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Title: The Exploitation of Methylthioadenosine Phosphorylase Deficiency in Cancer Therapy

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Methylthioadenosine (MTA) is a naturally occuring nucleoside formed during the synthesis of polyamines in mammalian cells. MTA is rapidly catabolised by MTA phosphorylase (MTAase), eventually yielding methionine and adenine. MTAase has been shown to be active in all normal cells, but deficient in some cells and tissues of malignant origin. A protocol has been developed which potentially exploits this metabolic difference between normal and malignant cells, focussing on MTA as the sole methionine source in a methionine deficient rodent chow. All normal cells in a rodent on this diet would possess MTAase and be able to catabolise MTA to obtain methionine. Introduced MTAase negative tumor cells would not be able to scavenge methionine from the MTA in the diet and would be selectively starved for methionine.

MTA as a replacement for methionine in the diet of mice could only partially support their growth, and could not selectively starve MTAase deficient tumors present in those mice. MTA as a replacement for methionine in the diet of rats could support an average of 35% of normal weight gain, and could not selectively starve MTAase negative tumors present in those rats. The effect of tumor on methionine metabolism is discussed in relation to the inability of MTA to provide selective starvation.

The Exploitation of Methylthioadenosine Phosphorylase Deficiency in Cancer Therapy

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INTRODUCTION

Methylthioadenosine (MTA) (Figure 1), is the principal structural component of the biological methyl donor S-Adenosylmethionine (SAM) (48). Upon the loss of the donated methyl group, SAM is converted to S-adenosylhomocysteine, which is then hydrolysed to adenosine and homocysteine by S-adenosyl hydrolase (25). After decarboxylation, SAM can also serve as a propylamino donor to putresine in the formation of polyamines. This formation appears to be ubiquitous in nature (48). Synthesis of the polyamines spermidine and spermine yields stoichiometric amounts of MTA: 1 molecule of MTA per molecule spermidine, two molecules of MTA per molecule spermine produced (35) (Figure 2). Spermidine synthase and spermine synthase reactions in mammalian cells represent the quantitatively most important pathways to MTA formation, with spermidine, spermine, and MTA favored (48). MTA enters cells more readily than SAM or S-adenosylhomocysteine. No surface adsorption of MTA occurs and it is rapidly taken up and metabolized (7).

MTA doesn't accumulate in mammalian cells. Concentrations of MTA are low compared to SAM, spermidine, and spermine levels (48, 58). The nucleoside is cleaved rapidly to adenine and metylthioribose-1-phosphate (MTR-1-P) by MTA phosphorylase (MTAase) (36). MTAase has been detected in the cytosol of all non-neoplastic cells of embryonic and adult mammals studied (58).

DellaRagione and coworkers purified MTAase 30,000 fold from human placenta. The enzyme exhibited a specific activity of 10.2 μM MTA

cleaved/min/mg protein, and a MW of 98,000 in three identical subunits. The K_m for MTA was 5 μM. With a lower affinity for phosphate, MTAase is probably saturated for phosphate *in vivo*. A high affinity for MTR-1-P and adenine favors MTA synthesis, but the rapid removal of these products*in vivo* probably prevents MTA synthesis (13). At saturating phosphate concentrations a Lineweaver-Burke plot indicated substrate inhibition at greater than 200 μM MTA. In phosphate-depleted erythrocytes no degradation of MTA occurred in the absence of exogeneous phosphate, indicating the reaction requires phosphate. Similar kinetics in ATP-depleted cells indicated the reaction is not energy dependent (7).

MTA binds to the enzyme first: analog studies imply an intact ribose ring is required, with direct involvement of the 6-amino and N3 atom in enzyme-substrate binding (12). Inorganic phosphate binds the enzyme after MTA, and then MTR-1-P is released, followed by adenine (58). The adenine liberated from MTA combines with phosphoribosylpyrophosphate to form 5'AMP and PP_i, catalysed by adenine phosphoribosyl transferase. The AMP is recycled to the adenine nucleotide pool (44) (figure 2).

In HL-60 cells labelled MTA is rapidly converted to MTR-1-P, which is then slowly converted to methionine. This conversion is complete in 4 hours, and appears to be stoichiometric (45). MTR-1-P is converted to methylthioribulose-1-phosphate by an isomerase partially purified from rat liver. Two protein fractions from rat liver cytosol are required to further convert 5-methylthioribulose-1-phosphate to 2-keto-4-methylthiobutyrate, with C1 of MTR-1-P released as formic acid (48), and the consumption of one mole of oxygen (54). 2-Keto-4-methylthiobutyrate is converted to methionine by a glutamine or asparagine dependent transamination (1) (figure 3). MTAase therefore provides complete salvage of all regions of decarboxylated SAM

except the aminopropyl group (58).

The MTA to methionine pathway may be important in maintaining methionine homeostasis *in vivo* where methionine, an essential amino acid, may be limiting (45). Only two pathways are known to recycle methionine in mammalian cells: salvage from the ribose moiety of MTA, or the methylation of homocysteine (a product of SAM) by methyltetrahydrofolic acid or betaine (9). Methionine metabolism in mammalian liver is regulated by changes in homocysteine distribution between remethylation to methionine by betaine:homocysteine methyltransferase or methyltetrahydrofolate:homocysteine methyltransferase, and transsulfuration to cysteine by cystathionine ß synthase (irreversible) (18). The MTA to methionine pathway provided by MTAase may exceed the homocysteine to methionine pathway in importance (6).

MTAase deficiency in some cultured murine malignant cell lines was first detected in 1977 (52). MTAase has since been shown to be lacking in many human and murine malignant cell lines of diverse origin (33). Five out of thirteen (38%) established human malignant hematopoietic cell lines were MTAase deficient (27). Seven out of 31 (23%) cell lines from leukemia, malignant melanoma, and breast cancer lacked MTAase, but none of sixteen normal tissue derived lines examined did (28).

The absence of MTAase activity is not restricted to cells in long term culture (4,32). Out of 8 patients with leukemia and 10 with solid tumors, one leukemia and three solid tumors (malignant melanoma, rectal adenocarcinoma, squamous lung carcinoma) were MTAase deficient (19). In another study, 10 out of 100 cases of leukemia lacked MTAase. The MTAase deficient leukemias were pathologically heterogeneous, however, all 10 were from male patients, while overall 60 out of the100 leukemia patients were male. The results

indicate MTAase deficiency may be X-linked (55). All non-leukemic blood fractions from patients in both studies were normal for MTAase activity (55,19).

Intracellular degradation of MTA in MTAase deficient cells is insignificant (33). Cells which lack MTAase, such as the CEM and K562 cell lines, must dump the MTA they produce. These lines excreted 0.58 and 0.70 nmol MTA/hr/mg protein, respectively, with 1 to 5 μM MTA accumulating in the media. Cellular levels of MTA were probably equal or greater than the media concentrations (27). MTA accumulation peaks prior to exponential growth and declines gradually during exponential and stationary growth phases. This correlates with the polyamine synthesis rate: maximum polyamine synthesis occurs prior to the onset of rapid growth (25). The addition of 3 μM MTA to cells in culture increased the incorporation of 3H-ornithine into putresine (catalysed by ornithine decarboxylase). Such a concentration could be achieved by cellular dumpage or intracellular build-up of MTA in MTAase deficient cells. If putresine is beneficial to cells, increased levels of putresine could provide selective pressure for achieving the MTAase deficient genotype *in vivo* or *in vitro* (27). MTAase deficient R1.1 clones and wild-type R1.1 cells grew at the same rate to equivalent density, had similar cloning efficiency, and were both lethal within 21 to 28 days of injection into mice so that MTAase deficiency does not appear to confer any disadvantage to these cells. Putresine and spermidine levels were similar in the deficient and wild-type cells, while spermine was lower in MTAase deficient cells by 10-17% (32).

MTAase deficient cells do not contain soluble enzyme inhibitors (58). L1210D cells lacked MTAase activity regardless of the substrate supplied (57,6). L1210D MTAase could not be induced by growth in MTA media, nor was MTA metabolized by any other pathways (57). No cross-reactive inert enzyme protein could be detected by immunoadsorption assay in MTAase deficient human cell lines (5). Possible genetic deletion of loci critical to both MTAase

activity and malignant transformation was suggested by cytogenetic studies of abnormalities of chromosome 9 in leukemia (8). Somatic cell hybridization (mouse:human) and isozyme analysis (isoelectric focussing) enabled assignment of the gene for MTAase to the human chromosome 9, with a regional location of 9pter to 9q12 suggested. No characteristic abnormalities of chromosome 9 in the MTAase negative cells studied by these authors was observed (4), and no consistent chromosomal abnormality was associated with the loss of enzyme activity in other studies as well (55). The MTAase deficient phenotype in mouse:mouse somatic cell hybrids segregated as a resessive characteristic: 5 out of 16 clones were MTAase negative (29). Of course, synthesis of defective mRNA may occur in deficient cells (5), or post-translational modification of the protein may provide inactivity (13).

Studies with an inhibitor of MTAase suggest that MTAase containing cells limit *de novo* adenine synthesis and require adenine from MTA for growth, while MTAase deficient cells rely solely on *de novo* adenine synthesis (34). CCRF-CEM and K562 cell lines both lack MTAase and have an increased rate of *de novo* purine synthesis not seen in the MTAase positive cell line studied. No association between increased purine synthesis and MTAase deficiency has been established, however, as the MTAase deficient cell line CCRF-HSB2 had a purine synthesis rate similar to the MTAase containing cells (23). More adenine may be synthesized *de novo* from MTA cleavage by MTAase than from adenosine or 2'deoxyadenosine cleavage by adenine phosphoribosyl transferase (30). Greater than 97% of endogenous adenine was produced by catabolism of MTA in human lymphoblasts in culture (4).

The action of MTAase on MTA therefore represents an important step in the recycling of both methionine and adenine. MTAase is also beneficial in that it removes MTA from cells, as MTA has been shown to have profound effects on cell growth and other processes (MTA less than 5 μM is non-toxic). The

presence or absence of MTAase is not a requisite for MTA growth inhibition (59). MTA greater than 25 μM dose-dependently inhibited L1210 (MTAase deficient) and WI-L2 (MTAase containing) cells, with toxicity preventing long term culture (6). 1.0 mM MTA blocked cell proliferation without affecting cell viability in MTAase positive cells (BW5147 and NS-1). Inhibition by MTA was dose-dependent, non-toxic, and reversible in BW5147 cells (59). MTA effects hematopoiesis by inhibiting proliferation without affecting either granulocytic or monocytic differentiation (41). MTA prevents terminal differentiation of murine erythroleukemia cells (46) and may be the mechanism for selective bone marrow response to granulopoietic or erythropoietic stimuli (41).

Due to its antiproliferative effect, it might be expected that MTA concentrations would be reduced in organs undergoing proliferation and polyamine accumulation. A significant decrease of hepatic MTA in rats killed 2 to 150 days after partial hepatectomy was observed by Ferrioli and Scalabrino (15). Polyamine biosynthetic enzyme levels in liver were either normal (ornithine decarboxylase), or were elevated (SAM decarboxylase) up to 100 days after partial hepatectomy (10 days only are required for regrowth). Hepatic MTA in rats fed a hepatocarcinogen were always lowered. MTA may control proliferation in eucaryotic cells, as its level decreases in liver characteristically when liver undergoes cell proliferation, either curbed (regeneration), or uncurbed (induced hepatoma). The decrease is maintained during and after cell proliferation, up to five months after partial hepatectomy. Decreased hepatic MTA may be associated with a possible MTAase activity increase at the start of rapid cell division as seen in some cultured cells (15).

As might be expected, MTA can influence the polyamine biosynthetic pathway responsible for its formation. MTA is a powerful inhibitor of mammalian spermidine and spermine synthase (12,48,11) and S-adenosylhomocysteine hydrolase (17,11). MTA rapidly and dose-dependently augmented ornithine

decarboxylase and SAM decarboxylase activity (33,37) with progressive accumulation of putresine and decarboxylated SAM in MTAase negative R1.1 Clone H cells. The augmentation was not mediated by the catabolic products of MTA or induced by non-specific changes in growth kinetics (33). MTA analog studies indicated the increase in SAM decarboxylase activity was due to an increased amount of enzyme protein promoted by an increase in both SAM decarboxylase mRNA and increased translational efficiency of that mRNA. The mRNA responses appeared to have been mediated by spermine depletion (37).

However, the biological effects of MTA cannot always be reversed by exogenous addition of spermidine or spermine, and inhibition by MTA of lymphocyte mediated cytolysis is unrelated to S-adenosylhomocysteine levels (62), so that competitive inhibition of polyamine biosynthesis or S-adenosylhomocysteine hydrolase is not necessarily the primary target of MTA action (37,39,40,41).

MTA inhibits transmethylation of eucaryotic proteins and procaryotic DNA and post-synthetic methylation of human cell DNA at low concentrations, *in vitro* (60,48,11). Although some researchers feel that this effect, though direct, is probably not "natural" *in vivo* (11), this inhibition could interfere with cell proliferation. MTA also competitively inhibited high affinity cAMP phosphodiesterase in S49 wild-type cells (MTAase positive). Perturbation of cAMP metabolism by MTA could allow cAMP to accumulate, causing cell growth arrest (40).

