Tris (pH 8.0), 5 mM $MgCl_2$, 3 mM 2-Mercaptoethanol, 0.25 µM [H^3] cAMP (300,000 cpm), enzyme extract and 250 µM methylthioadenosine.

Comparison of the cAMP response in S49 wild type and mutant cells after exposure to 5'-methylthioadenosine. S49 wild type and S49 protein kinase-deficient cells that had been exposed to 100 μM methylthioadenosine for 48 hrs exhibited markedly elevated levels of cAMP (Table II.2). The response in the wild type strain was a 17-fold increase in the intracellular concentration of the nucleotide. Nearly the same intracellular concentration of cAMP was found in the S49 protein kinase-deficient cells after 48 hrs in medium containing methylthioadenosine, representing a 6-fold enhancement in the cAMP levels. Under these same conditions, the S49 adenylate cyclase-deficient mutant cells did not contain detectable levels of cAMP. This is in agreement with the data of Coffino et al. (22) who also could not measure detectable levels of cAMP in this strain. Higher concentrations of methylthioadenosine were not used in these studies due to its extreme cytotoxicity to the wild type strain.

Table II.2. Effect of methylthioadenosine addition on cAMP pool size in S49 wild type and mutant cells.

	cAMP, pmol/10^7 cells		
Additions	S49 wt	S49 cyc$^-$	S49 pk$^-$
None	0.30	not detectable	1.15
Methylthioadenosine (100 μM)	5.56	not detectable	6.26

Cultures were seeded at 1 x 10^5 cells/ml in Dulbecco's modified Eagle's medium in the presence or absence of 100 μM 5'-methylthioadenosine. After 48 hrs. the cells were harvested by centrifugation and the nucleotides extracted in perchloric acid. The solution was cleared of the perchlorate salts by freon:alamine extraction, and cAMP levels were subsequently determined by the competition binding assay of Amersham. Cells were S49 wild type (S49 wt), S49 adenylate cyclase deficient (S49 cyc$^-$) and S49 protein kinase deficient (S49 pk$^-$). Results are the average of duplicate experiments where values did not vary more than 10%.

Discussion

The naturally occurring nucleoside, methylthioadenosine, has been described as a growth regulatory substance. Reports have demonstrated the cytostatic effects of this compound in murine T and B lymphocytes (3), as well as SV40 infected mouse fibroblasts (4). It has also been found to retard the progression of lymphocytes undergoing blastogenesis (2). Previous studies designed to elucidate its mode of action indicate that methylthioadenosine may exert its effects via the potent inhibition of polyamine biosynthesis (5,6). Pegg et al. (4) and Raina et al. (20), however, have observed that the biologic effects of methylthioadenosine cannot be reversed by the exogenous addition of the polyamines spermidine or spermine. Inhibition of S-adenosylhomocysteine hydrolase activity also has been proposed as the primary site of action (21). Zimmerman et al. (8), however, found that the inhibition by methylthioadenosine of lymphocyte-mediated cytolysis was unrelated to S-adenosylhomocysteine levels. On the basis of these results, it was suggested that other target sites must be considered. Zimmerman and co-workers (8) first proposed that this alternate site may be the high affinity cAMP phosphodiesterase. Although this work described the competitive inhibition of the enzyme by methylthioadenosine it did not assess the significance of this site of action to the inhibitory action of the nucleoside.

A central aim of this study was to use mutants defective in cAMP related functions to aid in determining the importance of cAMP in the growth inhibitory properties of methylthioadenosine. Like methylthioadenosine, high levels of cAMP arrest cellular growth (11) and influence a wide variety of biochemical processes (23). The effects of this regulatory nucleotide are thought to be mediated through the activity of a cAMP-dependent protein kinase (12). From this knowledge we predicted that if cAMP mediated the effects of methylthioadenosine, then mutant cells incapable of synthesizing cAMP (S49 adenylate cyclase-deficient) or incapable of responding to elevated levels of the nucleotide (S49 protein kinase-deficient) should have reduced sensitivity to methylthioadenosine. Our data show that these mutants are nearly ten-fold more resistant to the cytotoxic action of the nucleoside than the parental wild type strain and that this difference was not due to differences in methylthioadenosine phosphorylase activity. Correlating with these effects were increased levels of cAMP (up to 17-fold) in the S49 wild type and S49 protein kinase-deficient cells while the S49 adenylate cyclase-deficient cell line did not contain measurable levels of the nucleotide. In vitro experiments also demonstrated that the inhibitory potency of methylthioadenosine towards the S49-derived cAMP phosphodiesterase (K_i = 62 μM) was superior to that reported by Zimmerman and coworkers (8) in homogenates of murine cytolytic lymphocytes (K_i = 225 μM).

Taken together, our results suggest that the perturbation of cAMP metabolism by methylthioadenosine represents a primary site of action of this compound in S49 cells. Mechanistically, it appears that methylthioadenosine competitively inhibits the cytosolic enzyme responsible for the degradation of cAMP thereby leading to enhanced levels of the regulatory nucleotide and subsequent growth arrest.

It has been suggested that methylthioadenosine phosphorylase may be a useful target for chemotherapeutic or immunosuppressive agents (24). The data presented here indicate that nonmetabolizable structural analogs of methylthioadenosine which are also potent inhibitors of the cAMP phosphodiesterase should increase the effectiveness of such agents.

In conclusion, the primary growth regulatory properties of methylthioadenosine are transmitted through the ubiquitous second messenger, cAMP. The significance of this finding and its relationship to the regulation of cell division by either or both of these compounds must await further investigation. Knowledge of possible changes in the metabolism of methylthioadenosine throughout the cell cycle should aid in this endeavor.

CHAPTER III

5-METHYLTHIORIBOSE: Its Effects and Function in Mammalian Cells

Michael K. Riscoe and Adolph J. Ferro

Summary

The growth responses of 5-deoxy-5-methylthioribose on a 5'-deoxy-5'-methylthioadenosine phosphorylase-containing cell line (BW5147) and the methylthioadenosine phosphorylase-deficient cell line (L1210D) were examined. Methylthioribose was shown to dramatically affect these cells, increasing their growth rate, saturation density and viability. It was also found that methylthioadenosine could satisfy the methylthio dependence of the enzyme deficient cell line, L1210D. A model is proposed to explain the selective growth of methylthioadenosine phosphorylase-deficient cells in medium lacking a methylthio donor but containing fetal calf serum. It is hypothesized that cellularly exported methylthioadenosine is degraded to methylthioribose in the presence of medium containing serum of high methylthioadenosine phosphorylase activity (e.g., fetal calf serum). The resultant methylthioribose can then be used to satisfy the methylthio requirement of these cells. To test this theory, various purified preparations of bovine liver methylthioadenosine phosphorylase were used to artificially increase the specific activity of methylthioadenosine phosphorylase in horse serum. In each case, it was demonstrated that only medium containing serum of enzyme activity nearly equal to that of the glutathione-stimulated fetal calf serum activity, supported the growth of methylthio dependent cells in the absence of methylthio compounds. The data suggest that the degradation of methylthioadenosine and subsequent formation of

methylthioribose represents an essential process in the growth of mammalian cells.

Introduction

Mammalian cells in culture require a medium that is usually supplemented with animal serum. Some cell lines, however, exhibit an additional dependence for a sulfhydryl compound together with fetal calf serum (25). Toohey and Cline (26) showed that the requirement for sulfhydryl compounds can be replaced in these cells by compounds containing labile methylthio groups and, when a labile methylthio compound was present, the fetal calf serum could be replaced by purified proteins such as bovine serum albumin. Furthermore, it has also been postulated that a methylthio requirement exists for all cells on the basis that cells lacking 5'-deoxy-5'-methylthioadenosine phosphorylase need a methylthio source (e.g. cysteine methyl disulfide - Cys-S-S-CH_3) to support their growth (38). Toohey (27) has also suggested that cells containing methylthioadenosine phosphorylase, an enzyme which rapidly degrades methylthioadenosine to adenine and 5'-deoxy-5'-methylthioribose-1-phosphate (28), normally alleviate this requirement through the further metabolism of the phosphorylated product. Collectively, these results suggest that the degradation of methylthioadenosine is required for cell division.

Methylthioadenosine, a product of the polyamine biosynthetic pathway, has been shown to stimulate cell growth at very low concentrations in methylthioadenosine phosphorylase-containing cells (29), whereas it is toxic at both high and low concentrations to methylthioadenosine phosphorylase-deficient cells (30). Kamatani et al.

(31) demonstrated that, unlike cells which can rapidly degrade methylthioadenosine, methylthioadenosine phosphorylase-deficient cells export large quantities of the nucleoside into the culture medium. In this report, we explain the significance of the methylthioadenosine export process by methylthioadenosine phosphorylase-deficient cells to the ability of these cells to proliferate in the apparent absence of methylthio groups.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Minimal Essential Medium (MEM), MEM Selectamine Kit, calf and horse serum were obtained from GIBCO. Fetal calf serum was obtained from K. C. Biologicals. 5'-deoxy-5'-methylthioribose-1-phosphate was isolated from the reaction mixture of the rat liver methylthioadenosine phosphorylase assay (32). For large quantities of methylthioadenosine the general procedure of Kikugana et al. was used (33). 5'-methyl-[C^{14}] methylthioadenosine was prepared from S-adenosyl-L-methyl-[C^{14}] methionine (34), which was purchased from Amersham Corp. Cysteine methyl disulfide (Cys-S-S-CH_3) was synthesized and purified as described by Smith et al. (35).

Cell lines and cell culture procedures

BW5147, obtained from the Salk Institute, is oubain resistant T-lymphoma cell line from AKR mice. During logarithmic growth these cells exhibit methylthioadenosine phosphorylase activity of 68 pmol/min/mg protein (K_m = 5 μM) (29). The cells were grown in non-agitated suspension cultures containing Dulbecco's Modified Essential Medium, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% horse serum. L1210D, a methylthio-requiring mouse leukemia cell line was supplied by Dr. J. I. Toohey. Eagle's Minimal Essential Medium supplemented with 1.1 mg% pyruvate, 3 mg% glutamine, 5 mg% asparagine

1×10^{-4} M Cys-S-S-CH_3 and 10% calf serum was used to grow L1210D cells in nonagitated suspension cultures. All cultures were incubated at 37 °C in a 5% CO_2 humidified atmosphere. Cell number and viability were determined by the exclusion of trypan blue on a Neubauer hemacytometer. All sera utilized in this study were heat inactivated at 56 °C for 30 min. prior to use.

Paper chromatography

For descending chromatography, Whatman No. 1 sheets and three solvent systems were used: A. ethanol:water:acetic acid (65:34:1), B. n-butanol:acetone:acetic acid:water (70:70:20:40) and C. n-butanol:acetone:water (70:70:40) at pH 7.2 (Table III.1). The radioactive spots were detected using a Packard Model 7201 scanner.

Table III.1

Chromatography Systems and R_f Values

Compound	A	B	C
5'-methylthioadenosine	.67	.71	.67
5-methylthioribose	.73	.83	.73
5-methylthioribose-1-phosphate	.25	.65	.20
methionine	.49	.61	.40
2-keto-4-methiobutyrate	.76	.79	.56

Synthesis and Quantitation of Methylthioribose

5-Methylthioribose was prepared by the acid hydrolysis of 5'-methylthioadenosine (20 mg) in 4 ml of 1 M HCl at 100°C (36). Separation of the thiopentose was accomplished through cation exchange chromatography (Dowex 50 H^+). Concentrated samples were spotted onto silica gel F_{254} sheets and developed in a system containing ethyl acetate/methanol (9:1), purity was confirmed by detecting the sulfur-containing compounds with potassium iodoplatinate spray. The Methylthioribose concentration was determined using ribose as a standard in the phloroglucinol procedure detailed by Ashwell (37). Briefly, 5 ml of a reagent containing: glacial acetic acid (110 ml), concentrated HCl (2 ml), 0.8% glucose (1 ml), and 5% ethanolic phloroglucinol (5 ml) is added to 0.4 ml standard solutions (4-40 g aldopentose). The tubes are heated at 100°C for 15 min and cooled on ice. The difference in absorbance (A_{552} - A_{510}) is proportional to the concentration. Methylthioribose has been stored at -80°C for 4 weeks without decomposition.

Partial purification of methylthioadenosine phosphorylase from bovine liver

The purification scheme was adapted from work completed by Toohey (38) and Cacciapuoti et al. (39). Bovine liver samples were homogenized in Hepes-buffered saline containing 5% DMSO and 1 mM 2-mercaptoethanol, and the cells disrupted by the freeze-thaw method. After centrifugation, the pH of the supernatant was adjusted to 4.4.

