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ALTERED METHIONINE AUXOTROPHY OF A HUMAN MELANOMA CELL LINE ASSOCIATED WITH EXPOSURE TO SERUM

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ABSTRACT

The salvage of methionine occurs in the cell via two pathways. Methionine is converted to S-adenosylmethionine which can then be involved in either transmethylation reactions or polyamine synthesis. In the transmethylation arm of methionine salvage, homocysteine serves as an intermediate and can be directly converted to methionine. During the synthesis of polyamines, the by-product 5'-deoxy-5'-methylthioadenosine is subsequently converted to methionine. The metabolism of methionine is central to transmethylation reactions and has been found to be altered in many transformed cells. Since the level of expression of certain genes has been found to change during the transformation of a cell and since the degree of methylation of a gene has been found to have an effect on its activity, the effect of alterations in methionine metabolism on gene expression have been the subject of study for some time.

The poorly metastatic human melanoma cell line MeWo has been found to be capable of synthesizing DNA in serum-supplemented, methionine-free medium containing 5'-deoxy-5'-methylthioadenosine. In contrast, the highly metastatic MeWo-LC1 cell line, derived from MeWo, exhibits a markedly reduced ability to synthesize DNA under conditions where methionine is replaced by 5'-deoxy-5'-methylthioadenosine in

the medium. Both cell lines contain 5'-deoxy-5'-methylthioadenosine phosphorylase activity and therefore, the difference in methionine auxotrophy between MeWo and MeWo-LC1 cells is not due to a lack of the enzyme necessary to degrade 5'-deoxy-5'-methylthioadenosine. However, when MeWo-LC1 cells are cultured in serum-free medium containing transferrin and supplemented with the nucleoside in place of methionine, DNA synthesis occurs normally.

The presence of serum in the culture medium appears to alter the response of MeWo-LC1 cells to 5'-deoxy-5'-methylthioadenosine. The decrease in DNA synthesis in the presence of serum is not likely due to increased extracellular metabolism of the nucleoside by 5'-deoxy-5'-methylthioadenosine phosphorylase or adenosine deaminase activity, or due to a sequestering of the nucleoside by serum components. The uptake of the nucleoside by MeWo-LC1 cells is not decreased in the presence of serum and therefore the substrate is likely to be available to the cell. Further, the incorporation of 5'-deoxy-5'-methylthioadenosine into cellular macromolecules appears to be unaffected by the presence of serum. However, under conditions where methionine is not limiting, the nucleoside has a greater inhibitory effect on DNA synthesis in MeWo-LC1 cells in the presence of serum than in serum-free medium supplemented with transferrin. The mechanism responsible

for the increased inhibitory effect of 5'-deoxy-5'-methylthioadenosine appears to be independent of polyamine and S-adenosylhomocysteine metabolism.

The component(s) responsible for the inhibitory effect of serum on the 5'-deoxy-5'-methylthioadenosine-dependent synthesis of DNA remains elusive, but is likely to be a protein. This study suggests that although cells may contain 5'-deoxy-5'-methylthioadenosine phosphorylase activity, they may nevertheless be incapable of DNA synthesis when methionine is replaced by 5'-deoxy-5'-methylthioadenosine in the culture medium. This may be as a consequence of an inhibitory effect of the nucleoside itself, a phenomenon that is potentiated by the presence of serum.

This work is dedicated to my parents, Dr. and Mrs. S. Krzaniak and especially, to my husband Richard in recognition of their encouragement, love and support.

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ABBREVIATIONS USED IN THE TEXT