While MTA and its analogs need not be degraded to inhibit growth (59,10), MTA may also inhibit cell growth through the production of one or both of its degradation products (43). Adenine in the medium inhibited *de novo* purine synthesis in both MTAase positive and negative cells similarly. Inhibition of *de*

novo purine synthesis in MTAase containing cells by MTA may be by cleavage to adenine with possible feedback inhibition by the purine on the synthesis of phosphoribosylpyrophosphate (22).

Altered MTA metabolism is a specific abnormality distinguishing some malignant cells from normal cells (55). Successful cancer chemotherapy ideally exploitsdefined metabolic differences between malignant and normal cells. The MTAase deficiency has been succesfully exploited in the past by selectively starving MTAase negative cells for purines. Medium which contains methotrexate and pyrimidines blocks *de novo* purine synthesis. Addition of MTA provides the only purine source. MTAase positive cells grow, while MTAase deficient cells die within three days under this treatment. Methotrexate, however, is toxic to normally rapidly proliferating cells such as intestinal mucosa, and bone marrow (28). Also, mammalian sera can contain MTAase activity which could break down the MTA present to adenine. This adenine would then be available for use by the MTAase negative cells (42).

Difluoromethylornithine (DFMO) (an irreversible inhibitor of ornithine decarboxylase) as a 3% dose in water retarded the subcutaneous growth of EMT6 murine mammary sarcoma in mice. DFMO is non-toxic and depletes the polyamines putresine and spermidine (38). MTA inhibits spermine formation in cells in culture (37). DFMO at 250 μM plus MTA at 500 μM in the medium synergized to cause profound depletion of all polyamines and inhibition of growth of MTAase deficient murine lymphoma R1.1 H cells. However DFMO plus MTA massively increased decarboxylated SAM. Polyamine deprivation may cause chromosomal aberrations, which may be enhanced by changes in histone acetylation due to high decarboxylated SAM, affecting cell growth regulation (61).

MTAase positive cells cultured in methionine-free medium could utilize

exogenous MTA as a source of methionine, MTAase deficient cells could not. The presence of MTAase activity in the serum used in the culture medium did not alter this differential effect (42). A protocol was then developed which focussed on MTA as a methionine source, and had the potential to avoid the problems associated with methotrexate or DFMO. MTA in a methionine-free diet would represent the only exogenous methionine source for an animal on that diet. All the normal cells in the animal would be MTAase positive and able to catabolise MTA and regain methionine. Any MTAase deficienct malignant cells present would not be able to scavenge methionine from the MTA in the diet and would be selectively starved for methionine. This protocol therefore represented a non-invasive, selective form of chemotherapy. Potential problems existed in the manipulation of methionine metabolism, as well as the indicated effects of excess MTA on cellular processes.

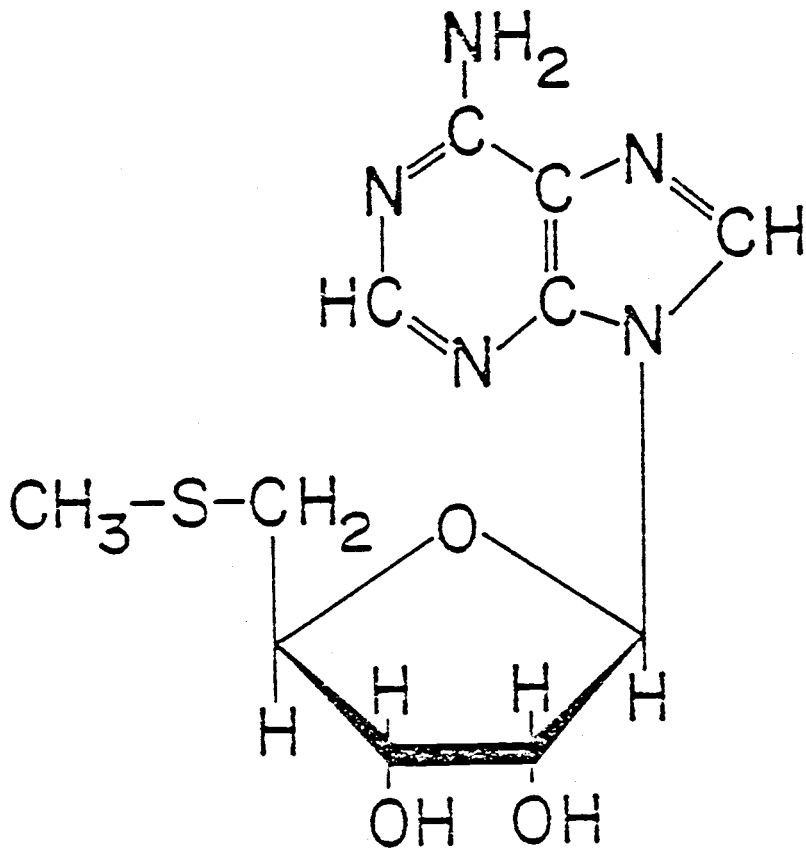

Figure 1. The structure of 5'-deoxy-5'-methylthioadenosine

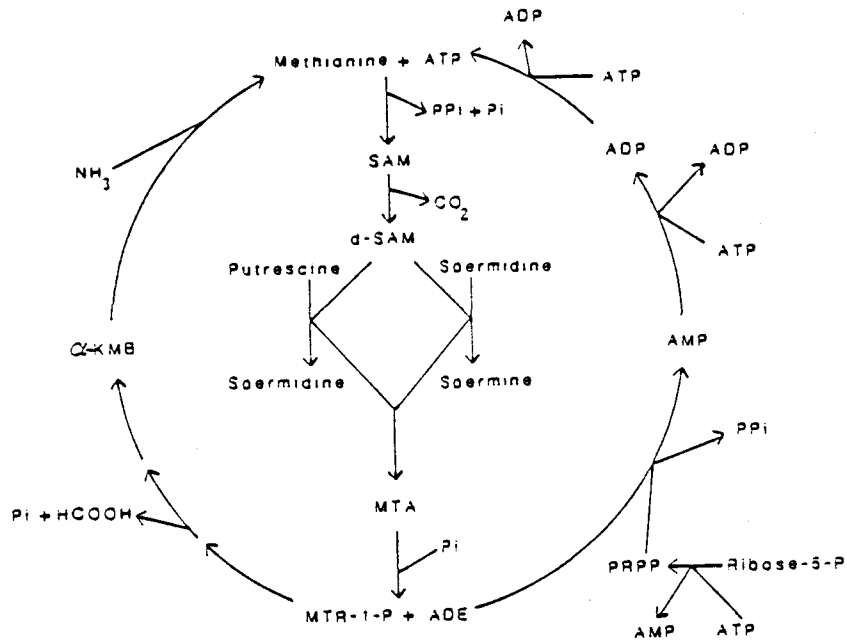

Figure 2. Summary of MTA metabolism in mammalian cells. Abbreviations: SAM, S-adenosylmethionine; d-SAM, decarboxylated SAM; MTA, 5'-methylthioadenosine; MTR-1-P, methylthioribose-1-phosphate; ADE, adenine; KMB, 2-keto-4-methylthiobutyric acid; PRPP, phosphoribosylpyrophosphate.

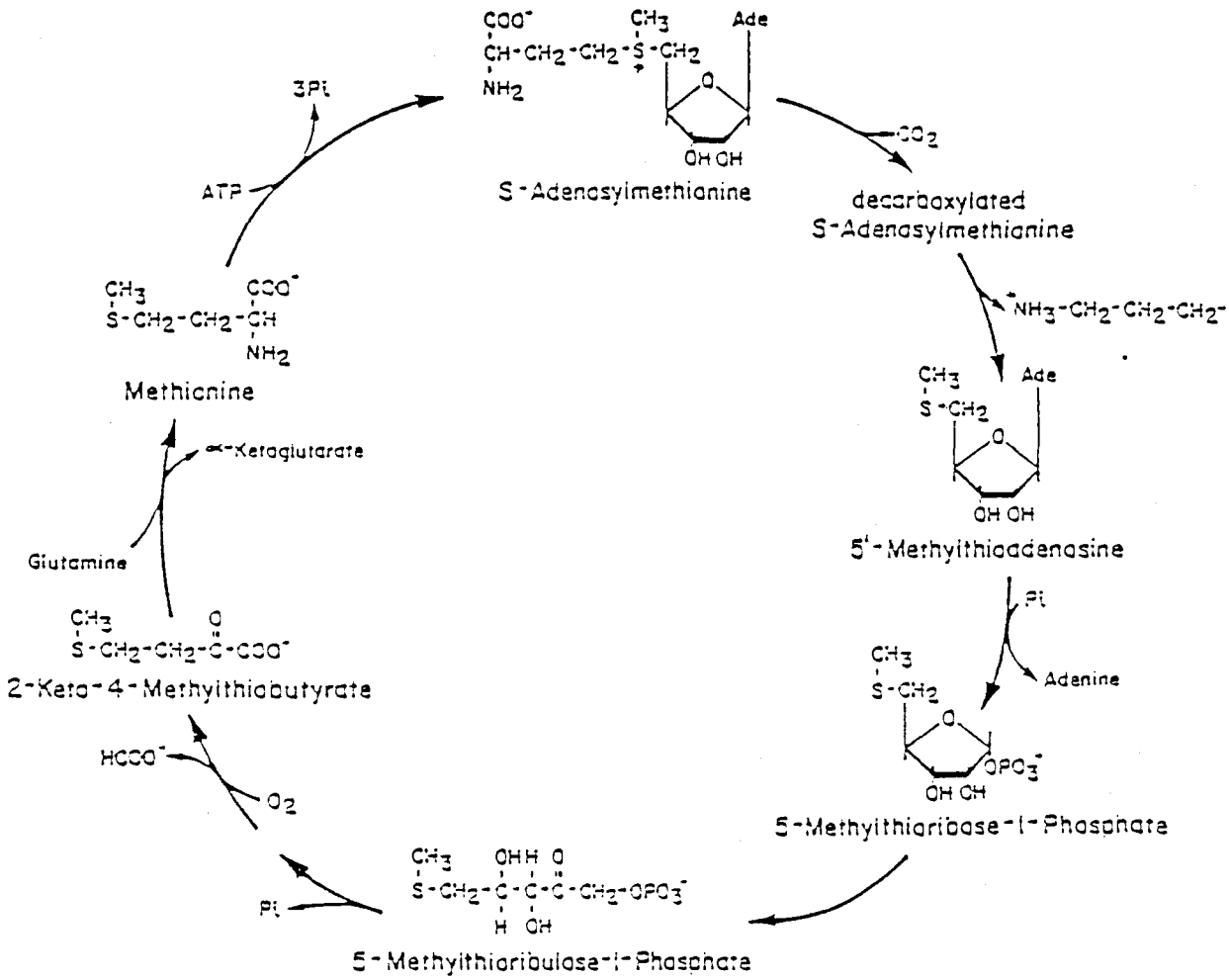

Figure 3. The methionine recycling pathway.

MATERIALS AND METHODS

In Vitro Studies:

Cell Culture: All media were obtained from M.A. Bioproducts, all sera were obtained from Hyclone. All cultures were held at 37°C in a ShelLab 5% CO_2 humidified incubator. Cell number and viability were determined by trypan blue exclusion on a Neubauer hemacytometer.

The following attached rat cell lines and media were used:

LC540 and FR cells were cultured in MEM Eagle with non-essential amino acids (NEAA), sodium pyruvate, Earle's balanced salt solution (EBSS), L-glutamine (L-Gln), 10% fetal bovine serum (FBS), and 0.5% PSF (50 units/ml penecillin, 50 mcgs/ml streptomycin, 0.125 mcgs/ml Fungizone). These cells were obtained from American Type Culture Collection (ATCC).

Walker 256 (ATCC) carcinoma cells were cultured in Medium 199 with EBSS, 10% horse serum (HS), and 0.5% PSF, and were a gift from Dr. John Fitchen, though originally obtained from ATCC.

Walker 256 (ADL) carcinoma cells, which could not be cultured, were maintained as an ascites tumor in Sprague Dawley rats. These cells were obtained from Arthur D. Little, Co (ADL).

Schmidt-Ruppin sarcoma cells were cultured in Medium 199 with EBSS, 20% FBS, and 0.5% PSF, and were a gift from Dr. John Fitchen.

Jensen's sarcoma cells were cultured in McCoy's 5A with 5% FBS and 0.5% PSF, and were also a gift from Dr. Fitchen.

The following suspended mouse cell lines and media were used:

BW5147 lymphoma cells were grown in MEM for suspension cultures with L-Gln, 10% HS, and 0.5% PSF. They were received from the Salk Institute.

L1210D leukemia cells were cultured in MEM for suspension cultures with L-Gln, 10% HS, 0.5% PSF, and 0.1 mM Cys-S-S-CH_3 as a methylthio source.