Following removal of the copious precipitate, the solution was incubated at 60 °C for 1 min, centrifuged (12,000 x g, 10 min), and neutralized. This procedure cleared the supernatant of 92% of the original protein and yielded a 2.8- fold purification. The preparation was subsequently concentrated to one-third its volume by Amicon ultrafiltration a 50,000 M.W. exclusion filter followed by separation on a Sephadex G-200 column (2 cm i.d. x 75 cm) (Figure III.1. The leading 90,000 M.W. fractions were collected and concentrated by ultrafiltration providing a 170-fold purification. After dialysis to remove free mercaptans, the preparation was applied to an organomercurial agarose column (BioRad 501, 1 cm i.d. x 15 cm) followed by successive elutions of: a) 10 ml 100 mM phosphate (pH 7.4); b) 60 ml 2 M KCl; c) 60 ml 1 mM 2-mercaptoethanol; d) 60 ml 2 M KCl and 10 mM dithiothreitol. Fraction d was concentrated by ultrafiltration to 2.8 ml and dialyzed for 2 hr against 4 changes of buffer containing 100 mM phosphate (pH 7.4), 1 mM DTT, and 5% DMSO. The final enzyme yield was 1.6% and the preparation proved to be 471-fold purified (Table III.2). On sodium dodecylsulfate/polyacrylamide gels, the denatured enzyme preparation exhibited a single intense band with M_r = 31,000 and four faint bands with higher molecular weight values (Figure III.2).

Figure III.1. Purification of methylthioadenosine phosphorylase on Sephadex G-200.

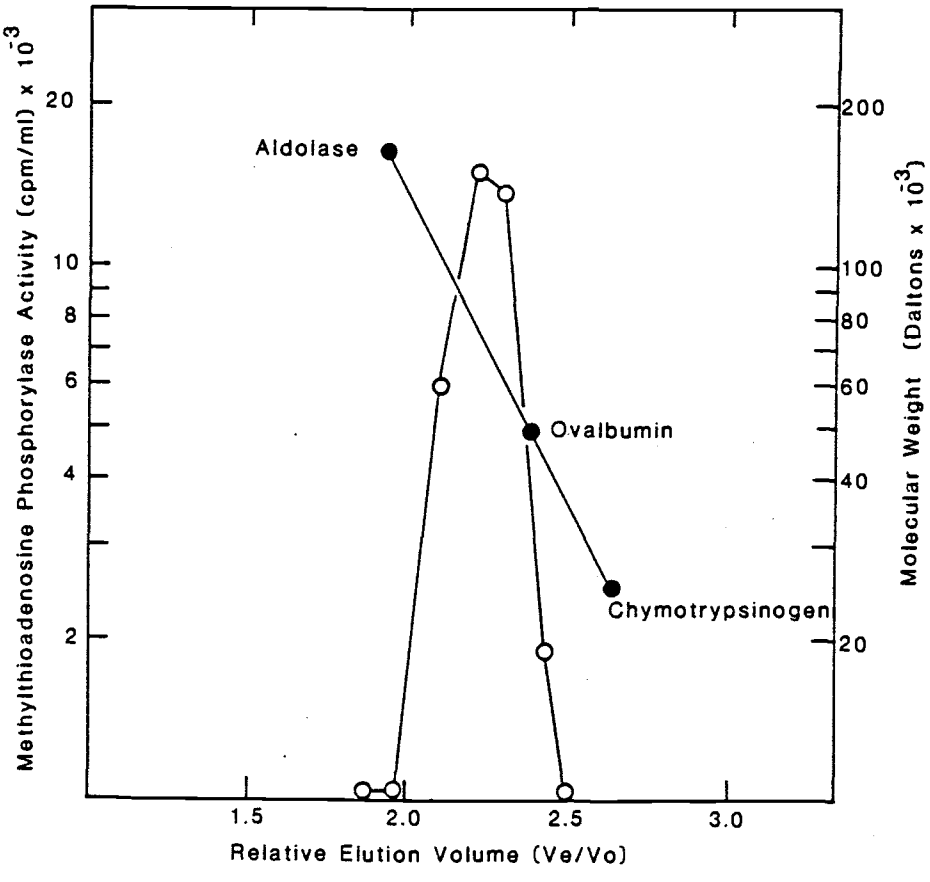
Methylthioadenosine Phosphorylase Activity (cpm/ml) x 10^{-3}
20
10
8
6
4
2
Aldolase
Ovalbumin
Chymotrypsinogen
200
100
80
60
40
20
Molecular Weight (Daltons x 10^{-3})
1.5
2.0
2.5
3.0
Relative Elution Volume (Ve/Vo)

Figure III.1

Figure III.2. SDS-polyacrylamide gel electrophoresis of purified 5'-methylthioadenosine phosphorylase.

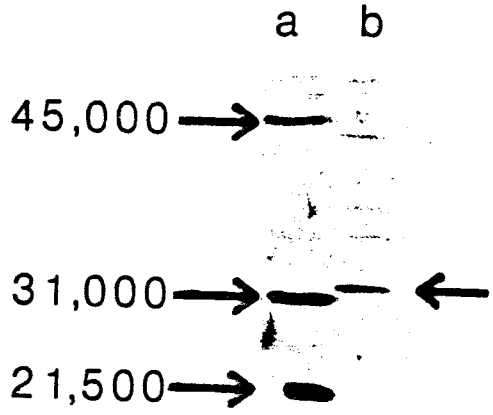

Figure III.2

Table III.2

Purification of 5'-methylthioadenosine Phosphorylase

Purification Step	Volume (ml)	Total Protein	Total Units[a]	Specific Activity (units mg/min)	Yield (%)	Purification Factor
Crude extract	41	3239	176.3	0.054	100	1
pH and heat treatment	64	228	42.9	0.149	24.3	2.8
Sephadex G-200	6	3.06	28.2	9.216	16.0	170.6
Organomercurial agarose	2.8	0.11	2.8	25.45	1.6	471.3

[a]Units are expressed a nmol of substrate converted per min.

Methylthioadenosine phosphorylase assays

Methylthioadenosine phosphorylase activity was determined using radioactively labeled substrate as previously described (40). All standard assay mixtures were incubated at 37°C for 30 min and protein determinations were made by the method of Lowry et al. (41).

Exhaustive dialysis of heat-denatured fetal calf serum

As described by Toohey (42), fetal calf serum was dialyzed in order against each of the following at 4°C: two changes of a solution containing 0.05 M NaCl and 0.1 M glutathione pH 7.2 over 24 hr, eight changes of PBS over 4 days, and two changes of MEM over 24 hr.

Results

Effect of methylthioribose on the proliferation and viability of BW5147 cells

Low concentrations of methylthioadenosine have been shown to stimulate the proliferation of methylthioadenosine phosphorylase-containing cells in culture (29). Since methylthioadenosine is rapidly degraded in these cells (43), the data suggest that the stimulatory effect may be mediated by a catabolite of this reaction, methylthioribose-1-phosphate or adenine. Methylthioribose-1-phosphate is a phosphorylated compound and is unlikely to be transported, and we have found that adenine is not stimulatory to growth at concentrations from 10 μM to 1 mM (data not shown). Since the formation of methylthioribose has been demonstrated in human erythrocytes incubated with methylthioadenosine (44), the ability of methylthioribose to affect the growth of BW5147 cells (methylthioadenosine phosphorylase-containing) was examined (Figure III.3). At concentrations as low as 10^{-4} M methylthioribose, a decrease in generation time from 16.5 hrs to 12 hrs was observed. This effect was more pronounced as the concentration of methylthioribose was increased to 1 mM. The cultures containing methylthioribose also exhibited up to a 2-fold increase in their final cell density at concentrations of methylthioribose above 25 μM. These data suggest that methylthioribose or a metabolite of methylthioribose may mediate the methylthioadenosine stimulatory effect.

Figure III.3 Growth response of BW5147 cells to methylthioribose. BW5147 cells were cultured in DMEM containing 10% horse serum supplemented with either: 0, none; ●, 10 μM; ■, 25 μM; or ▲, 1.0 mM.

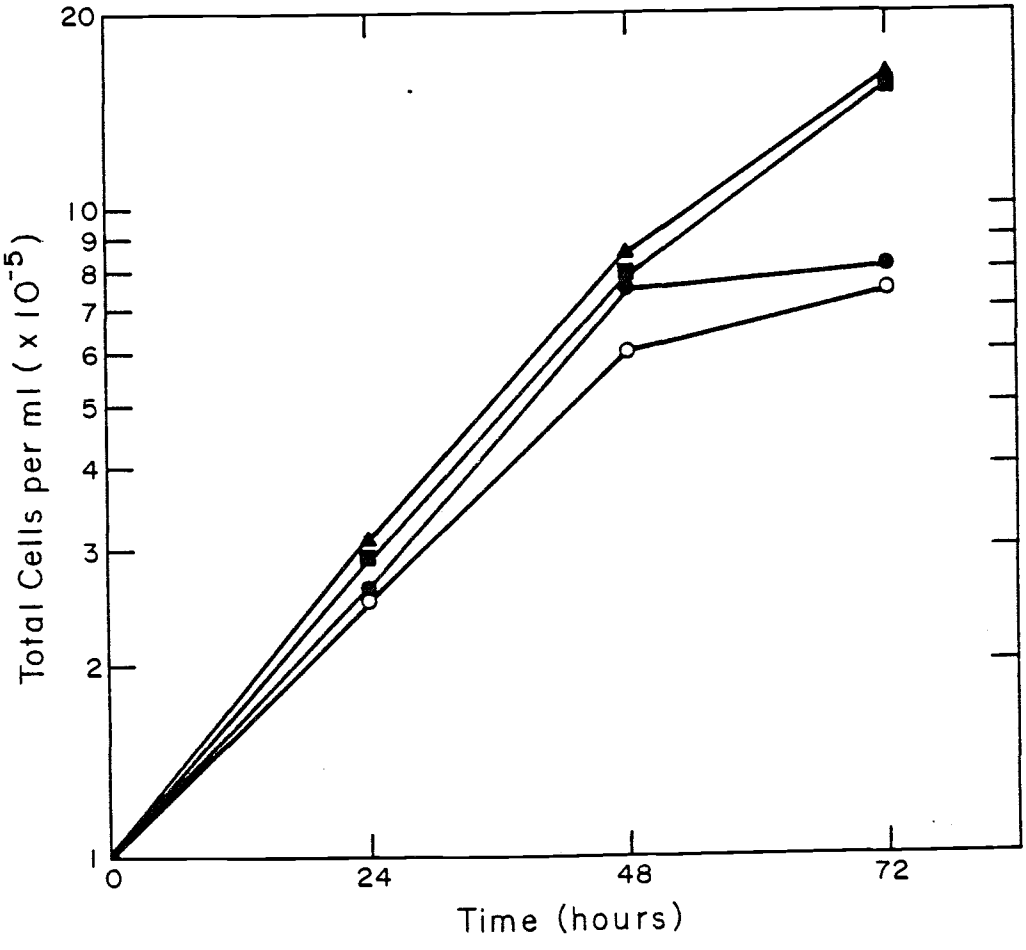

Figure III.3

The ability of methylthioribose to affect the viability of BW5147 cells in culture as measured by the Trypan Blue dye exclusion method was also examined (Figure III.4). The differences between the culture in which no additions were made, and those supplemented with methylthioribose were evident after 3 days; 21% of the cells in the control culture remained viable (data not shown), whereas 65% and 73% of the cells were viable in the culture containing 250 μM and 500 M methylthioribose, respectively. These dose responsive effects were most apparent at 1 mM methylthioribose where greater than 99% of the cell population excluded the dye after 72 hrs. Taken together, these results show that methylthioribose causes some striking biologic effects in mammalian cells leading to increased saturation density, viability, and growth rate.

Methylthioribose is not a source of methionine in BW5147 cells

Backlund and Smith (45) reported a system in rat liver which converted methylthioadenosine to methionine via methylthioribose-1-phosphate. Shapiro et al. (46) have reported a similar system in E. aerogenes involving methylthioribose. To investigate the possibility that intermediates of the methionine salvage pathway may be involved in the methylthioribose and methylthioadenosine-mediated effects, we tested the ability of methylthioribose and methylthioadenosine, respectively, to serve as a methionine source for BW5147 cells (Figure III.5). Cell proliferation was observed in methionine-deficient medium containing up to 250 μM methylthioadenosine. Under

Figure III.4 Stimulation of the viability of BW5147 cells by methylthioribose. BW5147 cells were cultured in DMEM containing 10% horse serum and variable amounts of methylthioribose. Results represent the average of duplicate experiments obtained after 72 hrs of culture growth.

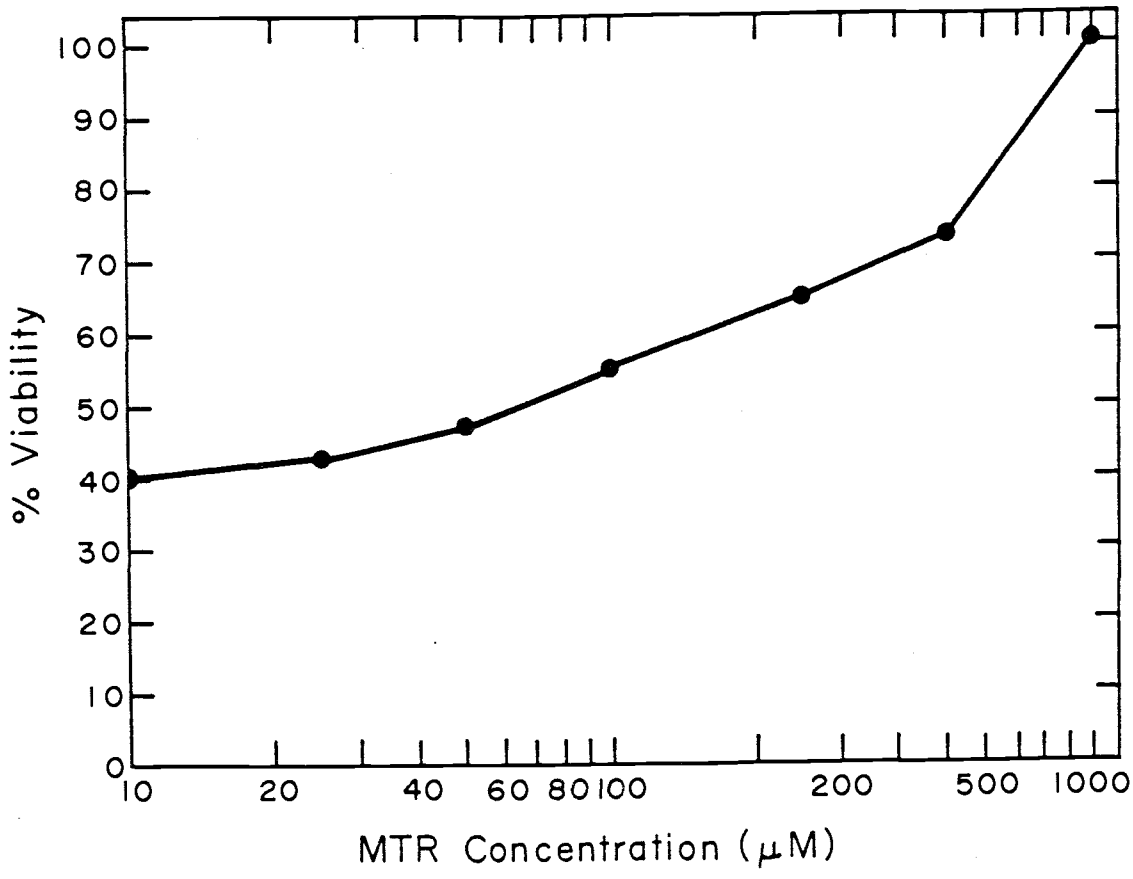
100
90
80
70
60
50
40
30
20
10
0
% Viability
10
20
40
60
80
100
200
500
1000
MTR Concentration (μM)

Figure III.4

these same conditions of deprivation, methylthioribose was found not to support cell growth at any of the concentrations tested. Furthermore, our efforts to detect methylthioribose kinase activity in these and other mammalian cells have proved negative. These data suggest that methylthioribose-mediated stimulatory effects are the result of events independent of methionine salvage.

Methylthioribose as a methylthio source in L1210 cells. The ability of methylthioribose to support the growth of a methylthioadenosine phosphorylase-deficient, methylthio-dependent cell line (L1210D) was examined (Figure III.6). In the absence of a methylthio donor, the cells did not proliferate. In the presence of 1×10^{-4} M Cys-S-S-CH_3, the cells proliferated for 3 days and rapidly lost viability. Methylthioribose (10 μM - 1 mM) also supported the growth of these cells in a dose-dependent manner, but unlike the Cys-S-S-CH_3 containing culture, methylthioribose-grown cells did not lose viability after 3 days. The Cys-S-S-CH_3 culture was 20% viable after 5 days, whereas the 1 mM methylthioribose-supplemented culture was 85% viable. These results demonstrate that the naturally occurring compound, methylthioribose, can support the growth of methylthio-dependent cells in the absence of any other methylthio source.

Figure III.5 Ability of 5'-methylthioadenosine and 5-methylthioadenosine and 5-methylthioribose to serve as a methionine source in BW5147 cells cultured in methionine-deficient medium. Cells were seeded at 1 x 10^5/ml into MEM containing 10% horse serum and various concentrations of methylthioadenosine (●) and methylthioribose (○). Cell number was determined after 72 h.

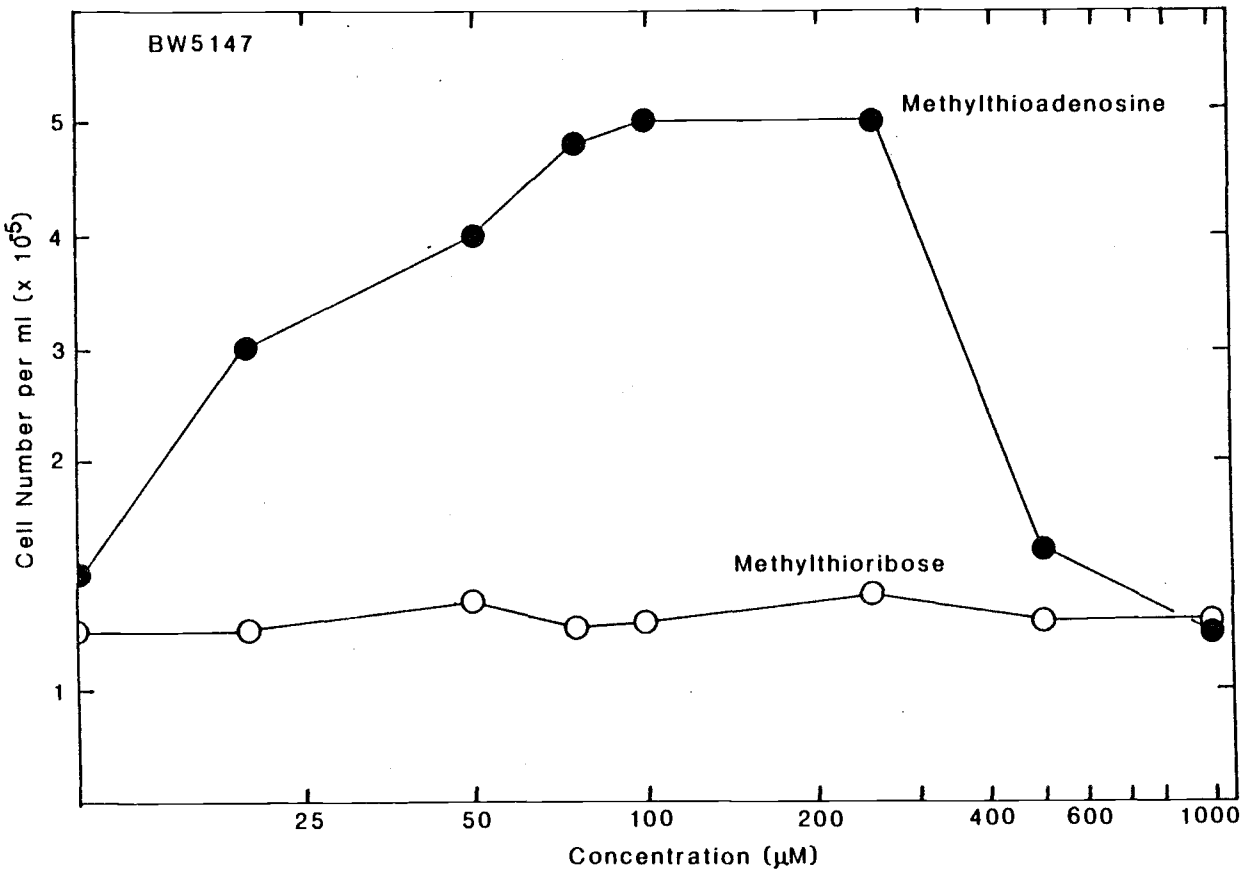
BW5147
Methylthioadenosine
Methylthioribose
Cell Number per ml (x 10^5)
1
2
3
4
5
25
50
100
200
400
600
1000
Concentration (μM)

Figure III.5

Figure III.6 Ability of methylthioribose to satisfy the methylthio requirement of L1210D cells. Cells were cultured in MEM containing 30 mg% glutamine, 10% calf serum, and either 1 x 10^{-4} M (Cys-S-S-CH_3) (▲) or the following concentration of methylthioribose: ○, none; ●, 100μ M; □, 0.5 mM; △, 1.0 mM.

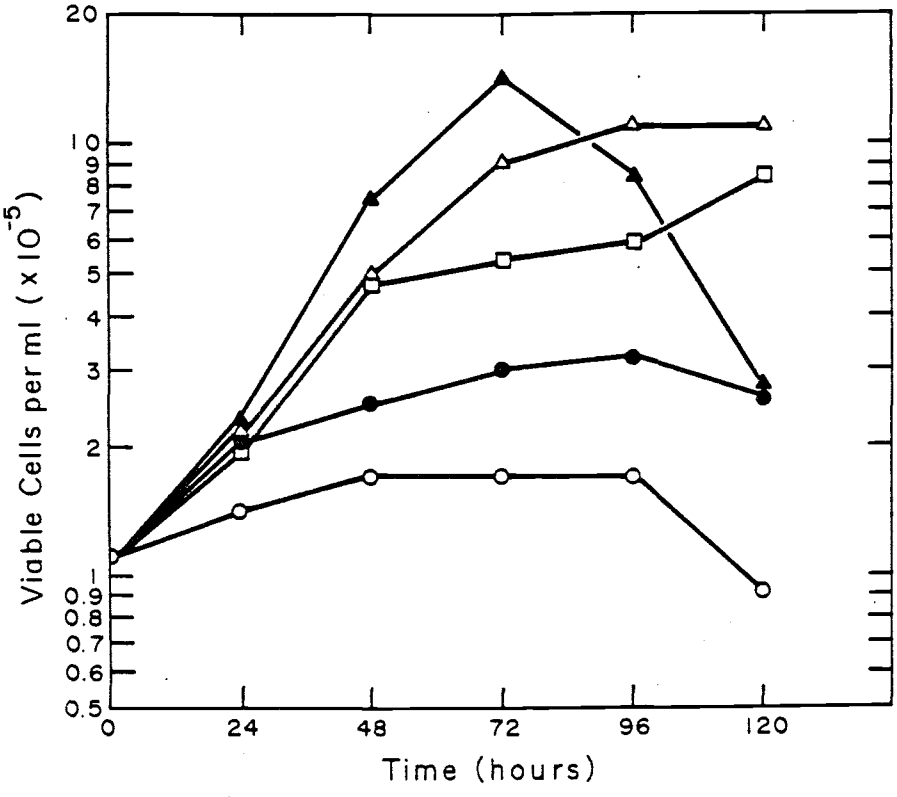
Viable Cells per ml (x 10^{-5})
20
10
9
8
7
6
5
4
3
2
1
0.9
0.8
0.7
0.6
0.5
0
24
48
72
96
120
Time (hours)

Figure III.6

Effect of various sera and methylthio donors on the proliferation of L1210D cells

Toohey (26) demonstrated that methylthio-dependent cells could grow in culture medium in the absence of a methylthio compound, but supplemented with fetal calf serum (10%) and one of a variety of sulfhydryl compounds, including glutathione (GSH). He also demonstrated that fetal calf serum is unique among sera in being able to do so (41, 42); that is, neither calf serum nor horse serum supplemented with GSH could support the growth of these cells. Experiments were performed to compare the ability of Cys-S-S-CH_3, methylthioribose, and GSH to support the growth of L1210D cells on various sera (Table III.3). None of the sera alone supported the growth of the cells and only fetal calf serum did so in the presence of GSH, confirming the findings of Toohey (42). Cys-S-S-CH_3 supported growth under all conditions tested, especially with 10% calf or fetal calf serum, in which case each of these cultures reached the saturation density of 1.4×10^6 cells/ml by 72 hrs. Methylthioribose supported proliferation only when serum was supplemented to the culture medium. Toohey (26) also found that Cys-S-S-CH_3 could support the proliferation of methylthio-dependent cells in the absence of serum when 0.3% bovine serum albumin was added to the medium. Under conditions in which bovine serum albumin was used as the protein source, only minimal growth was observed in the presence of methylthioribose. Unlike Cys-S-S-CH_3, methylthioribose has not been able to sustain the continuous passaging of L1210D cells in the absence of serum. The data

Table III.3 Effect of various sera and methylthio compounds on the growth of L1210D cells.

Additions	Cells per ml (x 10^{-5}) at 72 hrs[a]			
	10% Horse serum	10% Calf serum	10% Fetal calf serum	0.3% BSA
Control (no addition)	1.1	1.6	1.3	1.1
Glutathione (2 x 10^{-3} M)	1.3	1.2	14.0	1.4
Cys-S-S-CH_3 (1 x 10^{-4} M)	10.6	14.0	14.0	8.75
Methylthioribose (1 x 10^{-3} M)	7.3	8.9	11.5	3.0

[a]Cultures were inoculated at 1 x 10^5 cells/ml.

data demonstrate that methylthioribose is like Cys-S-S-CH_3 in that growth occurs when any serum is utilized and it is dissimilar to Cys-S-S-CH_3 in that only the latter compound will support the active proliferation of cells in the absence of serum.