Cell Extracts: Cell cultures at 1-4 x10^6 cell/ml, and 90% or higher viability were centrifuged and the cell pellet was washed with 5 ml breakage/storage buffer (3 mM DTT in 0.05 M potassium phosphate buffer, pH 7.2). The cells were then suspended in 0.5 ml breakage/storage buffer and freeze-thawed three times in dry ice/ethanol. Extracts were then microfuged (16,000 RPM) for 2-4 minutes, and the supernatent was stored at -20^{o}C until use.

Protein Assay: Protein concentration of cell extracts was determined by the method of Bradford (3). Absorbance was determined on a Beckman Spectrophotometer Model 25 set at 595 nm. A standard curve was generated using bovine serum albumin at 1.09 µg/µl protein. Extract protein concentration was interpolated from sample absorbance using this curve.

Compounds: 5'-[methyl-^{14}C]-methylthioadenosine was prepared by hydrolysis of S-adenosyl-L-[methyl-^{14}C]-methionine obtained from Research Products International, and was diluted with cold methylthioadenosine (Sigma) to 0.37 mM, 5-6 x10^6 cpm/µmol.

Enzyme Assay: MTA phosphorylase activity was determined by measuring the conversion of 5'[methyl-^{14}C]-methylthioadenosine to 5'-[methyl-^{14}C]-methylthioribose-1-phosphate by ion exchange chromatography. The following method, modified from Ferro et.al. (16), was used: the reaction mixture contained cell extract, buffer (0.5 M HEPES, 25 mM KH_2PO_4, 3 mM DTT, pH 7.2), and 0.37 mM $^{14}CH_3$-MTA, and was incubated at 37^{o}C for 60 minutes. Reactions were stopped with 1.8 M TCA (at 4^{o}C) and microfuged at 16,000 RPM for two minutes to remove precipitate. Supernatent was put over Dowex 50X4 columns, and $^{14}CH_3$-MTR-1-P was eluted with 3 mls

ddH_2O and counted in standard cocktail on a Beckman LS3801 liquid scintillation counter.

MTA Growth Studies: W256 (ATCC) cells (MTAase deficient) were seeded at 8.9 $x10^4$ cells/ml into each of the following types of culture conditions: (1) 10 mls of Medium 199 as a normal control; (2) 7.5 mls Medium 199 with 2.5 mls 2 mM MTA for a final MTA concentration of 0.5 mM, to test the effect of MTA on cell growth and proliferation, or (3) 7.5 mls Medium 199 with 2.5 mls ddH_2O added to test the effect of dilution of the media that occurs in culture 2.

Schmidt-Ruppin cells (MTAase positive) were seeded at 8.9 $x10^4$ cells/ml into each of the same three types of Medium 199. All six cultures were provided with 10% FBS, 0.5% PSF, and EBSS, and were incubated for 10 days. Cells were examined visually each day, and were trypsinized and counted each three to four days. Each culture was provided with fresh media of the appropriate type when resuspended for counting. As the diluted cultures produced a slightly higher number of detached but healthy cells than did control cultures, the culture media from these flasks was centrifuged and the cell pellet was added back to the fresh media added before counting.

In Vivo Studies:

Animals: All rats were Sprague-Dawley males or females, between 3.5 weeks old (40-50g) and 5.5 weeks old (125-175g). All mice were DBA males, 8 weeks old (18-22g). Animal weights were consistent within each experimental group. All animals were received from Simonsen, CA. All animals were caged separately and fed and watered ad lib. They were kept on a 12 hour light-dark cycle and held at 68^oC. Animals were weighed periodically, and their weight, condition, and food and water consumption was recorded.

Chow: Methionine deficient chow was obtained in a meal form from Bioserve, NJ, with less than 0.01% methionine, actual analysis. L-methionine (Sigma) or methylthioadenosine (Sigma) was added to the desired mg per gram of chow dose (sucrose was added to mouse chow in some cases at 2 mg/g), and the mixture was ground in an electric coffee grinder. The chow was then lightly moistened with ddH_2O, reground to uniformity, and hand-pressed through a 10 ml syringe body to form pellets. Methionine deficient chow was also obtained already pelleted from Bioserve and was fed directly in methionine starvation experiments. All chow was kept refrigerated when not in use. Growth on the methionine deficient chow plus MTA was compared either to methionine deficient chow plus methionine or a standard rodent pellet feed provided by Oregon State University refered to as standard chow.

Injections: All injections were done using 1 ml tuberculin syringes with 25G needles. Earlier rat studies used subcutaneous injections on the right leg, later studies used subcutaneous injections on the right flank. All mice were injected intra-peritoneally in the right lower abdomen. Between 6 $x10^6$ and 3 $x10^7$ cells/ml were injected into each rat, and 7 $x10^5$ to 1 $x10^6$ cells/ml were injected into each mouse. Innoculum volumes were consistant within each experiment, usually a volume of 0.25 ml, although volumes did range between 0.05 and 1.0 ml. The carrier consisted of sterile 0.9% NaCl. Injection controls consisted of an appropriate volume of sterile phosphate buffered saline. Tumors were measured in mm using calipers and size was determined using the formula: L x W x D x $\pi/6$ = cc (50).

RESULTS

In Vitro Studies:

MTA Phosphorylase Assays: Table I shows MTAase activities for the various cell lines as determined by column chromatography. LC540 and Jensen sarcoma cells were considered MTAase negative with activities of 5 pmol/min/mg or less. Walker 256(ATCC) was always MTAase negative when grown in culture, however, an extract of this cell line as a solid tumor showed significant activity at 122 pmol/min/mg. This may have been due to contamination of the tumor sample by sera, red blood cells, or other normal tissues which can contain the phosphorylase. Walker 256(ADL) was positive for the enzyme in all forms. L1210D cells were not assayed here, but are well documented as MTAase deficient (57,6,52,29). "D" refers to an L1210 clone dependent on a methylthio source for growth. The parent line, L1210, is also MTAase deficient.

Effect of MTA on cells containing or deficient in MTAase: As shown in Figure 4, 0.5 mM MTA is more toxic to MTAase deficient Walker 256(ATCC) cells than to MTAase containing Schmidt-Ruppin cells. By day 10 the Walker 256(ATCC) culture exposed to MTA was depressed in cell number to 7% of control, while MTA reduced viability in W256(ATCC) to 66%. In the Schmidt-Ruppin culture, inhibition by MTA was overcome, reaching 68% of control growth by day 10, up from a low of 44% of control on day 6. Viability in Schmidt-Ruppin cells remained close to 100%. Growth in media diluted by distilled water rather than 2 mM MTA show 26% and 23% inhibition in Walker 256(ATCC) and Schmidt-Ruppin cultures, respectively, so that the effect of media dilution seems less important than the effect of MTA (data not shown).

In Vivo Studies:

Mice (Figure 5): Five mice were used in this experiment. One mouse fed standard chow maintained a stable weight throughout the experiment, with only a slight gain overall. A mouse fed methionine deficient chow consistently lost weight, and the coat condition and general health of this animal deteriorated within the first 10 days on this diet. MTA supplementation of methionine-free chow could not support positive growth in three mice at dosages between 2.5 and 7.5 mg/g, with coat condition and general health of the MTA-fed mice beginning to deteriorate by day 12 to 14. Raising MTA chow doses on day 12 (from 2.5 to 5.0 mg/g), and again on day 32 (from 5.0 to 7.5 mg/g for one mouse only) did not prevent weight loss in these animals. However, the rate of weight loss for the MTA-fed mice was much lower than the rate of weight loss for the methionine-starved mouse. The experiment was terminated on day 36.

MTA Effect on MTAase Deficient Tumors in Mice: Seven animals were used in this experiment. Four were held on 6.0 mg MTA/g methionine-free chow, two of these were injected with L1210D cells. Two mice were held on 5.0 mg methionine/g methionine-free chow, one of these was injected with L1210D cells. The seventh mouse was held on standard chow and recieved no injection. The two mice on 6.0 mg MTA/g methione-free chow diets that were injected on day 15 with L1210D cells failed rapidly from day 25 and died on day 27, day 12 post injection (Table II). The time to death for all three mice injected with L1210D cells was consistent with the time to death for mice on standard chow previously given similar L1210D injections. The mouse on a 6.0 mg MTA/g diet that was not injected also died, on day 29, of apparent starvation. MTA, as a replacement for methionine, could not prevent or delay death from MTAase negative L1210D cells injected into mice, nor could it support adequate growth in mice whether or not the mice were carrying

L1210D tumors. As shown in Figure 6, mice given 5.0 mg methionine/g methionine-free chow achieved growth comparable to mice on standard chow, at or above 0% weight change, agreeing with other reports of maximum dietary improvement at methionine doses between 4.7 and 9.4 mg/g (20). Both methionine-dosed and standard chow fed mice maintained normal coat condition and good health during this experiment, with the methionine-dosed, L1210D injected mouse only declining in health within the 24 hours prior to its death from the tumor. This animal was injected on day 15 and maintained a weight similar to that before injection until day 27, when it lost weight rapidly until its death on day 28, day 13 post injection. All mice on 6.0 mg MTA/g methionine-free chow lost weight slowly but consistently after the first four days of the diet. Coat condition and general health began to deteriorate within the first nine days on the MTA diet.

Rats: In the first experiment, five rats were used: one on standard chow, four on methionine-free chow with 0.625 to 8.3 mg methionine/g chow supplemented. The optimal rat methionine dose was not determined (Figure 7), but it appeared to lie between 2.5 and 8.3 mg methionine per gram chow. It is given as 0.6% in the literature (20). Coat condition deteriorated in the two lower methionine doses given.

In the next trial five rats were used: one on standard chow, one each on 2.25 or 2.00 mg methionine/g methionine-free chow, one each on 5.00 or 2.50 mg MTA/g methionine-free chow. As shown in Figure 8, 2.25 mg/g methionine appeared to provide growth comparable to both 2.5 and 8.3 mg methionine/g doses (77.4%, 69.6%, and 69.5% of control, respectively), though still below optimal. 2.0 mg methionine/g chow was too low a dose, with 66% of control weight gain and deterioration of coat condition. 5.0 mg MTA/g chow provided growth comparable to that achieved on 2.5 or 2.25 mg methionine/g chow (76.3% of control vs 69.6% or 77.4%). 2.5 mg MTA/g chow was too low a dose

to provide adequate methionine nutrition.

In the last diet experiment 10 rats were used (Figure 9): two each on standard chow, on methionine-free chow, or on methionine-free chow supplemented with 6.0 mg methionine per gram. The remaining four rats were held on methionine-free chow with 5.0, 7.5, 10.0, or 12.0 mg MTA/g chow. The 5.0 mg MTA/g dose provided only 17.29% of normal growth, while the 7.5, 10.0, and 12.0 mg MTA/g doses all provided comparable growth at 37.49%, 40.37%, and 31.75% respectively. Of these three rats, the one receiving 12.0 mg MTA/g consumed the highest gram amount of MTA and gained the least weight, the rat receiving 10.0 mg MTA/g consumed an intermediate amount of MTA and gained the most weight, while the rat receiving 7.5 mg MTA/g consumed the lowest amount of MTA and gained the most weight. All three of these rats were the same age at the start of the diet but the rat receiving 12.0 mg MTA/g weighed 20 g less than either the 7.5 or 10.0 mg MTA/g dosed rats. Again, the methionine deprived rats lost weight consistently during the course of the experiment, with the associated deterioration of general condition. The methionine dose of 6.0 mg/g supplied approximately 55% of the growth achieved on standard chow in the same time period.

General effect of MTA on the growth of rats: Wide variability existed within the amount of weight gained by individual rats on MTA or methionine supplemented methionine-free chow, even among rats of the same age or size at the start of the diet. MTA provided on average only 35% of normal growth regardless of dose, rat weight or age, presense of tumor, or comparison to either standard chow or methionine supplemented methionine-free chow fed controls.

In comparing all MTA treated animals, including rats carrying tumors, no difference in weight gain between diet-only rats and diet-plus-tumor rats could

be found (p>.1). While rats above an initial weight of 80 grams took longer to achieve weight gain when first placed on MTA diets than did rats below an 80 gram initial weight (p=.04) (Table III), no trend in either chow consumption or weight gain was observed to be associated with either animal age or weight at the start of the diet.

While the five highest MTA mg/day consumption rates did provide three of the highest percentages of control growth achieved, no obvious correlation between MTA dose and chow consumption could be determined, and no significant difference was found between growth achievement at the 5.0 mg MTA/g dose and either the 7.5 or the 8.0 mg MTA/g doses (p>.1 in both cases). However, the variablity in the amount of growth gained was lower for the 7.5 and 8.0 mg MTA/g dosages than it was for the 5.0 mg MTA/g dose, indicating these doses were perhaps more appropriate for consistent growth within a group of animals.