Differences in the 5'-methylthioadenosine phosphorylase activities among sera

Cacciapuoti et al. (39) found that reduced sulfhydryl compounds are required for optimal activity of methlthioadenosine phosphorylase in rat placenta. To determine possible enzymatic differences, methylthioadenosine phosphorylase activities of sera were measured in the presence and absence of GSH (Table III.4). As noted by Kamatani et al. (47) horse serum exhibited no detectable enzyme activity while fetal calf serum contained high activity. We found that heat-denatured calf and fetal calf sera contain 59 and 54 units (pmol/min) per ml of activity, respectively. Of these three sera, only the fetal calf serum activity was appreciably stimulated by the presence of 2 mM GSH. Clearly, a 2.6 fold increase in activity was demonstrated under these conditions. Human serum also exhibited high methylthioadenosine phosphorylase activity. The exhaustive dialysis protocol utilized by Toohey (see Materials and Methods) to remove methylthio groups associated with fetal calf serum destroyed the ability of GSH to stimulate methlythioadenosine phosphorylase activity. Whether this procedure inactivates a different form of the enzyme or only its ability to respond to reduced sulfhydryls is not known.

Table III.4 5'-Methylthioadenosine phosphorylase activities in various sera in the presence and absence of 2 mM GSH.

Additions	MTA Phosphorylase Activity[a] (units/ml)			
	Horse Serum	Calf Serum	Fetal Calf Serum	Human Serum
Control (no additions)	0	59	54	ND[c]
+ Glutathione (2×10^{-3} M)	0	70	141	99
+ Glutathione (2×10^{-3} M)	ND[c]	ND[c]	62	ND[c]

[a]5'-Methylthioadenosine phosphorylase activity was determined as described in Materials and Methods. Reactions containing up to 20 mg protein were incubated for 30 min at 37° C and stopped by the addition of ethanol. One unit of activity is defined as the conversion of 1 pmol of substrate in 1 min.

[b]The dialysis procedure is described in Materials and Methods.

[c]Not determined.

The production of methylthioribose from methylthioadenosine in fetal calf serum

Methylthioadenosine phosphorylase activity in fetal calf serum in the presence of 2 mM GSH is greater than in any other serum examined in this study. An investigation into the metabolism of methylthioadenosine in fetal calf serum under these conditions was undertaken to explain the relationship between the export of methylthioadenosine from methylthio-dependent cells and their ability to proliferate in a medium containing fetal calf serum (10%) and 2 mM GSH as a methylthio source. 5'-methyl-[$^{14}CH_3$]methylthioadenosine was incubated under standard methylthioadenosine phosphorylase conditions with 2 mM GSH and either 0.3% bovine serum albumin (control) or 30% fetal calf serum. The reactions were allowed to proceed for 1 hr and stopped with ethanol. Separation of the reaction products from the radioactive substrate was accomplished by descending paper chromatography in a solvent system consisting of ethanol:water:acetic acid (65:34:1). The distribution of radioactivity in the products was analyzed by radiochromatogram scanning (Figure III.7). The R_f values for methylthioadenosine, methylthioribose and methylthioribose-1-phosphate are 0.71, 0.83 and 0.65, respectively. The degradation of methylthioadenosine and the appearance of two products, identified as methylthioribose-1-phosphate and methylthioribose, was demonstrated. The identification of the two products was verified in two other solvent systems. To quantitate the radioactivity under the peaks,

Figure III.7 Radiochromatogram scans of the products formed after incubation of 5'-[methyl-^{14}C]methylthioadenosine with fetal calf serum. The control vessel (A) contained purified bovine serum albumin. The experimental protocol was as described in Materials and Methods.

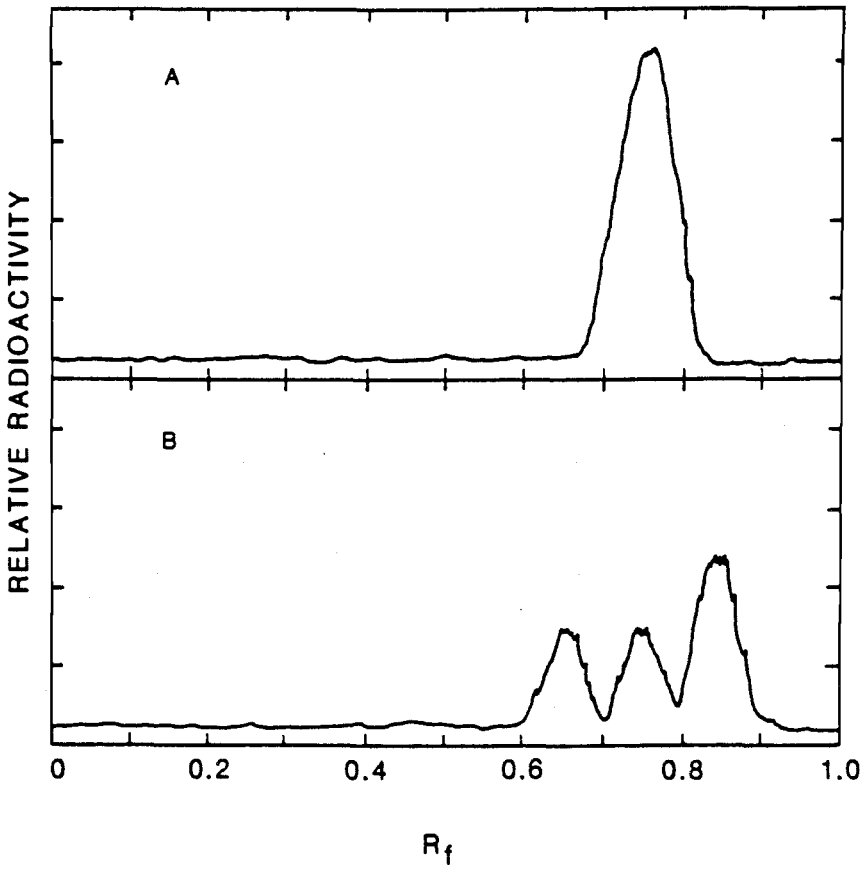

Figure III.7

these spots were cut out and the radioactivity determined by scintillation counting (Table III.4). In the absence of fetal calf serum, methylthioadenosine remained unreacted. However more than 40% of the original substrate was converted to methylthioribose after only 1 hr of incubation in fetal calf serum and GSH. In fact, under these conditions methylthioribose was found to be the major product of the reaction. These data indicate that fetal calf serum methylthioadenosine phosphorylase activity stimulated by GSH can be considered to be a methylthioribose "generating" system when methylthioadenosine is present.

Growth of methylthio dependent cells on methylthioadenosine phosphorylase

Kamatani and Carson (31) demonstrated that methylthioadenosine accumulated in the culture medium from cells deficient in this enzyme. More recently, White and co-workers (42) have demonstrated that methylthioadenosine is exported from L1210D cells and also accumulates in the culture medium. In each of these cases 10% horse serum, was utilized. The correlation between the ability of the various sera to support the growth of methylthio-dependent cells and their enzymatic ability to convert cellularly exported methylthioadenosine into methylthioribose was examined. Methylthioadenosine phosphorylase was partially purified from bovine liver (see Materials and Methods). Samples of the enzyme preparations from different steps of this purification procedure (2.8, 170, and 471 fold purified) were utilized in this study. Increasing quantities of these preparations

Table III.5 Distribution of radioactivity after incubation of 5'-[^{14}CH$_3$]methylthioadenosine with fetal calf serum and glutathione.

Addition	Radioactivity[a] (% of total)		
	MTR-1-P	MTA	MTR
None	-	25,300 (100%)	-
30% FCS + 2 mM GSH	7,300 (25%)	7,000 (28%)	10,900 (43%)

[a]Aliquots of deproteinized reaction mixtures were applied to paper chromatograms (Whatman No. 1) and the substrate and products were separated using a solvent system consisting of; Ethanol:Water:Acetic acid (65:34:1). The radioactive compounds were located with a chromatogram scanner, cut out and the radioactivity determined by scintillation counting.

were added to culture medium containing 10% horse serum thus providing the serum with a range of methylthioribose-generating capacities between zero and 40 units per ml of medium. The effect on the growth of L1210D cells by varying the methylthioadenosine phosphorylase activities in a culture medium devoid of an apparent methylthio source is shown in Figure III.8. After 96 hrs of incubation, only a slight increase in cell number was observed in the control culture (1.6 to 2.0 x 10^5 cells/ml) supplemented with 10% horse serum and 2 mM GSH. Increasing the methylthioadenosine phosphorylase activity in the medium led to enhanced proliferation in each culture, regardless of the enzyme purity. In cultures containing 5 units/ml of methylthioadenosine phosphorylase activity in their medium (equal to that found in calf serum with GSH), an increase in cell number from 1.6 to 2.5 x 10^5 cells/ml was noted after 4 days; whereas, when this activity was adjusted to nearly equal that of fetal calf serum (14 units/ml), the cell number increased more than 5-fold in each culture over the same period. At methylthioadenosine phosphorylase activities of 20 or 40 units/ml, the effects were even more dramatic, as the cell number reached saturation at 1.0 and 1.1 x 10^6 cells/ml, respectively.

The data suggest that the ability of fetal calf serum and 2 mM GSH to satisfy the methylthio dependency of L1210 cells is due to the increased capacity of the medium components to degrade exported methylthioadenosine into methylthioribose, which can then be used to satisfy the methylthio requirement. Here we demonstrate the ability

Figure III.8 Growth response of methylthio-dependent cells (L1210D) to culture medium of differing 5'-methylthioadenosine phosphorylase activity. L1210D cells were inoculated at 1.5×10^5 cells/ml into medium containing 2 mM GSH and 10% horse serum. Adjustment of the activity (units/ml medium) was accomplished by the addition of partially purified enzyme preparations. 2.8-fold (▲), 170-fold (■), and 471-fold (●). (Arrows indicate the activities found in medium containing 2 mM glutathione and 10% fetal calf serum or 10% calf serum.) Cell number was determined after 96 hrs of incubation.

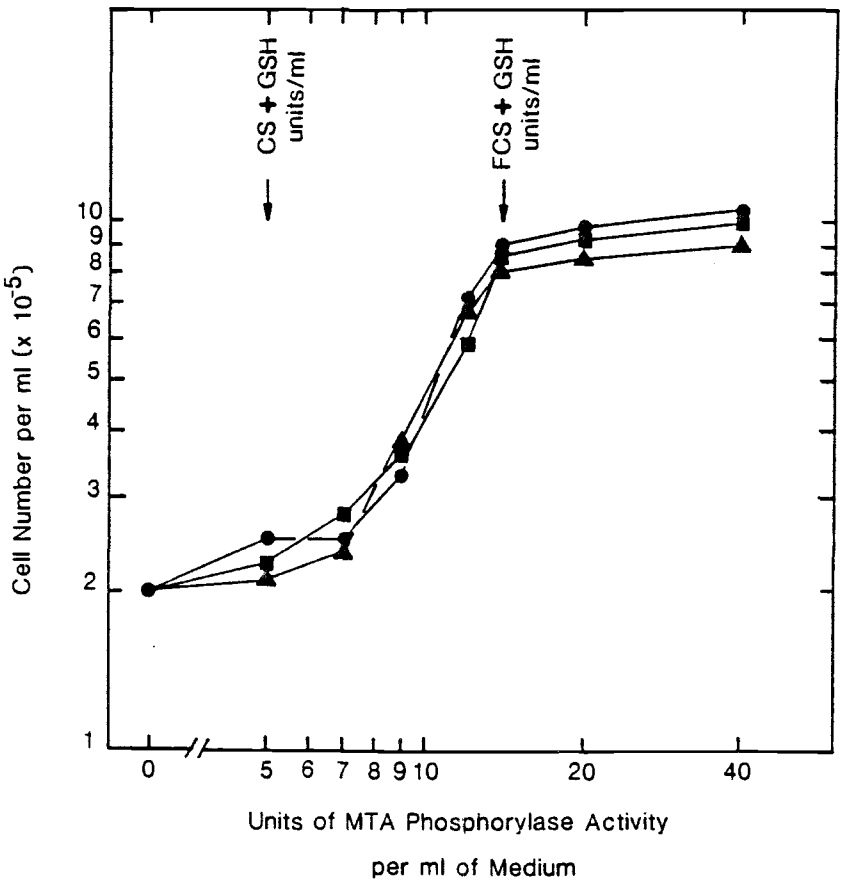
CS + GSH units/ml
FCS + GSH units/ml
Cell Number per ml (x 10⁻⁵)
10
9
8
7
6
5
4
3
2
1
0
5
6
7
8
9
10
20
40
Units of MTA Phosphorylase Activity
per ml of Medium

Figure III.8

of horse serum to satisfy this requirement only when its methylthioadenosine phosphorylase activity is artificially increased to nearly equal that of GSH-stimulated fetal calf serum.