No apparent tissue damage was seen in light microscopic examination of liver, stomach, and intestine samples from MTA-fed animals (data not shown) (T. Traweek, personal communication). MTA provided on average only 35% of normal growth regardless of dose, rat weight or age, or presense of tumor, and appears to be a non-toxic but inefficient methionine source in the diet of rats. See Figures 10 and 11. Interestingly, young animals held on methionine-free chow with MTA supplementation retained a juvenile appearance. They had soft fluffy white coats, while animals of the same age that had been held for the same length of time on either methionine-free chow with methionine supplementation, or standard chow, achieved smoother yellowish adult coats as well as greater size.

MTA Effect on MTAase Positive Tumors in Rats: Six rats were used in this experiment. Three rats were held on 6.0 mg methionine/g methionine-free

chow and two of these were injected with 1.13 x10^6 W256(ADL) cells sc. Three rats were held on 7.5 mg MTA/g methionine-free chow and injected with 1.13 x10^6 W256(ADL) cells sc. The number of cells innoculated did not appear to control either survival time or maximum tumor size (Figures 12). With the phosphorylase-containing Walker 256(ADL) cells there was no difference between the average tumor size in rats on the7.5 mg MTA/g diet, and the average tumor size in rats on the 6.0 mg methionine/g diet ($p>.05$). However, the MTA dosed rats did not survive as long as the methionine dosed rats. An exact comparison of time to death due to tumor was not possible, as the methionine dosed rats were sacrificed after the majority of the MTA dosed rats had already died. These rats were judged healthy enough to have survived at least one more day had they not been sacrificed. The MTA dosed rat surviving to the termination of the experiment was in very poor health when sacrificed.

As indicated in Figure 13, rats gained weight as expected on methionine or MTA diets, despite presense of tumor. Also, rats in each diet group (methionine or MTA) gained similar amounts of weight. As expected, MTA as a replacement for methionine in the diet of rats could not prevent or delay the growth of MTAase containing tumor cells injected into those rats.

MTA Effect on MTAase Negative Tumors in Rats: In the final experiment 12 rats were divided into groups of four. One group was placed on standard chow; three rats in this group were injected with W256(ATCC) cells sc. The second group was given methionine-free chow with 6.0 mg methionine/g and all four were injected with W256(ATCC) cells sc. The remaining group was held on 8.0 mg MTA/g methionine-free chow and all four were injected with W256(ATCC) cells sc. Correlation of tumor size by caliper measurement to tumor weight in grams appeared to be accurate ($R=0.97$).

Figure 14 shows the variability in tumor size within the standard chow fed

group. All tumors increased in size throughout the experiment until the final two to six days. Note the delay in achievement of measurable size for these tumors, day 18 to 24 p.i. compared to day 12 p.i. for most other tumors (see Figures 15 and 16). No tumors were rejected in the standard chow-fed rats.

Tumor growth in methionine-fed rats, shown in Figure 15, is also highly variable. One rat rejected his tumor 16 days (day 34 p.i.) after it was first measurable, after it had achieved a maximum size of 0.59 cc.The three remaining methionine dosed tumors increased in size throughout the experiment, with slight decreases in size during the last two days. No correlation of tumor size to the amount of methionine consumed was observed.

As shown in Figure 16, there was great variability in tumor size within the MTA-dosed group. One MTA-fed rat rejected his tumor 8 days (day 20 p.i.) after it became of measurable size. This tumor reached a maximum size of 0.25 cc before decreasing in size and disappearing altogether. A second rat's tumor achieved a maximum size of 5.56 cc before steadily becoming smaller. This tumor was considered to have regressed, in that it lost 40% or more of its maximum tumor volume (50). This tumor was down to 0.38 cc on the day all animals were sacrificed, and would most likely have been rejected within days. The other MTA-dosed tumors increased in size continuously throughout the experiment. No correlation between tumor size and the amount of MTA consumed was observed.

Figure 17 compares all three average tumor size curves; curve-fitting for the MTA average gives a slope of 0.42 (R=0.98). Methionine showed a slope of 1.25 (R=0.97), and standard chow a slope of 0.55 (R=0.96). Both MTA- and standard-fed rats had a slower tumor growth rate than did methionine-fed rats, but, due to the variability within each diet group, there is no significant difference in tumor size for any of the diet treatments ($p>.1$ for all comparisons).

The largest tumors constituted a large percentage of final body weight, as illustrated in Figures 18, 19, and 20, and shown in Table IV. There was no difference in mean in mean percentage of final body weight between the various diet treatments, again due to the high variance within each diet group: MTA vs methionine or standard chow $p>.1$, methionine vs standard chow $p=.077$. Animals with tumors of 22.28, 23.51, and 27.71% of body weight were in failing condition by the final day of the experiment, and were most likely within 24 to 48 hours from death due to tumor. This included two methionine-fed rats and one MTA-fed rat, with the MTA animal in the worst condition.

On removal from the rat carcasses, the tumors and carcasses were visually examined. Only one of the nine tumors was necrotic, and that only in the area which had penetrated the skin over it. Vascularization was most extensive in the largest tumors (37 to 61 g), normal in the mid-range tumors (7 to 23 g), and nonexistant in the smallest tumors (0.19 and 0.82 g). There was no obvious metastisis of the tumor in any of the animals.

Figure 21 shows the average weight gained by this group. In general, standard chow-fed animals gained the greatest amount of weight, methionine-fed rats gained a comparable amount (87% of standard chow gain), and the MTA-fed rats gained on average only 37% of the amount gained by the standard chow fed animals.

Table I. MTAase activity in mouse and rat cell lines

CELL TYPE	SPECIFIC ACTIVITY pmol/min/mg
BW5147	661.16
FR	570.0
	493.78
JENSEN	5.0
LC540	4.95
SCHMIDT-RUPPIN	94.0
W256(ATCC)	0.86
	0
	0
W256(ADL), FRESH ASCITES	395.49
W256(ADL), FROZEN ASCITES	337.44
W256(ADL), TUMOR	278.19

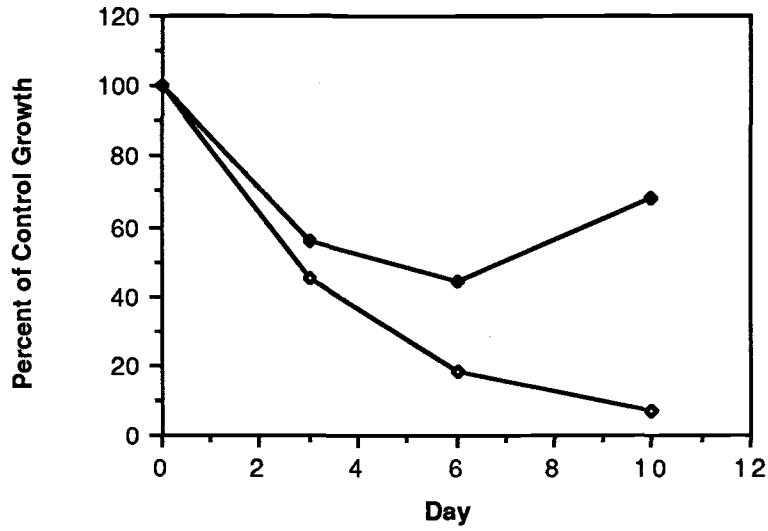

Figure 4. Effect of 0.5 mM MTA on cells containing or deficient in MTAase. W256 (ATCC) cells (MTAase deficient) were seeded at 8.9 x10^4 cells/ml into 10 mls of Medium 199 as a normal control. (◇) W256 (ATCC) cells seeded at 8.9 x10^4 cells/ml into 7.5 mls Medium 199 with 2.5 mls 2 mM MTA for a final MTA concentration of 0.5 mM, presented as percent of control culture growth. Schmidt-Ruppin cells (MTAase positive) were seeded at 8.9 x10^4 cells/ml into 10 mls of Medium 199 as a normal control. (◆) Schmidt-Ruppin cells seeded at 8.9 x10^4 cells/ml into7.5 mls Medium 199 with 2.5 mls 2 mM MTA for a final MTA concentration of 0.5 mM, presented as percent of control culture growth. All six cultures were provided with 10% FBS, 0.5% PSF, and EBSS, were incubated for 10 days, and were trypsinized and counted each three to four days.

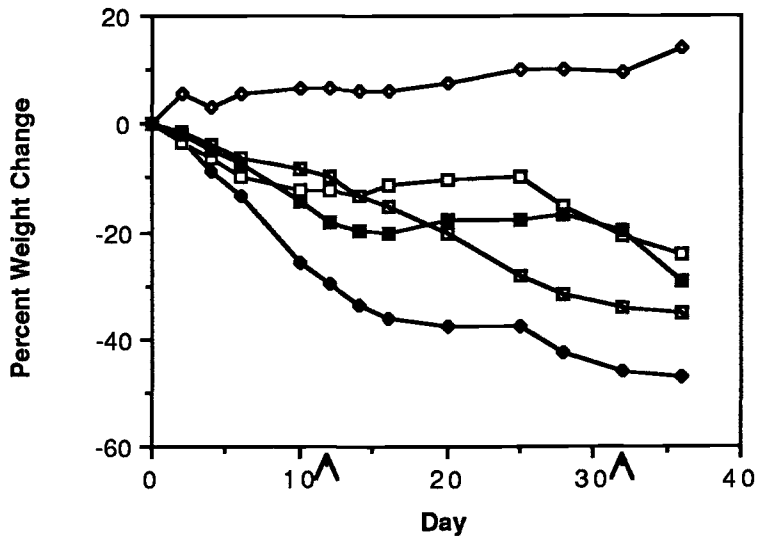

Figure 5. Weight change in mice on methionine-free chow with MTA added. Each curve represents one mouse weighing 18 to 22 g at the start of the diet on (◇) standard chow, (◆) methionine-free chow with no supplementation, or methionine-free chow with (▫) 2.5 mg MTA/g chow, raised to 5.0 mg MTA/g and then to 7.5 mg MTA/g, consecutively, on the days indicated by the two arrows; (■) 2.5 mg MTA/g chow, raised to 5.0 mg MTA/g on day 12 as indicated by the first arrow, or (▧) 2.5 mg/g MTA with no changes

Table II. Survival time for mice with L1210D tumors

Dosage	Cells Injected i.p.	Day of Diet Start	Day of Injection	Day of Death,p.i.	Day of Death
Standard Chow	1.1×10^6	0	0	11	11
Standard Chow	7.1×10^5	0	0	17	17
5.0 mg Met/g MF* Chow	1.0×10^6	0	15	13	28
6.0 mg MTA/g MF Chow	8.2×10^5	0	15	12	27
6.0 mg MTA/g MF Chow	1.0×10^6	0	15	12	27

* MF indicates Met-Free chow

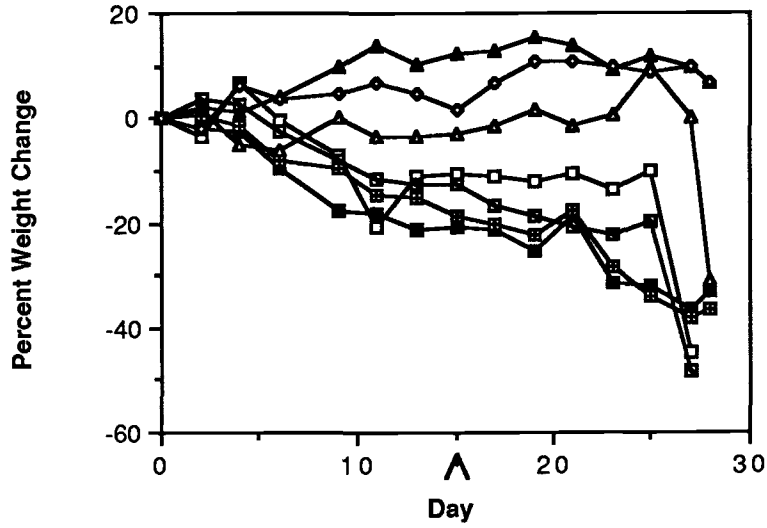

Figure 6. Weight change in mice with L1210D tumor. Each curve represents one mouse weighing 18 to 22 g at the diet start on: (◇) standard chow, no L1210D injected; (■) and (▣) methionine-free chow with 6.0 mg MTA/g chow, no L1210D injected; (▫) methionine-free chow with 6.0 mg MTA/g chow, 8.2 x10^5 L1210D injected; (▧) methionine-free chow with 6.0 mg MTA/g chow, 1.0 x10^6 L1210D injected; (▲) methionine-free chow with 5.0 mg methionine/g chow, no L1210D injected; or (△) methionine-free chow with 5.0 mg methionine/g chow, 1.0 x10^6 L1210D injected. All injections were given intraperitoneally, on day 15 as indicated by the arrow.

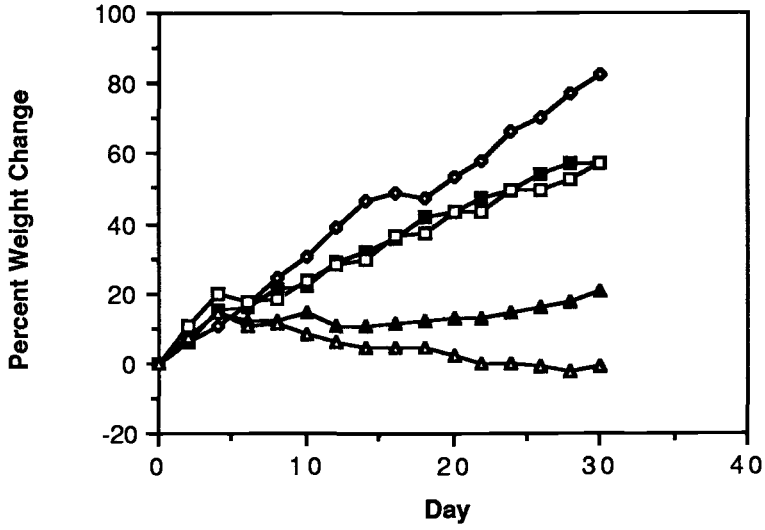

Figure 7. Weight change in rats on methionine-free chow with methionine added. Each curve represents one rat weighing 113 to 120 g at the start of the diet on (◇) standard chow; or methionine-free chow with (■) 8.3 mg methionine/g chow; (□) 2.5 mg methionine/g chow; (▲) 1.25 mg methionine/g chow; or (△) 0.625 mg methionine/g chow.

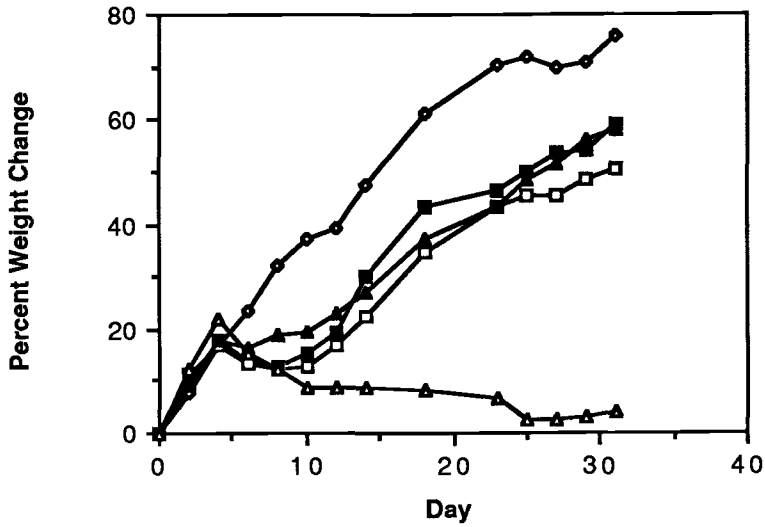

Figure 8. Weight change in rats on methionine-free chow with either methionine or MTA added. Each curve represents one rat weighing 97 to 107 g at the diet start, on (◊) standard chow; or methionine-free chow with (■) 2.25 mg methionine/g chow; (□) 2.00 mg methionine/g chow; (▲) 5.00 mg MTA/g chow; or (△) 2.50 mg MTA/g chow.

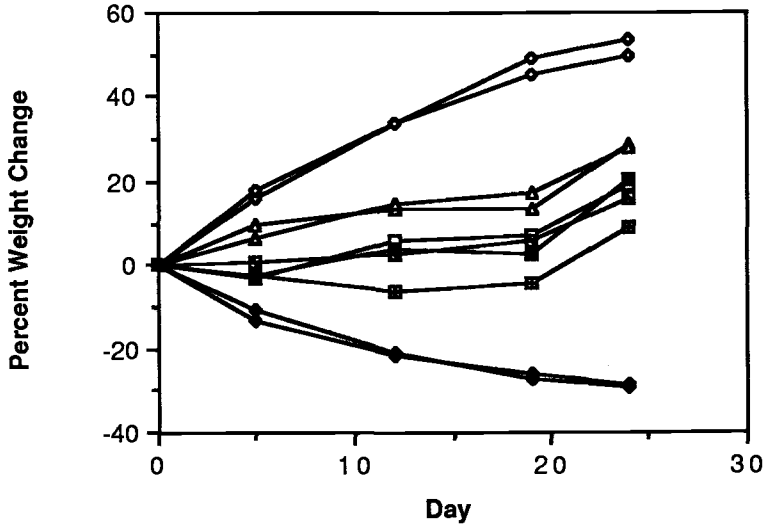

Figure 9. Weight change in rats on methionine-free chow, or methionine-free chow with methionine or MTA added. Each curve represents one rat weighing 168 to 196 g at the start of the diet on: (◊) and (◊) standard chow; (♦) and (♦) methionine-free chow with no additions; or methionine-free chow with: (△) and (△) 6.0 mg methionine/g chow; (⊞) 5.0 mg MTA/g chow; (□) 7.5 mg MTA/g chow; (■) 10.0 mg MTA/g chow; or (⊠) 12.0 mg MTA/g chow.

Table III. Effect of MTA dose on weight gain

MTA mg/g	Day of 1st wt.Gain	Start of Diet Wt. g	Start of Diet Age Days	MTA mg/day	Chow g/day	% Control	Mean
2.5	8	124.19	36	22.79	9.12	5.1	
5.0	4	115.48	36	82.20	17.44	76.3	41.26±31.02
	15	107.07	38	53.18	10.63	30.2	
	24	168.30	39	40.10	8.02	17.29	
7.5	2	79.85	29	41.40	5.52	32.03	32.96± 6.44
	2	52.0	22	55.83	7.44	42.75	
	2	82.37	29	22.59	3.01	20.33	
	4	57.0	22	50.98	6.79	31.96	
	4	47.0	22	42.61	5.68	34.19	
	6	78.0	29	71.21	9.49	34.80	
	8	86.0	29	85.84	11.44	30.16	
	12	196.81	39	47.81	6.37	37.49	
8.0	2	71.83	28	77.89	9.74	54.42	39.42± 8.77
	2	72.00	26	72.88	9.25	42.12	
	4	72.00	26	85.50	10.86	42.14	
	4	78.16	28	76.62	9.57	30.80	
	8	71.00	26	68.63	8.72	34.20	
	10	73.00	26	70.56	8.90	32.84	
10.0	12	194.85	39	59.30	5.93	40.37	*36.06± 6.09
12.0	5	175.99	39	87.79	7.32	31.75	

* Mean given for doses 10.0 and 12.0 mg/g combined

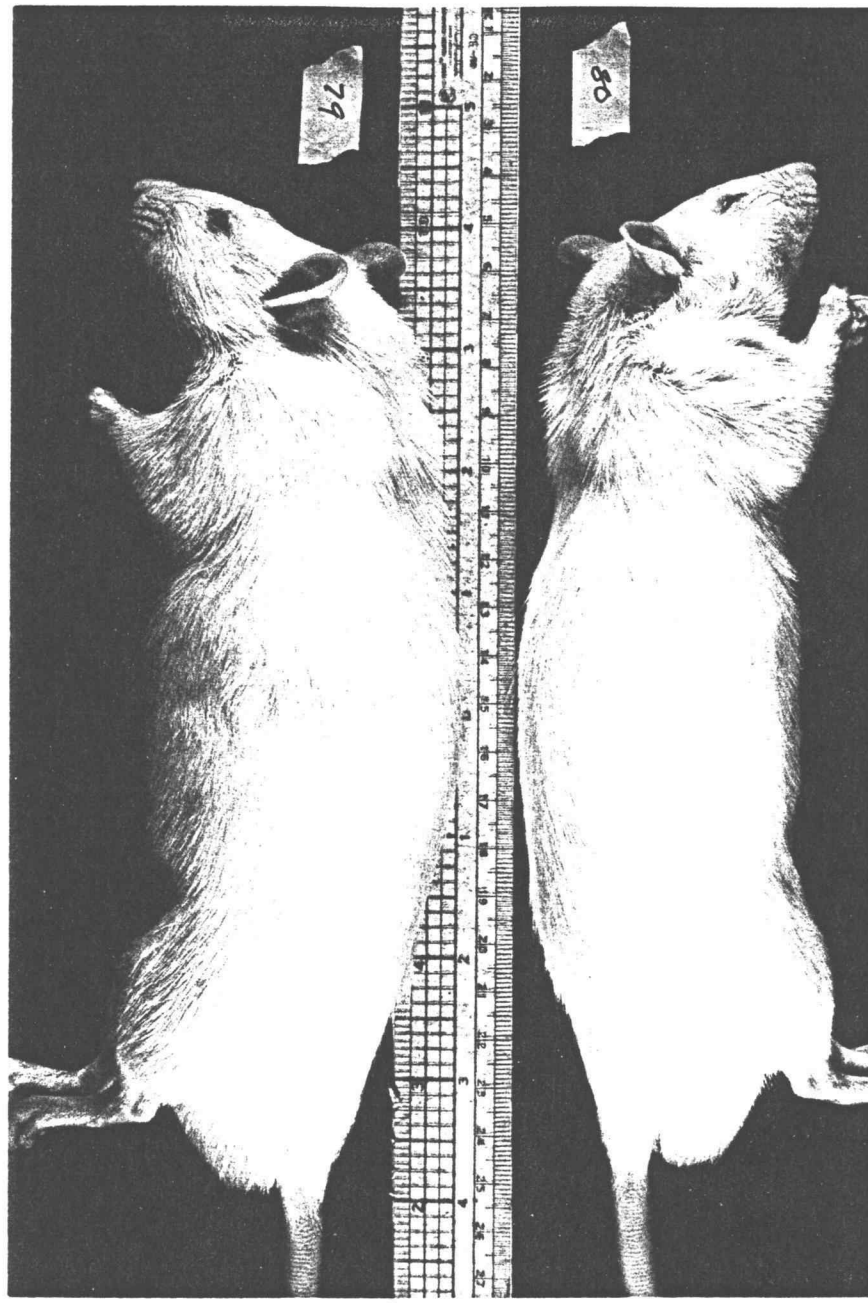

Figure 10. Comparison of growth of rat on standard chow (#79) with growth achieved by rat on methionine-free chow supplemented with 6.0 mg methionine/g chow (#80). #79 weighed 68 g and #80 weighed 71 g at the start of the diet. Both rats have normal adult appearance, but the standard chow fed rat is 25% larger and more robust than the methionine supplemented methionine-free chow fed rat after 42 days on the diet.

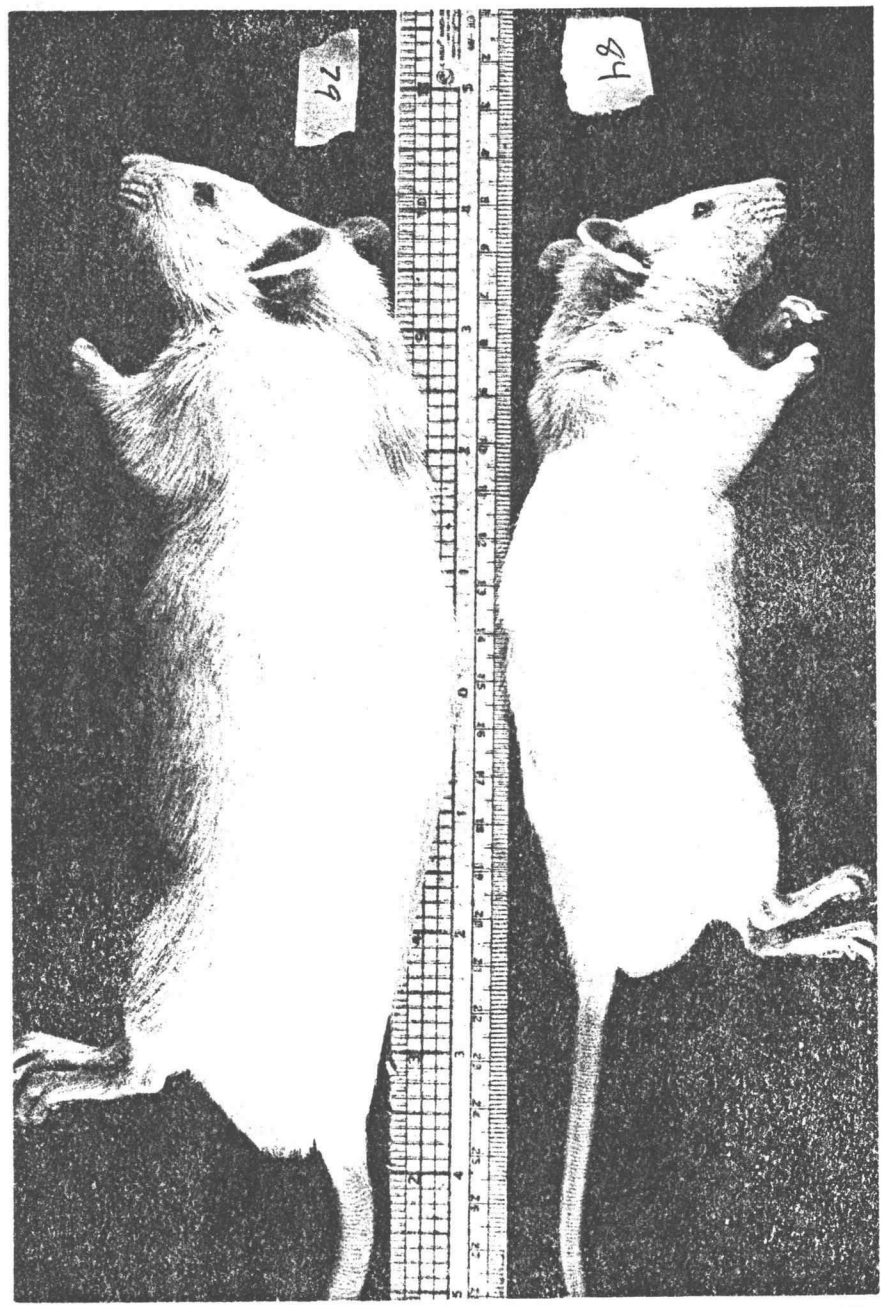

Figure 11. Comparison of growth of rat on standard chow (#79) with growth achieved by rat on methionine-free chow with 8.0 mg MTA/g chow (#84). #79 weighed 68 g and #84 weighed 71 g at the diet start. The standard chow fed rat is 69% larger than the MTA supplemented methionine-free chow fed rat after 42 days on the diet. The MTA fed rat also has retained a juvenile-appearing coat.