Discussion

Low concentrations of methylthioadenosine stimulate proliferation in certain cell systems. That this effect is only exhibited in methylthioadenosine phosphorylase-containing and not in methylthioadenosine-deficient cells suggests that the degradation of methylthioadenosine is required for this response. There have been reports of the isolation of methylthioribose from Escherichia coli (48) and yeast (49) and of its in vitro formation in soluble enzyme extracts from methylthioadenosine (50); however, its significance in or effects on mammalian cells has never been described. We have demonstrated that the addition of low concentrations of methylthioribose to the culture medium increased the growth rate, saturation density and viability of the methylthioadenosine phosphorylase-containing BW5147 cell line.

The mechanism by which methylthioribose evokes these responses does not appear to involve methionine. Even though methylthioribose is known to be a precursor of methionine in Enterobacter aerogenes (45), only methylthioribose-1-phosphate and not methylthioribose may serve as a precursor of this amino acid in mammalian tissues (44). Furthermore, 200 μM methionine was present in all of the media used and thus cannot be considered to be in limiting supply. Instead, Toohey et al. (26) demonstrated that alkylthio groups are essential for cell division and have provided evidence that the metabolism of methylthioadenosine was required for the generation of these groups.

It was also demonstrated that cells incapable of degrading methylthioadenosine exhibited a methylthio dependency. Toohey (51) has previously suggested that 2-keto-4-methylthiobutyrate, an intermediate in the methionine salvage pathway (44), is the critical methylthio donor in mammalian cells. We have found that methylthioribose cannot satisfy a methionine deficiency in either BW5147 or L1210D cells, therefore, its effects appear to be distinct from methionine salvage. The ability of methylthioribose to support the growth of L1210D cells represents the first demonstration of the use of a naturally occurring compound to satisfy the methylthio requirement.

To date, the methylthio dependency has been relieved by the addition to the growth medium of a combination of 10% fetal calf serum and a reduced sulfhydryl compound or 0.3% bovine serum albumin and the labile methylthiodonor, Cys-S-S-CH_3. It has been hypothesized that labile methyl mercaptans are present in fetal calf serum as disulfides of serum proteins and that these are released and transported into the cell by the sulfhydryl compounds (41). The theory is supported by the observation that an extensive long-term dialysis protocol designed to remove these bound mercaptans from serum also renders the serum incapable of supporting the growth of methylthio requiring cells in the presence of GSH.

An alternate interpretation of this phenomenon exists. The presence of methylthioadenosine phosphorylase activity in fetal calf serum has been previously noted (47). Cacciapuoti et al. (38) and Zappia et al. (52) have demonstrated that enzyme activity from human

placenta and prostate gland is greatly influenced by the presence of reduced thiols. We have shown that methylthioadenosine phosphorylase activity in heat-inactivated fetal calf serum is markedly increased, 2.6 fold, in the presence of 2 mM GSH. Also of interest is that this stimulation could not be observed after the exhaustive, long term dialysis procedure described by Toohey. These findings are of importance in light of recent studies by Kamatani and Carson (31). These investigators found that cells unable to metabolize methylthioadenosine actually excrete large quantities of this nucleoside into the culture medium. Not surprisingly, the accumulation of this compound was measured in medium containing 10% horse serum, which is totally devoid of methylthioadenosine phosphorylase activity. Under our standard in vitro assay conditions for the methylthioadenosine phosphorylase activity of fetal calf serum, we found that more than 70% of the original substrate had been degraded to methylthioribose-1-phosphate after 1 hr. In fact, most of the phosphorylated sugar (60%) had been subsequently converted to methylthioribose.

Collectively, these data suggest an alternate model for the growth of methylthioadenosine phosphorylase-deficient cells in medium supplemented with fetal calf serum and reduced sulfhydryl compounds (Figure III.9). According to this model, intracellular methylthioadenosine, which is formed primarily during the synthesis of polyamines (31), is excreted into the surrounding medium. The elevated methylthioadenosine phosphorylase activity of GSH-stimulated fetal calf serum rapidly degrades the methylthioadenosine to methylthiori-

Figure III.9 Proposed metabolic pathway describing the growth of a 5'-methylthioadenosine phosphorylase-deficient cell line in medium containing fetal calf serum and glutathione but in the absence of compounds containing labile methylthio groups.

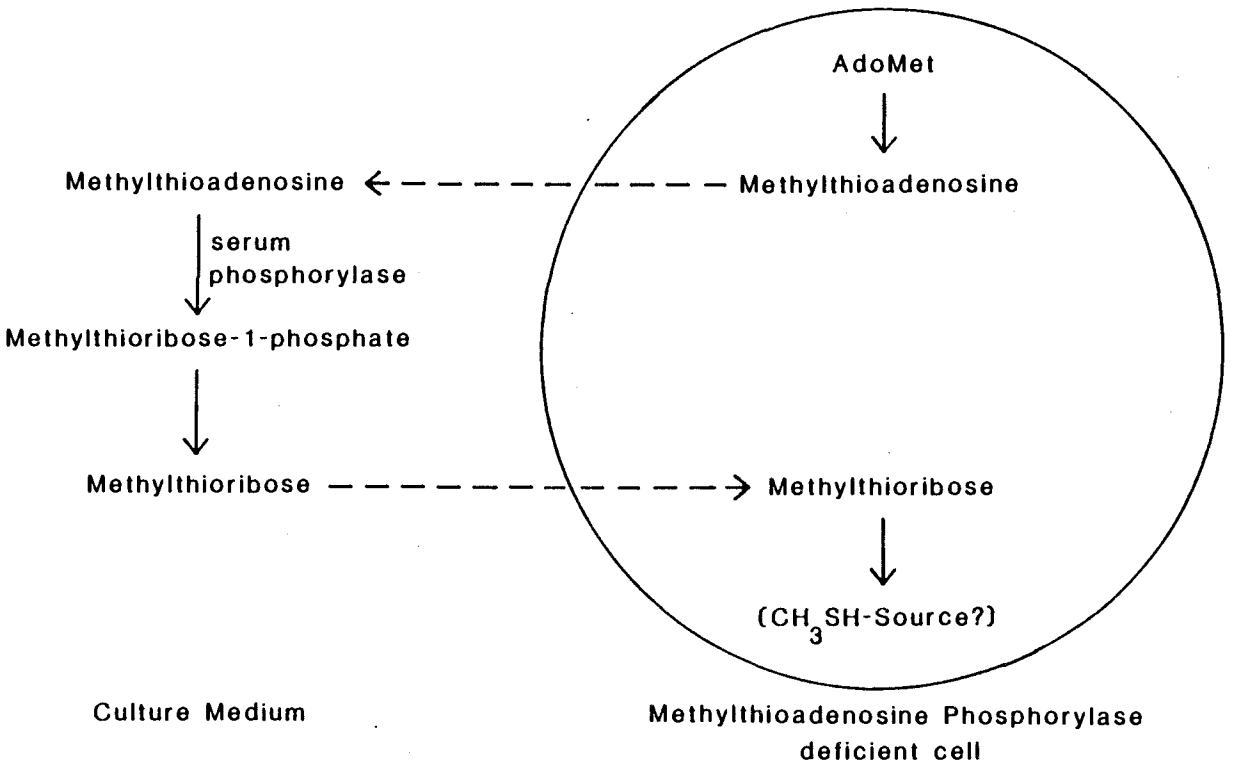
AdoMet
Methylthioadenosine
Methylthioadenosine
serum
phosphorylase
Methylthioribose-1-phosphate
Methylthioribose
Methylthioribose
(CH3SH-Source?)
Culture Medium
Methylthioadenosine Phosphorylase
deficient cell

Figure III.9

bose-1-phosphate, which is subsequently converted to methylthioribose. The external formation of methylthioribose from cellularly derived methylthioadenosine can then satisfy the methlythio requirement of methylthioadenosine phosphorylase-deficient cell.

In support of this theory is the inability of these cells to thrive on medium containing serum of low or no methylthioadenosine phosphorylase activity, e.g. calf serum, horse serum or fetal calf serum not supplemented with GSH. In fact the growth of the L1210D cell line was accomplished by simply adjusting the methylthioadenosine phosphorylase specific activity to approximately equal the level observed in the GSH-stimulated fetal calf serum condition.

The results presented in this report may aid in the understanding of observations made previously. Kamatani et al. (47) have described the passage of methylthioadenosine phosphorylase-deficient cells in medium containing fetal calf serum but in the apparent absence of a methylthio donor. If these investigators utilized non-heat-denatured fetal calf serum in these studies (unstimulated activity = 105 units/ml) then our model involving cellularly exported methylthioadenosine and the subsequent generation of methylthioribose by the high serum methylthioadenosine phosphorylase activity may explain this observation.

Kamatani et al. (47) have also described a procedure for the selective killing of methylthioadenosine phosphorylase-deficient tumors by the inhibition of de novo purine biosynthesis in the presence of exogenously provided methylthioadenosine. The chemother-

apeutic regimen is based on the selective ability of normal cells to salvage the adenine moiety of methylthioadenosine needed for DNA synthesis. Since we have found high levels of methylthioadenosine phosphorylase activity in human serum, we question the application and selectivity of this scheme in vitro. Our work suggests that an alternate scheme designed to select for the ability of normal cells to salvage methionine from methylthioadenosine may be useful in the treatment of enzyme deficient tumors.

Finally, several reports have also described the inhibition of cell growth by inhibitors of methylthioadenosine phosphorylase (28, 39, 42, 53) or polyamine biosynthesis (54, 55, 56). In light of our observation on the importance of the generation of methylthioribose for cell growth, we suggest that these inhibitors may also starve the targeted cells for methylthioribose or one of its metabolites and thereby represent an important mode of action for toxicity by these compounds.

In conclusion, we have shown that methylthioribose can satisfy the methylthio requirement of methylthioadenosine phosphorylase-deficient cell line. We have demonstrated that the stimulated methylthioadenosine phosphorylase activity of fetal calf serum in the presence of GSH is capable of converting the cellularly exported methylthioadenosine of an enzyme deficient cell into methylthioribose which then may be utilized as a methylthio source. Also, that labile methylthio donors can support the growth of methylthioadenosine phosphorylase-deficient cells in the absence of serum whereas methyl-

thioribose cannot suggests that a serum component(s) is needed for further processing of methylthioribose.

CHAPTER IV

The Presence of Methylthioribose in Serum

Authors: Michael K. Riscoe, John H. Fitchen,
Donald A. Griffin and Adolph J. Ferro

Summary

A naturally occurring sulfur-containing sugar, 5-deoxy-5-methylthioribose, was isolated from commercially available fetal calf serum. Methods used to compare the isolated material to an authentic standard were chemical analysis, paper, gas, and thin-layer chromatography, enzyme specificity, and mass spectrometry.

A method was developed for the analysis of methylthioribose in biological fluids. The procedure utilizes dansylhydrazine as a fluorescent labeling reagent and its separation and quantitation by reverse phase high performance liquid chromatography. Good separation of most common sugars was obtained within 30 min. after injection while the detection limit for methylthioribose was determined to be 1-2 nmol (Absorbance 254 monitor). This method was used to determine the levels of the thiopentose in various pooled sera. Fetal calf serum exhibited the highest concentration (11.8 μM while human and horse serum contained 5.6 μM and 2.3 μM, respectively. The possible origin of serum methylthioribose is also discussed.

Introduction

5-Deoxy-5-methylthioribose was first discovered in 1925 as a product of the chemical hydrolysis of 5'-deoxy-5'-methylthioadenosine (49). Since this observation, it has been shown that many biological systems contain the enzymatic capacity to form methylthioribose from S-adenosylmethionine via methylthioadenosine (Figure IV-1). In most bacteria and plants, methylthioadenosine is cleaved directly to

---

Procaryotes:

Methylthioadenosine--------- Methylthioribose + Adenine

Eucaryotes:

Methylthioadenosine------ Methylthioribose-1-phosphate-----
Methylthioribose + Adenine

Figure IV-1. Formation of 5-methylthioribose in biological systems.

methylthioribose and adenine by the hydrolytic action of a specific nucleosidase (57). In human erythrocytes (44) and fetal calf serum (58), the methylthio sugar is synthesized following the phosphorolytic splitting of the parental nucleoside to yield adenine and 5-methylthioribose-1-phosphate.

Little is known about the function or further metabolism of methylthioribose in biological systems. In Enterobacter aerogenes, Shapiro and Barrett (46) demonstrated that the methylthio and ribose

portions of the molecule are recycled into methionine. The essential first step in this recycling pathway appears to be its conversion to methylthioribose-1-phosphate by the enzyme, methylthioribose kinase (59). In contrast, mammalian cells apparently do not contain methylthioribose kinase (58) and methylthioribose is not an intermediate in their methionine salvage pathway (45). In recent studies we have shown that methylthioribose stimulates the growth rate and viability of cultured mammalian cells (58). The present report describes the presence of low concentrations of methylthioribose in the serum of higher animals. In addition we present a sensitive procedure which can be used to accurately measure the levels of this substance in biological fluids.

Materials and Methods

Materials

Dansylhydrazine, phloroglucinol, and standard sugars were obtained in reagent grade from Sigma Chemical. Acetonitrile (spectral grade) and bis (trimethylsilyl) acetamide were purchased from Aldrich Chemical Co. Fetal calf serum was a product of Irvine Scientific (Lot Number: 210631). Human serum was pooled from three healthy volunteers.