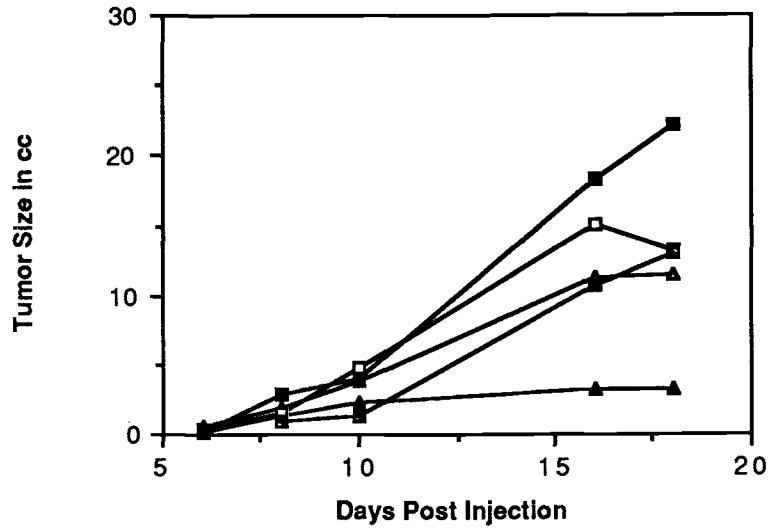

Figure 12. MTAase positive W256(ADL) tumor growth in rats on methionine-free chow with methionine or MTA added. Each curve represents the tumor size achieved in one rat, weighing 47 to 62 g at the start of the diet, on methionine-free chow with: (△) and (▲) 6.0 mg methionine/g chow; or (□), (■) and (▧) 7.5 mg MTA/g chow. All rats were injected with 1.13 x10^6 W256(ADL) cells subcutaneously, on day 4 of the diet. The body weight achieved by these rats is presented in the following figure.

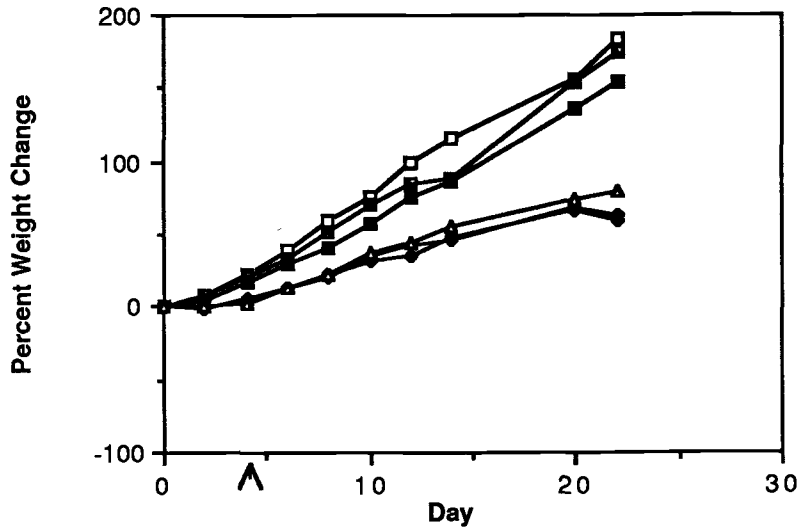

Figure 13. Weight change in rats carrying MTAase positive W256(ADL) tumors, while on methionine-free chow with methionine or MTA added. Each curve represents one rat weighing 47 to 62 g at the start of the diet, on methionine-free chow with: (□) 6.0 mg methionine/g chow and no W256(ADL) cells injected; (■) and (▣) 6.0 mg methionine/g chow and W256(ADL) cells injected; or (◊), (♦) and (△) 7.5 mg MTA/g chow with W256(ADL) cells injected. All rats injected recieved 1.13 x10^6 cells subcutaneously, on day 4 of the diet, as indicated by the arrow. Tumor sizes achieved in these rats are presented in Figure 12.

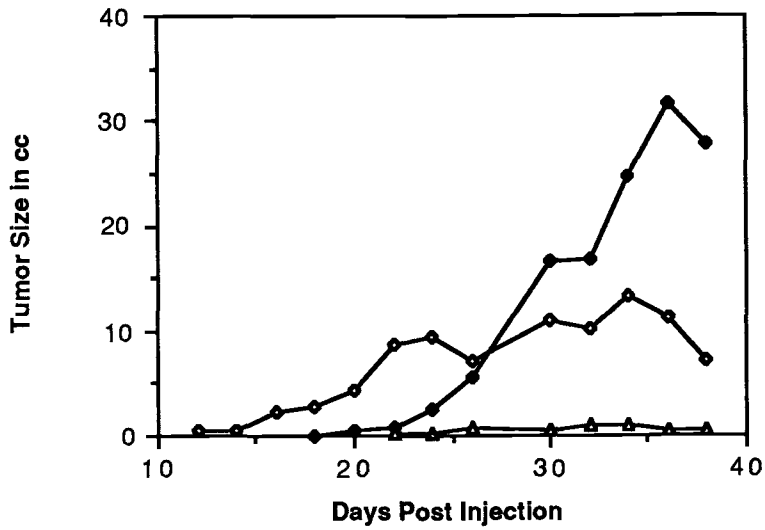

Figure 14. MTAase negative W256(ATCC) tumor growth in rats on standard chow . Each curve represents the tumor size achieved in one rat, weighing 65 to 69 g at the start of the diet, on standard chow. Each rat was injected subcutaneously with 2.2 x10^6 W256(ATCC) cells on day 4 of the diet. Average tumor size achieved in these rats is presented in Figure 17. Average body weight gained in these rats is presented in Figure 21.

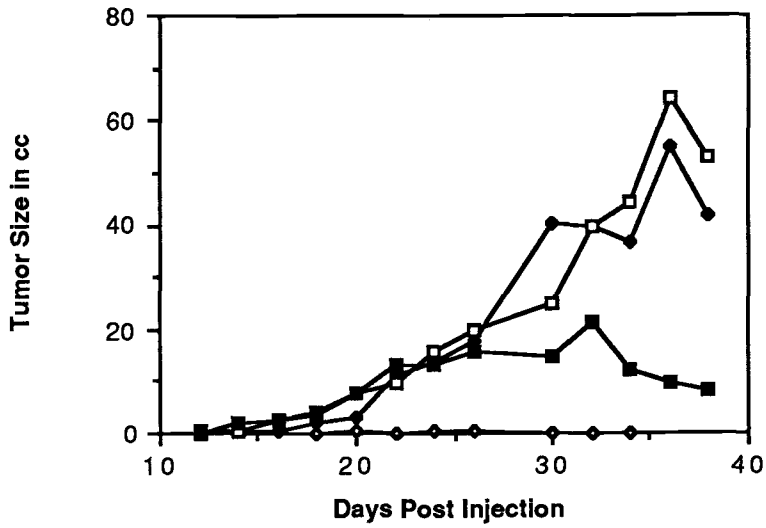

Figure 15. MTAase negative W256(ATCC) tumor growth in rats on methionine-free chow with methionine added. Each curve represents the tumor size achieved in one rat weighing 70 to 71 g at the start of the diet, on methionine-free chow with 6.0 mg methionine/g chow. Each rat was injected subcutaneously with 2.2 $x10^6$ W256(ATCC) cells, on day 4 of the diet. Average tumor size achieved in these rats is presented in Figure 17. Average body weight gained by these rats is presented in Figure 21.

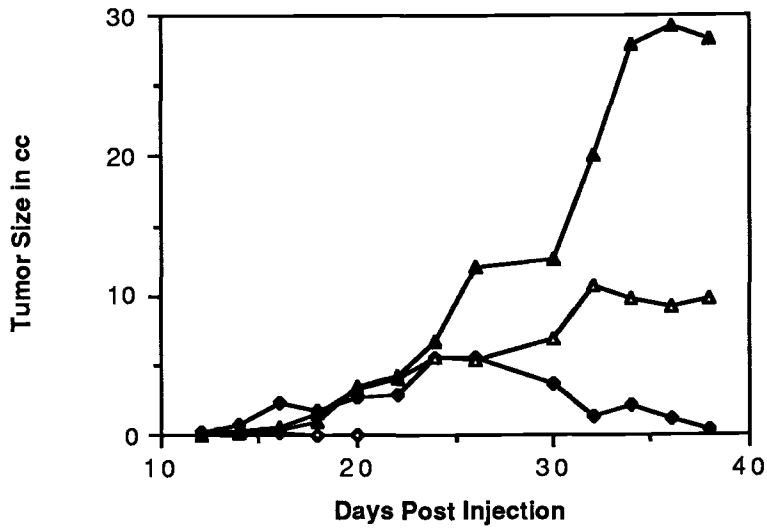

Figure 16. MTAase negative W256(ATCC) tumor growth in rats on methionine-free chow with MTA added. Each curve represents the tumor size achieved in one rat weighing 71 to 73 g at the diet start, on methionine-free chow with 8.0 mg MTA/g chow. Each rat was injected subcutaneously with 2.2 x10 W256(ATCC) cells, on day 4 of the diet. Average tumor size achieved in these rats is presented in Figure 17. Average body weight gained by these rats is presented in Figure 21.

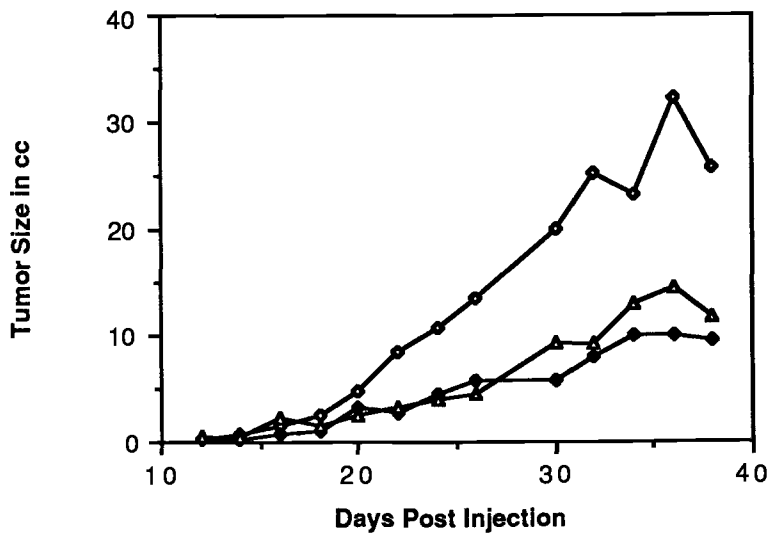

Figure 17. Average MTAase negative W256(ATCC) tumor size in rats on standard chow or methionine-free chow with methionine or MTA added. (△) average tumor size achieved in three rats on standard chow; (◇) average tumor size achieved in four rats on methionine-free chow with 6.0 mg methionine/g chow; (◆) average tumor size achieved in four rats on methionine-free chow with 8.0 mg MTA/g chow. All rats weighed 65 to 73 g at the diet start and were injected subcutaneously with 2.2 $x10^6$ W256(ATCC) cells on day 4 of the diet. Average body weight gained in each diet group is presented in Figure 21.

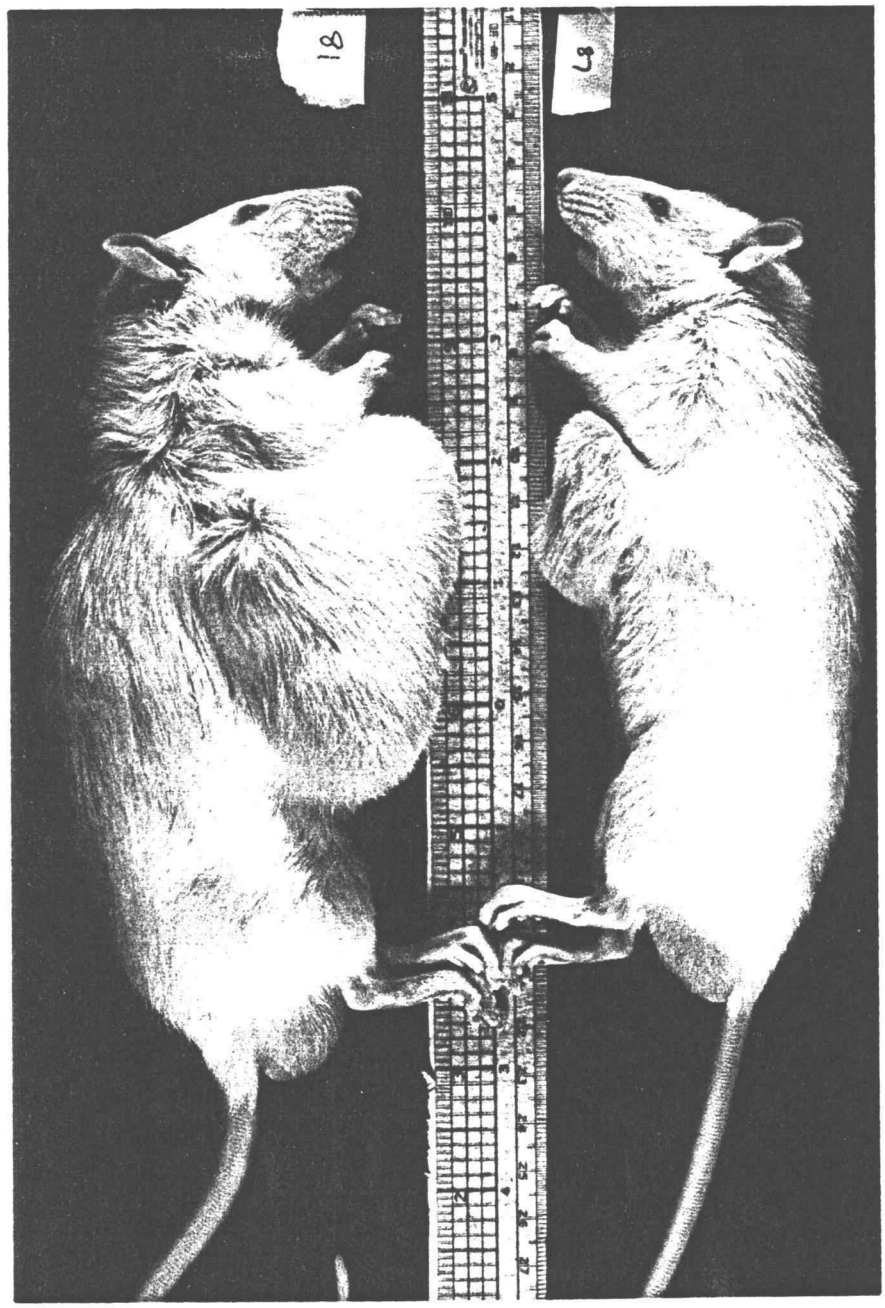

Figure 18. Comparison of the MTAase negative W256(ATCC) tumor size achieved in a rat on methionine-free chow with 8.0 mg MTA/g chow (#87), with the tumor size achieved in a rat on methionine-free chow with 6.0 mg methionine/g chow (#81) after 38 days of growth. #87 weighed 72 g and #81 weighed 70 g at the diet start. Both rats were injected subcutaneously with 2.2 $x10^6$ W256(ATCC) cells on day 4 of the diet.

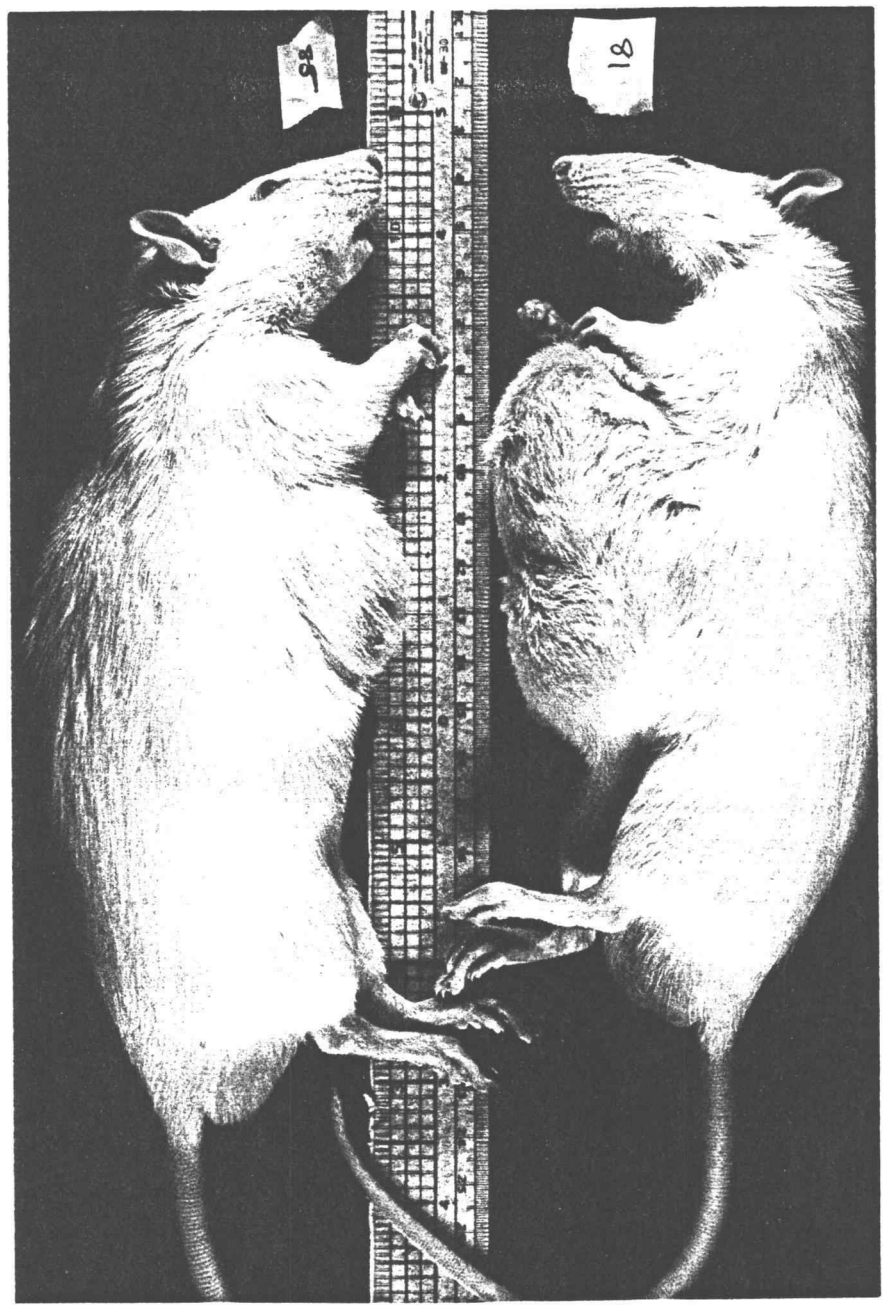

Figure 19. Comparison of the MTAase negative W256(ATCC) tumor size achieved in a rat on standard chow (#85) with the tumor size achieved in a rat on methionine-free chow with 6.0 mg methionine/g chow (#81) after 38 days of growth. #85 weighed 69 g and #81 weighed 70 g at the start of the diet. Both rats were injected with 2.2 $x10^6$ W256(ATCC) cells subcutaneously on day 4 of the diet.

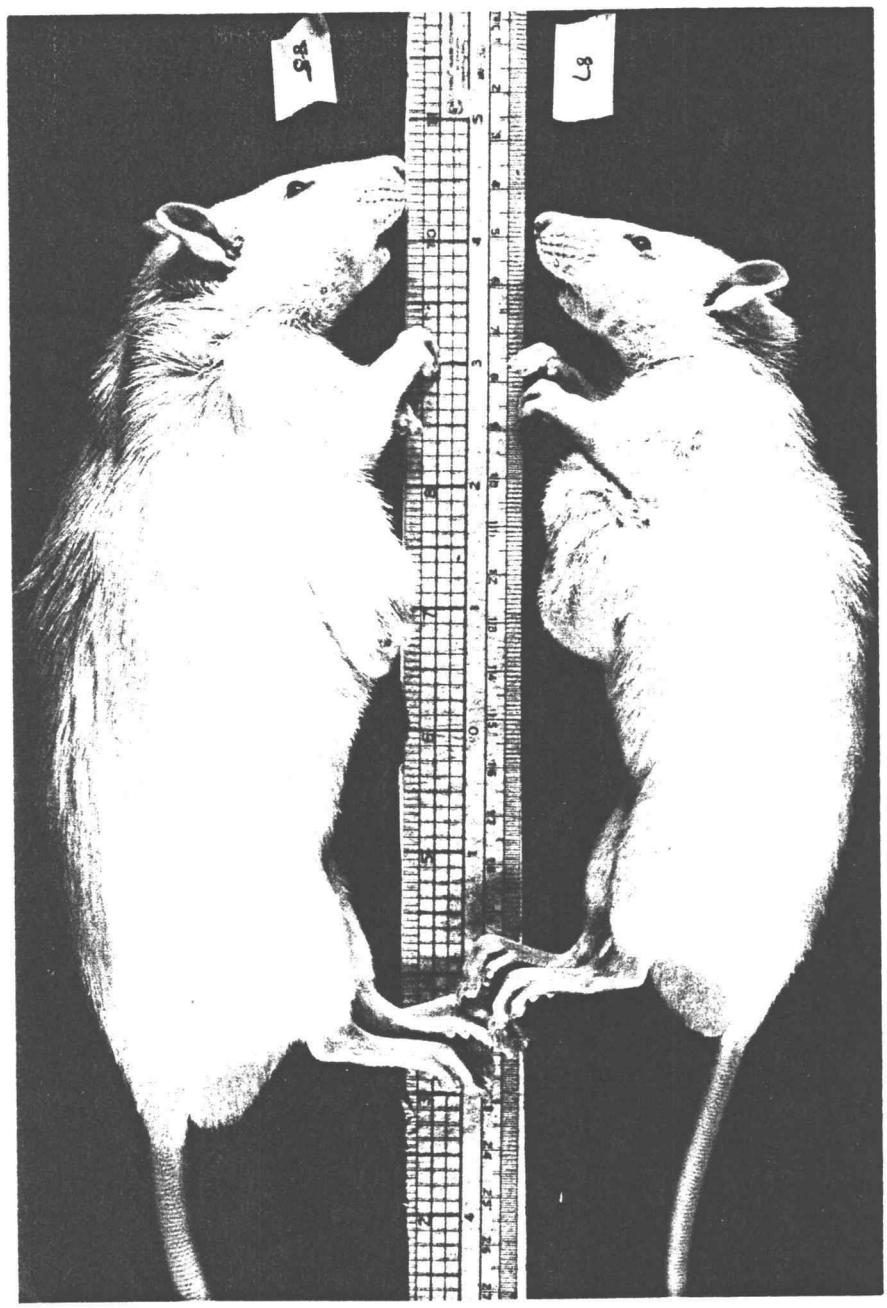

Figure 20. Comparison of the MTAase negative W256(ATCC) tumor size achieved in a rat on standard chow (#85) with the tumor size achieved in a rat on methionine-free chow with 8.0 mg MTA/g chow (#87) after 38 days of growth. #85 weighed 69 g and #87 weighed 72 g at the start of the diet. Both rats were injected with 2.2 $x10^6$ W256(ATCC) cells subcutaneously on day 4 of the diet.

Table IV. Tumor size as percentage of body weight

Dose mg/g	Body Weight, g	Tumor Weight, g	Percentage
6.0 Met	274.19	61.10	22.28
6.0 Met	257.23	60.49	23.51
6.0 Met	252.62	8.63	3.42
8.0 MTA	170.29	0.82	0.48
8.0 MTA	165.15	9.38	5.68
8.0 MTA	134.97	37.40	27.71
Standard Chow	275.72	0.19	0.07
Standard Chow	269.43	23.45	8.70
Standard Chow	262.64	7.03	2.68

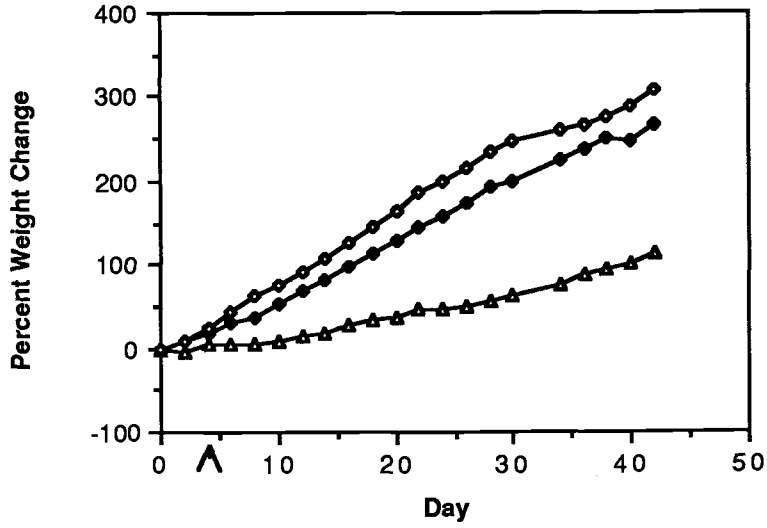

Figure 21. Average body weight gain in rats with MTAase negative W256(ATCC) tumors, held on standard chow or methionine-free chow with methionine or MTA added. (◊) average body weight gain of three rats on standard chow; (♦) average body weight gain of four rats on methionine-free chow with 6.0 mg methionine/g chow; (△) average body weight gain of four rats on methionine-free chow with 8.0 mg MTA/g chow. Rats weighed between 65 g and 73 g at the start of the diet. All rats were injected with 2.2 x10^6 W256(ATCC) cells subcutaneously, on day 4 of the diet as indicated by the arrow. Average tumor size achieved in each of the diet groups is presented in Figure 17.