Synthesis and Quantitation of Methylthioribose Standard

Standard 5-methylthioribose was prepared by the acid hydrolysis of 5'-deoxy-5'-methylthioadenosine in 4 ml of 1 M HCl at 100°C (36). Separation of the thiopentose was accomplished through cation exchange chromatography (Dowex 50-H^+). Concentrated samples were spotted on silica gel thin layer sheets (20 cm x 20 cm, EM Labs) and developed in a system containing ethyl acetate/methanol (9:1). Purity was confirmed by detecting the sulfur-containing compounds with potassium iodoplatinate spray. The determination of the concentration of methylthioribose was by the method of Ashwell (37) using ribose as the standard and phloroglucinol as the chromophor. Briefly, 5 ml of a reagent containing: glacial acetic acid (110 ml), concentrated HCl (2 ml), 0.8% glucose (1 ml), and 5% ethanolic phloroglucinol (5 ml) is added to 0.4 ml standard solutions (4-40 µg aldopentose). The tubes are heated at 100°C for 15 min. and cooled on ice. The difference in absorbance (A_{552}-A_{510}) is proportional to the concentration. We have stored the compound at -80°C for up to 1 month without significant decomposition to methylthioribose sulfoxide.

Extraction and Purification of Unknown Material from Fetal Calf Serum

Aliquots of fetal calf serum (100 ml) were deproteinized by the addition of 10 ml of 1.5 N perchloric acid and the coagulum removed by centrifugation. The supernatant was neutralized by the addition

of 1.0 N KOH and the precipitated salts were cleared by low speed centrifugation. The remaining solution was subjected to ultrafiltration (Exclusion limit: 500 M.W., Amicon) and the eluant was subsequently lyophilized. To minimize the salt concentration for chromatography, the residue was extracted twice with 2 ml volumes of absolute ethanol. Samples were applied to silica gel thin layer chromatography plates and developed in ethyl acetate:methanol (9:1). Fractions comigrating with standard methylthioribose (R_f = 0.75) were cut-out, scraped and the unknown material eluted from the gel with 2 ml of absolute ethanol. To obtain greater purity for chemical characterization, this material was also subjected to a second dimension of thin layer chromatography on silica gel sheets (methanol:chloroform, 85:15 v/v). Fractions comigrating with standard methylthioribose (R_f = 0.74) were scraped and eluted into water at 4°C overnight. The eluted material was lyophilized and used in the initial GC-mass spectral analysis.

Reagents and Reactions for Chemical Characterization

Reactions utilizing spray reagents were performed directly on the thin-layer chromatography plates. Ninhydrin, 0.15% in ethanol with a few drops of pyridine, was used as a spray reagent for amino groups. Platinic iodide reagent was prepared by mixing equal volumes of 1.1% aqueous KI and 0.135% $PtCl_4$ solution (60) prior to use. Reaction with 3,5-dinitrosalicylic acid was performed as described previously (61). In testing for ionizable groups, samples were

applied to short cation (Dowex 50-H^+) or anion (Dowex-1-X8) exchange columns (0.7 i.d. x 2 cm) and the compound eluted with 2 ml of distilled water or 0.01 N sodium formate (pH 5.0), respectively. Binding of the material to either column was followed by the dansyl-hydrazine/HPLC method described below.

Mass Spectrometry

Trimethylsilyl (TMS) ethers of standard methylthioribose and of the unknown material were prepared with bis (trimethylsilyl) acetamide (62). GC-mass spectral analysis of the unknown material comigrating with standard methylthioribose after the second dimension of chromatography was performed. A Finnigan model 4023 GC-MS computer system with a 4500 source upgrade (quadrupole mass spectrometer) using an electron ionization energy of 70 eV and a source temperature of 190°C were employed. The glc column (7% OV-101, 10' x 2 mm) conditions used to obtain the separation utilized a programmed temperature gradient from 125 to 200°C at a rate of change of 8°C per min.

Chromatographic Systems

Ascending and descending paper chromatography was performed with Whatman No. 1 paper. The solvent systems were: butanol-1:acetone:acetic acid:water (70:70:20:40, descending), ethanol:water:acetic acid (65:34:1, ascending). Thin layer chromatography was performed with silica gel sheets (EM Labs). The systems employed were:

methanol:chloroform (85:15), ethyl acetate:methanol (9:1), and chloroform:iso-amyl alcohol:acetic acid (70:30:0.5). Unless otherwise stated, sulfur-containing compounds were detected by reaction with iodoplatinate. Dansyl derivatives were visualized with a mineral lamp.

Methylthioribose Kinase Assay Conditions

5-Methylthioribose kinase was purified 10-fold from Enterobacter aerogenes by established methods (63) and was a gift of Dr. Kevin Marchitto. The assay mixture contained, as described previously, 50 mM imidazole-HCl (pH 7.3), 0.26 mM [γ-^{32} P]ATP (4×10^7 cpm/μmol), 2.0 mM $MgSO_4$, 10 mM dithiothreitol, methylthioribose kinase and the extracted material from fetal calf serum. Reaction mistures were incubated at 30^{o}C for 2 hr and stopped by the addition of ethanol. After chromatography in ethanol:water:acetic acid (65:34:1), 1 cm strips were cut out and the distribution of radioactivity determined by scintillation counting.

Dansylation of Sugars for HPLC Analysis

The general procedure described by Alpenfels (64) was used with modifications suggested by Mopper and coworkers (65). Briefly, each reaction mixture contained up to 2 μmol of reducing sugars (standards) or unknown material in 100 μl of water, to which was added 10 μl of 10% (w/v) trichloroacetic acid and 50 μl of a 5% (w/v) dansylhydrazine/acetonitrile solution. After heating at 65^{o}C for 10

min, 5.0 ml of water was added to the solution. The samples were cleared of salts by passing through a Waters C_{18} Seppak cartridge which was preactivated with acetonitrile (2 ml) and water (2 ml). The loaded cartridge was rinsed with 5.0 ml of 5% acetonitrile and the dansylhydrazones were subsequently eluted with 6.0 ml of 60% acetonitrile. (Elution with 20% acetonitrile, as suggested by Alpenfels, will not release dansyl-methylthioribose from the Seppak cartridge.)

To examine various sera directly for methylthioribose the following modifications were used. Serum (5.0 ml) was deproteinized by the addition of 1.5 N perchloric acid. After centrigutation, the solution was neutralized with 1 N KOH and the precipitated salts were cleared by a second centrifugation. The supernatant was then concentrated to 0.1 ml by evaporation and subsequently derivatized as previously described. Quantitation of the sugar dansylhydrazones was accomplished through the use of HPLC. A Beckman Model 110A solvent delivery system equipped with a Waters C_{18} bondapack column (30 x 4 mm i.d.), a Beckman Model 164 variable wavelength detector, and a Gilson Model FL-1A fluorometer (300 nm excitation/465 nm emission) were employed. An isocratic 22% acetonitrile:0.02 M formic add mobile phase at a flow rate of 1 ml/min constituted the conditions used for the separation throughout the study.

Results

Chemical Identification

All chemical tests were performed on material which comigrated with authentic methylthioribose after two-dimensional chromatography. Treatment of this substance with ninhydrin indicated the absence of primary or secondary amino groups. A carbonyl group was present, however, since this same compound reacted with the 3,5-dintrosalicylic acid reagent. Treatment of the plate with platinic iodide solution yielded a positive reaction at the same R_f as the methylthioribose standard (0.74) indicating the presence of sulfur within the molecule. The non-ionic character of the compound was demonstrated by its inability to be retained by either anion or cation exchange resins. A summary of the functional group analyses is shown in Table IV-1.

Chromatographic Characterization

The material isolated from fetal calf serum and its dansylhydrazone were found to co-chromatograph with authentic methylthioribose (and the dansyl derivative) in the chromatographic systems shown in Table IV-2.

5-Methylthioribose kinase (59) is the only enzyme known to metabolize methylthioribose. The unknown substance was incubated with [γ-^{32} P]ATP and a ten-purified preparation of the kinase under standard assay conditions. Chromatographic separation of the radio-

Table IV-1. Chemical reactions of unknown material.

Reagent	Functional Group Test For[a]	Reaction Order
Iodine vapor	- general organic	+
Ninhydrin spray	- primary amine	-
Platinic iodide spray	- oxidizable sulfur	+
Ion exchange chromatography	- ionizable group	-
3,5-Dinitrosalacylic acid	- reducing sugars	+
Ultraviolet absorbance	- conjugated bond system	-

[a]Methods were as described in Materials and Methods section.

Table IV-2. R_f values of unknown material and some standards in paper and thin-layer chromatography solvent systems.

	Paper		Thin-layer		
Compound	Butanol-1:Acetone Acetic acid:Water (70:70:20:40)	Ethanol:Water: Acetic acid (ascending) (65:34:1)	Methanol: Chloroform (85:15)	Ethyl Acetate: Methanol (9:1)	Chloroform: Amyl alcohol: Acetic acid (70:30:0.5)
5-Methylthioribose	0.71	0.77	0.71	0.75	-
Unknown material	-	-	0.71	0.75	-
Methionine	0.49	0.62	0.37	origin	-
5'-Methylthioadenosine	0.67	0.63	0.64	0.27	-
2-Keto-4-methiobutyrate	0.75	-	0.79	0.04	-
5-Methylthioribose-1-phosphate	0.25	0.55	origin	origin	-
ATP	-	0.15	-	-	-
Inorganic phosphate	-	0.49	-	-	-
Dansyl-methylthioribose	0.92	0.80	-	-	0.65
Dansyl-unknown	0.92	0.80	-	-	0.65
Dansyl-glucose	0.72	0.17	-	-	0.13
Dansyl-ribose	-	-	-	-	0.50
Dansyl-xylose	-	-	-	-	0.23
Dansyl-rhamnose	-	-	-	-	0.27
Dansyl-arabinose	-	-	-	-	0.19

Figure IV-2. Enzymatic formation of methylthioribose-1-phosphate from the serum extracted material. Reaction conditions were as described in the Methods section for the methylthioribose kinase activity. (O), Control with γ-[P^{32}]ATP and kinase preparate; (●), Reaction containing γ-[P^{32}]ATP, enzyme and unknown material.

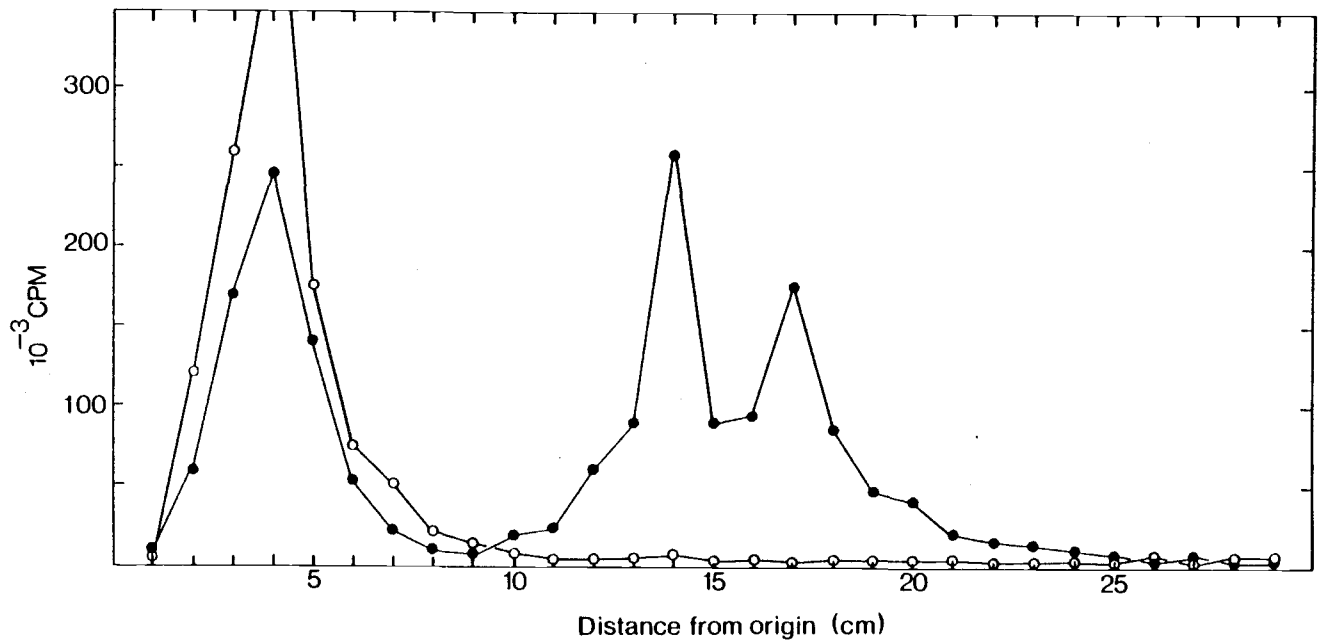

Figure IV.2

active substrate and reaction products is presented in Figure IV-2. In a control experiment, the radiolabelled ATP remained unreacted in the presence of the kinase preparate. After incubation with the material isolated from serum, the formation of a compound which comigrates with authentic 5-methylthioribose-1-phosphate (R_f = 0.55) was detected. Inorganic phosphate (R_f = 0.49) was also formed and is presumed to be due to non-enzymatic degradation of the chemically labile methylthioribose-1-phosphate.