DISCUSSION

A protocol was developed which focussed on MTA as a methionine source. MTA in a methionine-free diet would represent the only exogenous methionine source for an animal on that diet. All the normal cells in the animal would be MTAase positive and able to catabolise MTA and regain methionine. Any MTAase deficienct malignant cells present would not be able to scavenge methionine from the MTA in the diet and would be selectively starved for the essential amino acid. These malignant cells would then be expected either to die off, or grow more slowly than otherwise.

The cell culture experiments described in this report confirm that MTA can depress cell proliferation at higher concentrations. MTAase containing cells are less sensitive to the presence of MTA, most likely due to their ability to catabolise the nucleoside. Other researchers had shown that MTAase positive cells cultured in methionine-free medium could utilize exogenous MTA as a source of methionine while MTAase deficient cells could not. The presence of MTAase activity in the serum used in the culture medium did not alter this differential effect (42).

To test the protocol, the ability of MTA to supply nutritional methionine first had to be determined. MTA as a methionine replacement in the diet of mice could only partially support growth in mice at dosages between 2.5 and 7.5 mg/g. These animals lost weight, but at a slower rate than methionine-deprived mice. On a molar basis, catabolism of MTA provides only one-half the methionine of an equal weight of the amino acid, so that these MTA doses are equivalent to 1.25 to 3.75 mg methionine/g chow respectively. Given the poor appetite of the mice provided with the MTA chow, the MTA doses given may have been too low to provide adequate growth. This conclusion is supported by deterioration of coat condition in the MTA-dosed mice similar to that seen in

methionine-deprived mice.

MTA, as the sole dietary methionine source, could not rescue mice from death due to the presence of an MTAase deficient leukemia (L1210D). The effect of the tumor, however, may have been exacerbated by the inability of the diet to provide adequate nutrition in mice. Also, L1210 cells grown as ascites in mice have been shown previously to exhibit low, variable MTAase activity. As L1210 cells are definitely MTAase negative (51), this effect was attributed to the presence of inflammatory cells (53) and may have been operative in this experiment.

In rats, chow consumed did not reflect dosage given, implying that the sulfurous odor of MTA did not affect the palatability of the MTA chow for rats as it appears to have for mice. MTA as a methionine replacement in the diet of rats provided adequate, but not optimal, growth at dosages between 5.0 and 12.0 mg/g methionine-free chow. No difference in growth achieved was observed for any dose within this range, however, greatest consistency in the amount of weight gained was seen with the 7.5-8.0 mg/g doses. MTA provided on average only 35% of the growth achieved by rats on standard chow, regardless of MTA dose, or rat weight or age. It is not clear if MTA is merely a substandard or inefficient methionine source, or if it has a more active role in preventing normal growth as the young rats stunted by MTA in size also retain a juvenile appearance. Rats of the same age given either standard chow or methionine-free chow with methionine supplementation achieve a normal adult appearance.

Supplementation of the methionine-free chow with methionine did not provide optimal growth either, however, with doses between 2.5 and 8.3 mg/g providing only 50% to 77% of growth on standard chow. This implies that standard rodent feeds supply some nutrient not present in the methionine-free chow. The effect of this missing nutrient on the experiments presented here is

unknown.

Several rat cell lines were determined to be MTAase deficient. Walker 256 carcinosarcoma was the MTAase deficient cell line chosen for this experiment. Originally discovered on the lower abdomen of a pregnant albino rat in 1928, W256 initially appeared as an adenocarcinoma but now appears to be highly anaplastic. W256 had a rapid, uniform growth rate and rare early regression (14). W256 was identified in 1983 as a methionine auxotroph, unable to use exogenous MTA or homocysteine as a methionine replacement for growth. The cell line posessed MTAase activity, formed methionine from MTA, and incorporated label from exogenous 3H-MTA into DNA, RNA, and protein (51). The W256 cells cultured in this laboratory were always MTAase deficient since their receipt from American Type Culture Collection (ATCC). The W256 cells recieved from Arthur D. Little, Co. (ADL) could not be cultured and were maintained as an ascites tumor in rats. They retained MTAase activity. Karyotype analysis of these two "identical" cell lines may reveal interesting information regarding the nature of the MTAase deficiency in the W256(ATCC) cells.

As expected, MTA in the diet of rats had no effect on the tumor size achieved by the MTAase positive W256(ADL) cells injected into those rats. These rats gained weight on methionine- or MTA-supplemented methionine-free chow diets, but the MTA-dosed rats gained only about one-third the weight gained by the methionine-fed rats. The MTA-fed rats declined in health, and did not survive as long as the methionine-dosed animals did.

Rats with MTAase negative W256(ATCC) tumors, despite gaining weight on MTA as a methionine replacement in the diet, did not achieve tumor sizes significantly smaller from those achieved on standard or on methionine-supplemented methionine-free chow. This conclusion is based on

comparing the average tumor size for all tumors in each diet group. The variability in tumor size within each diet group is high, but not unexpected. Subcutaneous W256 tumors have been shown previously to fall into discontinuous size catagories, grouping into large and small tumors (49), with little to no necrosis in the large tumors (50). Size variability of tumor was due to the position of growth (attached to muscle versus skin), level of vascularization, and suseptibility of the animal, rather than innoculation error (49). Rat size or sex had no effect on tumor size (49). These observations are supported by the results presented here, in that within each diet group are large (>12.00 cc) and small (<10.00 cc) tumors, as well as tumors that regress in size, although regression could not be linked with diet type. The small tumors achieve similar size with the three diet treatments. The large tumors are of comparable size in the MTA-dosed chow and the standard chow groups as well. Supplementation of methionine-free chow with methionine at 6.0 mg/g however, supported tumors almost twice the size of those seen in the standard chow group. Vascularization was extensive in all the large tumors so that this difference in size appears to be due the methionine in the diet.

Tumor utilization of an amino acid such as methionine can create a concentration gradient from host cells towards tumor cells, starving the host for that amino acid (24). Tumor tissue is insensitive to host starvation (50). This implies the addition of methionine to the methionine-free diet may selectively support tumor growth over body growth. The methionine dosage given may have been excessive, without being toxic, so that excess methionine was available for increased tumor growth.

The presence of tumor may also influence methionine synthesis. Ehrlich ascites (EAC) tumor in mice activated methionine synthesis in liver using homocysteine as substrate and methyltetrahydrofolate as the methyl group donor. Methyltetrahydrofolate reductase and methionine synthase were higher

in livers of EAC bearing mice of all ages than in healthy mice (24). Enhanced hepatic methionine biosynthesis with methyltetrahydrofolate as donor was also reported in rats with some hepatomas (24). This implies the tumor can influence methionine production from homocysteine in the normal tissuesof its host.

Homocysteine, as a product of SAM in methylation reactions, is limited by the size of the SAM pool. The size of the SAM pool is limited by methionine as SAM is formed from methionine and ATP. MTA and homocysteine accumulate simultaneously in MTAase deficient cells' medium (25). Homocysteine accumulation estimates the transmethylation rate: maximum occurs during the mid-exponential growth phase, close to the time of cell (DNA) replication (21). Rapidly dividing MTAase deficient malignant cells can be assumed to be producing much homocysteine as high rates of transmethylations are characteristic of tumor cells (24). Also, choline was present in the methionine-free chow used and by conversion to betaine can remethylate homocysteine through the action of betaine:homocysteine methyltransferase. However, total SAM pools are probably not large enough to continue to supply methionine from homocysteine for any length of time when exogenous methionine is not supplied.

It is possible that normal cells take up MTA, convert it to methionine by MTAase action, only to have the rapidly dividing MTAase deficient tumor selectively draw the methionine from normal tissues, for the support of the tumor, and in effect starving the normal cells for an essential amino acid. This might explain why rats on MTA diets retained the small body size typical of those diets and yet grew MTAase deficient tumors as large as those on rats recieving standard chow.

MTAase is known to be present in some mammalian blood sera. Although rat

sera has not been analysed, if MTAase is present, it would allow for the removal of MTA excreted by MTAase deficient cells and its degradation to MTR-1-P, but does not necessarily provide for further catabolism. MTR-1-P can not be absorbed by cells and will degrade to methylthioribose (MTR). Is it possible that some source of kinase activity also is present which would phosphorylate the MTR after it is taken up by cells and return it to the methionine recycling pathway?

High rates of transmethylations are characteristic of tumor cells (24), and methionine has a major role in biological methylations. Lipotrope (methionine and choline) deficient diets induce increased liver N2 guanine tRNA methyltransferase activity, with other base specific tRNA methyltransferases remaining unchanged. This pattern is also seen within one week of administering acetlyaminofluorene (AAF), a carcinogen, in rat diet.. Methyl group supplemented diets diminish the effects of some carcinogenic chemicals *in vivo* including AAF, implicating altered metabolism of methyl groups in carcinogenesis (56).

Lipotrope deficient diets also enhance action of several hepatocarcinogens and decrease the SAM:S-adenosylhomocysteine ratio in the livers of rats. A possible relationship between methyl deficieny and hepatocarcinogenesis is again indicated as this ratio determines whether substrates will be normally methylated (47). MTA supplementation might therefore be beneficial to animals on a methionine-deficient diet as a methyl group source as well as a methionine source. The toxicity of high methionine doses, however, seems to involve the methyl group rather than the homocysteine moiety as similar growth depression and tissue damage occur when high levels of other methyl-containing compounds are fed (2). No obvious tissue damage was seen in the animals exposed to MTAase deficient malignancies or to MTA diets or both, however.

Regulation of eucaryotic gene expression is often dependent on DNA hypomethylation. Inhibition of DNA methylation (as results from chemical carcinogens or methyl deficient diets perhaps) with resulting alterations in gene expression might induce or support tumor formation. However, the response is not universal: a methionine and choline deficient diet that lowered hepatic DNA methylations and enhanced liver tumor formation in F344 rats, did not do so in C3H mice, even in those mice treated with a carcinogen (47). It is possible this difference in response is also reflected in the difference between the response of rats and mice to the MTA diet used in this experiment.

States of increased cell proliferation such as cancer are commonly associated with accelerated rates of polyamine synthesis (33), and the concomittant increased production of MTA (26). Patients with malignant tumors have high serum polyamine concentrations and excrete larger amounts of polyamines in the urine than do normal subjects. Amounts of urinary MTA in malignant patients, however, were not significantly different from those of normal patients (31). Plasma MTA, on the other hand, rose dramatically in mice bearing an MTAase deficient mutant of R1.1 cells, but not in mice bearing wild-type MTAase positive R1.1 cells; both groups' tumors achieved significant mass and progressed fatally. Plasma MTA also increased in mice injected with MTAase deficient L1210 cells (5). MTAase negative malignancies therefore appear to dump high levels of MTA into the bloodstream. This plasma MTA increase could concievably affect the host animal. The lack of an increase in MTA in urine suggests MTA is being taken up or degraded at some point, and supports the possibility that the MTA excreted by MTAase negative cells is taken up by normal cells and catabolised.

In conclusion, MTA as a methionine replacement in the diet of mice could only provide marginal nutrition, and could not support their growth. MTA as a methionine replacement in the diet of mice carrying MTAase deficient ascitic

tumors could not increase the longevity of those mice. In rats, MTA as the sole dietary methionine source could only provide 35% of the growth achieved by rats on standard chow. MTA as a methionine replacement in the diet of rats carrying MTAase deficient tumors could not affect the size of those tumors. present in either rodent. These MTAase deficient malignancies appear to be resistant to selective starvation for methionine, perhaps by manipulating methionine metabolism in their host such that the host starves before the tumor does.

That methionine can be obtained from other sources in the body, such as homocysteine produced from SAM, or from normal cells' degradation of the MTA provided as the sole exogenous source of methionine, may preclude the selective starvation of the MTAase deficient malignancy in the protocol as it is designed. MTA therapy may still be beneficial in combination with other forms of chemotherapy including methotrexate and DFMO.

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