Mass Spectroscopy

Trimethylsilyl ethers of authentic methylthioribose and the isolated material were prepared as described in the Methods section. These derivatives were subjected to a gas chromatographic temperature gradient separation and mass spectra were obtained of the component which appeared at 5 min 50 sec in each scan (Figure IV-3). Mass spectra of methylthioribose (Appendix - 2) and the extracted material were virtually identical and were similar to other trimethylsilyl sugars. The molecular ion peak of 396 was absent, as is typical of trimethylsilyl sugars. The most intense ion at highest mass was observed at m/e 381 and corresponds to the loss of a methyl group from the parent compound (M - 15). The ion of m/e 61 results from the fragmentation of the ribose structure between carbons 4 and 5 of the pentose ring (66). Rather intense peaks at m/e 73 and 147 were observed in both spectra as is common in the spectra of trimethylsilyl ethers of carbohydrates (67).

Figure IV.3. Mass spectrum of the trimethylsilyl derivative of the unknown material isolated from fetal calf serum (B) and standard methylthioadenosine (A).

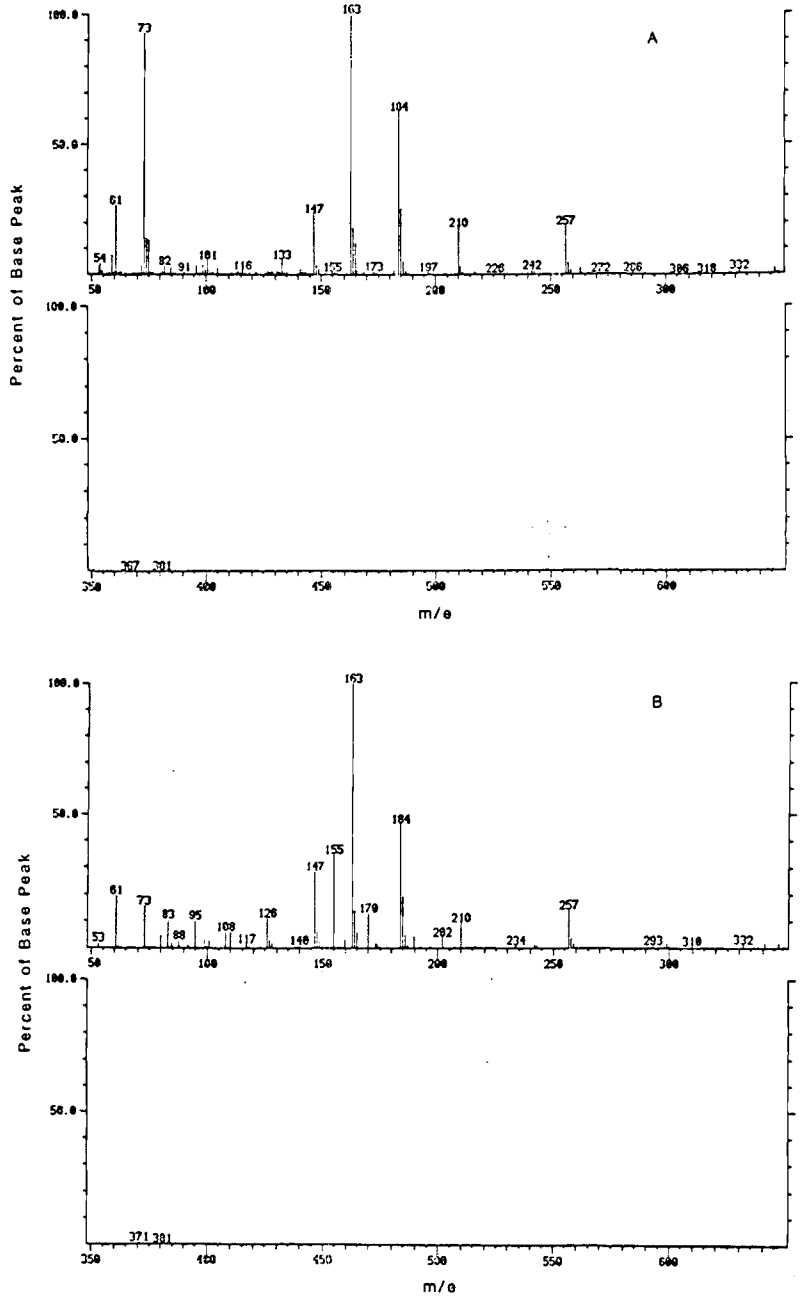

Figure IV.3

Detection and Quantitation of 5'-methylthioribose by HPLC

The procedure used to determine the levels of methylthioribose in serum was adopted from the method described by Alpenfels (64) with certain modifications as suggested by Mopper and Johnson (65). Retention times for the various sugar dansylhydrazones were: galactose, 8.2 min; glucose, 10.0 min; mannose, 11.5 min; arabinose, 11.5 min; xylose, 11.7 min; fructose, 12.2 min; ribose, 13.5 min; fucose, 15.5 min; rhamnose, 18.0 min; and methylthioribose, 27.1 min. The HPLC separation of these compounds is presented in Figure IV-4. The limit per injection was determined to be 1-2 nmol from the calibration graph (Figure IV-5), although greater sensitivity can be obtained through the use of a fluorometer. The u.v. monitor provided a linear response to methylthioribose concentrations between 5.0 nmol and 100.0 nmol.

The HPLC separation method was used to facilitate the detection of methylthioribose in serum samples. A typical HPLC scan of the serum-extracted reducing sugars is shown in Figure IV-6. The thiopentose was detected in all three sera tested (Table IV-3). Fetal calf serum exhibited the highest concentration (11.5 μM) while human and horse serum contained 5.6 μM and 2.3 μM, respectively. To demonstrate further that the material was dansyl-methylthioribose, this fraction was collected and chromatographed by HPLC in two solvent systems: isocratic, 30% methanol:water and a 10-40% acetonitrile:

Figure IV.4. Separation of dansyl derivatives of biologically important reducing sugars. HPLC conditions: column, Bondapak C_{18} (3.9 mm i.d. x 30 cm); 22% acetonitrile in 0.02 M formic acid at 1 ml/min; detector, absorbance at 254 nm. The injected sample contained: galactose (1), glucose (2), mannose (3), arabinose (3), fructose (4), ribose (5), fucose (6), rhamnose (7), reagent (8), and methylthioribose (9).

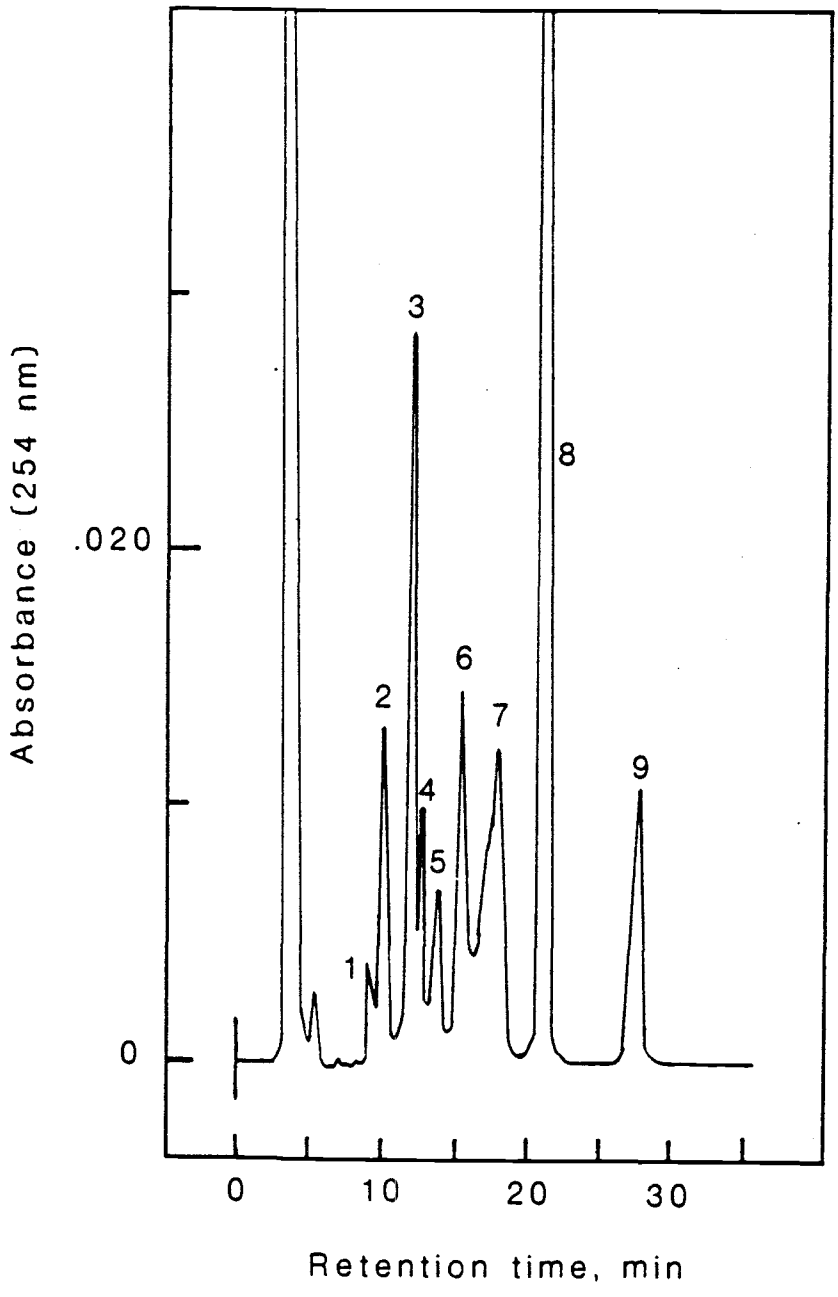
Absorbance (254 nm)
.020
0
1
2
3
4
5
6
7
8
9
0
10
20
30
Retention time, min

Figure IV.4

Figure IV.5. Calibration graph for the HPLC analysis of dansyl-methylthioribose.

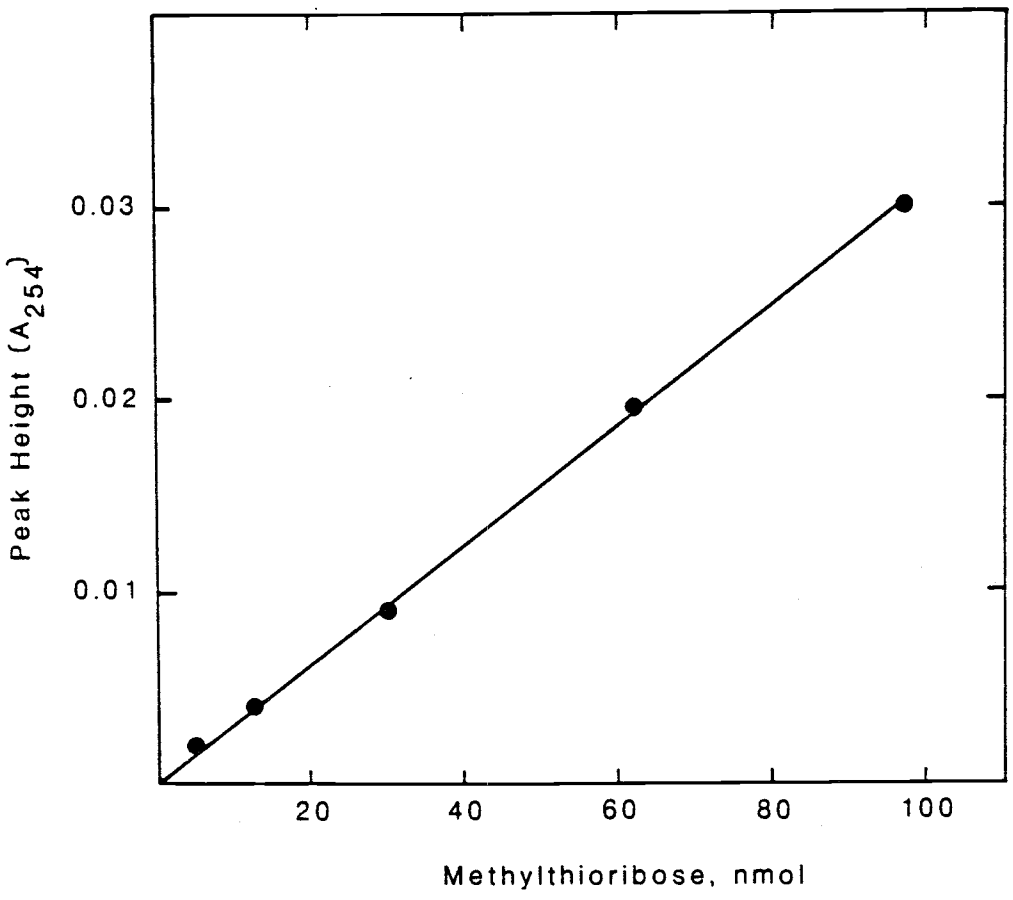
Peak Height (A_{254})
0.03
0.02
0.01
20
40
60
80
100
Methylthioribose, nmol

Figure IV.5

Figure IV-6. HPLC profile of the dansyl derivatives of reducing sugars in serum.

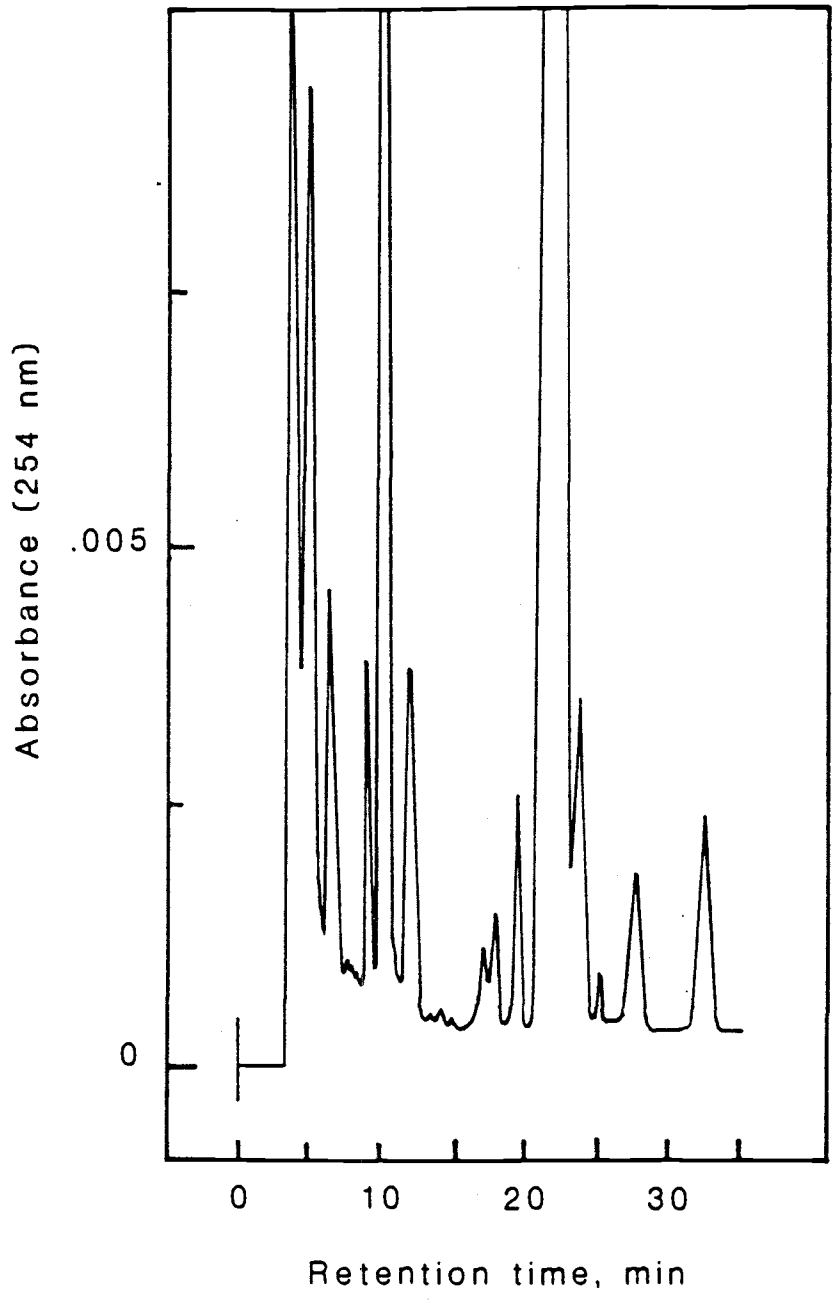

Figure IV.6

Table IV.3. Concentration of methylthioribose in various sera.

Serum Source	Concentration, μM[a]
Fetal Calf	11.5 ± 0.55
Human	5.6 ± 0.42
Horse	2.3 ± 0.23

[a]Concentrations of methylthioribose were determined as described in Methods section. The reported values represent the average of three determinations and the standard deviation.

0.02 M formic acid linear gradient system. In each case the material co-chromatographed with authentic dansyl-methylthioribose.

Discussion

The natural occurrence of methylthioribose was first demonstrated in cultures of Escherichia coli B by Schroeder and coworkers (66). Recently, Zappia et al. (44) have shown that methylthioribose is exported from human erythrocytes incubated with methylthioadenosine. However, we have also reported the synthesis of methylthioribose via methylthioribose-1-phosphate in serum (58).

In this communication we present chemical and physical evidence to establish the presence of methylthioribose in human, fetal calf and horse serum samples. Methods used for the comparison of this substance to our synthetic standard included paper, gas, thin layer and ion exchange chromatography, and mass spectroscopy. High performance liquid chromatographic analysis of its dansyl derivative indicated that it was present in micromolar concentration in each of the sera tested.

The significance of methylthioribose in the serum of higher animals is not known. The methylthio and ribose portions of the molecule are recycled into methionine in Enterobacter aerogenes (46) but this process does not appear to occur in mammalian tissues (58,45). Toohey et al. (25, 27) first suggested that the degradation of methylthioadenosine is required for the growth of some mammalian cell lines. Comparing methylthioadenosine phosphorylase-deficient and-

containing cell lines, our previous studies proved that methylthioribose, a product of methylthioadenosine phosphorylase metabolism in higher animals, can satisfy this methylthio requirement (58). Furthermore, micromolar concentrations of methylthioribose were found to stimulate the growth rate, viability and saturation density of the phosphorylase-containing BW5147 cell line.

The origin of serum methylthioribose is unknown, but several possibilities exist. First, since this compound is a normal product of methylthioadenosine degradation via methylthioadenosine nucleosidase in plants (68), the thiopentose in serum may arise from the diet. However, no measurements of methylthioribose levels in plant tissues have been reported. Also, some bacteria within the normal gastrointestinal flora are also known to excrete the sulfur-containing sugar (48). Third, like Escherichia coli, mammalian cells may excrete methylthioribose. Zappia and coworkers (44) demonstrated that in human erythrocytes, the supplementation of methylthioadenosine resulted in the transport and internal metabolism of the nucleoside to methylthioribose-1-phosphate and adenine, and the export of methylthioribose. Finally, methylthioadenosine is exported from methylthioadenosine phosphorylase-deficient (47) and containing cells (43), the latter occurring only when the enzyme activity is inhibited. It is also known that most sera have the capacity to degrade methylthioadenosine to methylthioribose and methylthioribose-1-phosphate (58). In light of these findings, any condition which substantially lowers the methylthioadenosine phosphorylase activity in vivo

may result in the presence of methylthioribose in serum. The fact that horse serum contains methythioribose, but not methylthioadenosine phosphorylase activity, suggests against this latter possibility.

In conclusion, methylthioribose, a biologically important metabolite of methylthioadenosine, is present in human serum, fetal calf serum, and horse serum in micro molar concentrations. Although its function in vivo is not known, methylthioribose has been shown to stimulate the growth of a variety of cell types in culture. Studies are in progress to determine the fate of methylthioribose in vivo and to examine the serum level of this sugar in neoplastic and other disease states.

Summary

In summary, I have studied the biologic effects and metabolism of two naturally occurring compounds, methylthioadenosine and methylthioribose. Three major accomplishments have resulted from my work:

1. A significant part of the growth inhibitory action of methylthioadenosine relies upon the molecule's ability to perturb cAMP metabolism.
2. Methylthioribose, a degradation product of methylthioadenosine, stimulates the growth rate of certain mammaliam cells. This substance can also satisfy a "methylthio" requirement in cells incapable of metabolizing the parental nucleoside.
3. Methylthioribose is present in human, horse, and fetal calf serum.

Many areas of potential interest have been left unexplored. Some of the questions that could be answered by further research in this area are;

1. Do the intracellular levels of methylthioadenosine correlate to the progression of cells through the cell cycle?
2. What is the metabolic fate of methylthioribose and is its fate the same in methylthioadenosine phosphorylase-deficient and containing cells?

3. Is the presence of methylthioribose in serum significant in some regulatory fashion, i.e., does methylthioribose play a role in hematopoiesis?
4. What is the source of methylthioribose in serum?
5. Since methylthioribose is the major degradation product of methylthioadenosine in human serum, is it possible that the levels of this sugar correlate with certain disease states? (i.e. MTAase-deficient leukemia's are known to excrete MTA and therefore MTR could accumulate in blood).
6. Finally, since methylthioribose kinase is not present in mammalian cells and therefore this sugar doesn't recycle into methionine, it is possible that ethylthioribose (metabolized into ethionine (a toxic analog of methionine!) could be used to selectively kill MTR kinase-containing microorganisms.

In conclusion, since methylthioadenosine and its products have opposing effects on cell proliferation, it seems reasonable to postulate that these molecules play an important role in the regulation of cell division.

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APPENDIX

A study on the specificity of the "methylthio" requirement of L1210D cells was performed. Various structural analogs of methylthioribose were synthesized and the ability of these compounds to support the growth of L1210D cells in the absence of a methylthio source was tested (Figure App. 1). These growth studies indicate that the methylthio requirement is actually a less specific "alkylthio" requirement. In addition, alterations in the sugar portion of the molecule are not tolerated by the L1210D cells.

Figure App.1, Ability of structural analogs of methylthioribose to satisfy the methylthio requirements of L120 cells. (iPTR, isopropylthioribose; iBTR, isobutylthioribose; BTR, butylthioribose; ETR, ethelthioribose; PTR, propylthioribose; MTarab, methylthioarabinose; 5-Cl Rib, 5-chlororibose.)

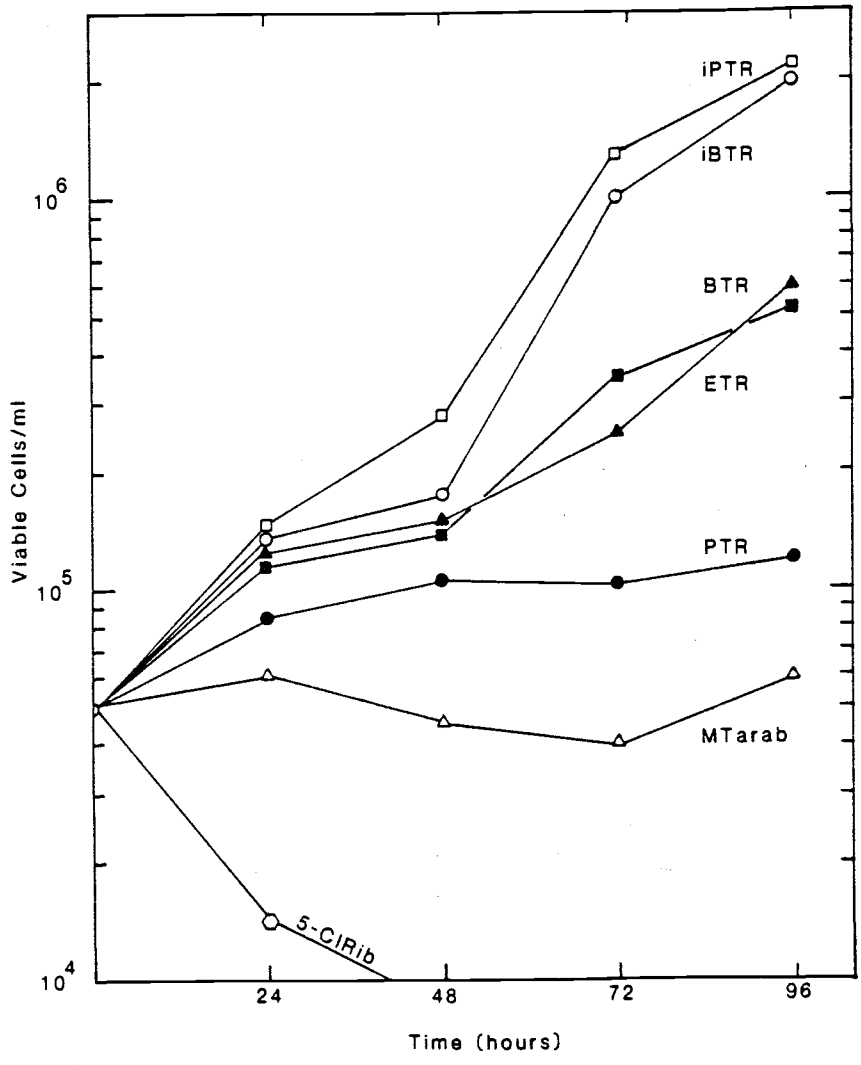
iPTR
iBTR
BTR
ETR
PTR
MTarab
5-ClRib
Viable Cells/ml
10^6
10^5
10^4
24
48
72
96
Time (hours)

Figure App.1