Ade: adenine

AdoHcy: S-adenosylhomocysteine

AdoMet: S-adenosylmethionine

Adox: pericdate oxidized adenosine

AMP: adenosine monophosphate

ATP: adenosine triphosphate

c-AMP: cyclic adenosine monophosphate

Ci: curie

2dA: 2'-deoxyadenosine

dATP: deoxyadenosine triphosphate

dCF: 2'-deoxycoformycin

dES: dialyzed equine serum

dFBS: dialyzed fetal bovine serum

DNA: deoxyribonucleic acid

DTT: DL-dithiothreitol

EDTA: disodium ethylenediamine tetraacetate

ESA: equine serum albumin

Hcy: homocysteine

KMTB: ketomethylthiobutyrate

5-mC: 5-methylcytosine

MeSAdo: 5'-deoxy-5'-methylthioadenosine

Met: methionine

MTR-1-P: methylthioribose-1-phosphate

RNA: ribonucleic acid

SDS: sodium dodecyl sulphate

Spd: spermidine

Spm: spermine

INTRODUCTION

The process of oncogenesis as well as the 'progression' of tumors from a relatively benign to a highly malignant state have been the subject of intense scientific study for many years. Many human cancers are believed to be caused by the abnormal regulation of developmentally important genes (Comings, 1973). Cancer may result when a normally inactive developmental stage-specific gene is activated producing a functional product in a cell type that would not normally contain any such product (Riggs & Jones, 1983).

Most normal cells have specific morphology and function, i.e. they are differentiated. As a result, they tend to lose their ability to proliferate. Usually, tissues undergo renewal via the proliferation of a precursor cell, known as a stem cell, followed by differentiation of its progeny (Hill & Tannock, 1987). Most cancers probably originate from the transformation of a precursor cell (Hill & Tannock, 1987). This can occur by a variety of mechanisms which result in the damage or alteration of cellular DNA, causing heritable changes in the cellular program (Hoffman, 1984; Hill & Tannock, 1987). Genes may be activated or inactivated by point mutations, chromosomal translocation to a location next to a more or less active promoter, resulting in changes in the level of transcription or, by the loss of

control regions or the entire gene (Darnell, Lodish & Baltimore, 1986; Phillips, 1987). As a consequence of such modifications, the level of encoded protein or a qualitative change in the protein itself, may occur (Darnell et al., 1986).

Site-specific DNA methylation has been found to have a silencing effect on genes and the disruption of this methylation can either activate a gene or alter the chromatin structure which also probably plays an important role in the regulation of gene expression (Cooper, 1983; Doerfler, 1983; Riggs & Jones, 1983). DNA methylation plays a fundamental role in governing gene expression during normal cell differentiation and therefore alterations in the methylation pattern of DNA may be one of the factors responsible for the aberrant expression of genes seen in cancer (Riggs & Jones, 1983). 5-Methylcytosine (5-mC) is formed by the methylation of cytosine and is the most common form of DNA modification in eukaryotes (Cooper, 1983). Generally, active genes are hypomethylated and since gene activation can result in cancer, the levels of 5-mC in transformed cell systems may be different from those in normal cells. Several studies have found that tumor cell DNA tends to show reduced methylation (Diala, Cheah, Rowitch & Hoffman, 1983; Liteplo & Kerbel, 1987; Liteplo & Munro, 1988).

Defects in transmethylation reactions, which are

Defects in transmethylation reactions, which are associated with alterations in methionine metabolism, have been observed in a wide variety of cancer cells (Hoffman, 1985). The metabolism of methionine (FIGURE 1, structure FIGURE 2) involves its conversion to S-adenosylmethionine (AdoMet, FIGURE 2), which subsequently serves as the methyl donor for a variety of transmethylation reactions and as a precursor for polyamine biosynthesis (Hoffman, 1984). The link between methionine metabolism and carcinogenesis is based on the observations that: (1) the methionine analogue ethionine causes neoplastic transformation in rat liver cells and its action can be partially relieved by the readministration of methionine (Brown, Wilson & Poirier, 1983; Hoover, Hyde, Wenk & Poirier, 1986) and (2) the administration of methyl-deficient diets (i.e. those lacking methionine and choline), results in the development of hepatomas and promotes chemical carcinogenesis in rats and some strains of mice (Mikol, Hoover, Creasia & Poirier, 1983; Locker, Reddy & Lombardi, 1986; Wainfan, Dizik, Hluboky & Baus, 1986; Wainfan, Dizik, Stender & Christman, 1989).

The major metabolic functions of methionine include its use in protein synthesis and the initiation of translation and conversion to AdoMet. The salvage of methionine within the cell occurs via two pathways (FIGURE 1). AdoMet is

Pathways of Methionine Synthesis

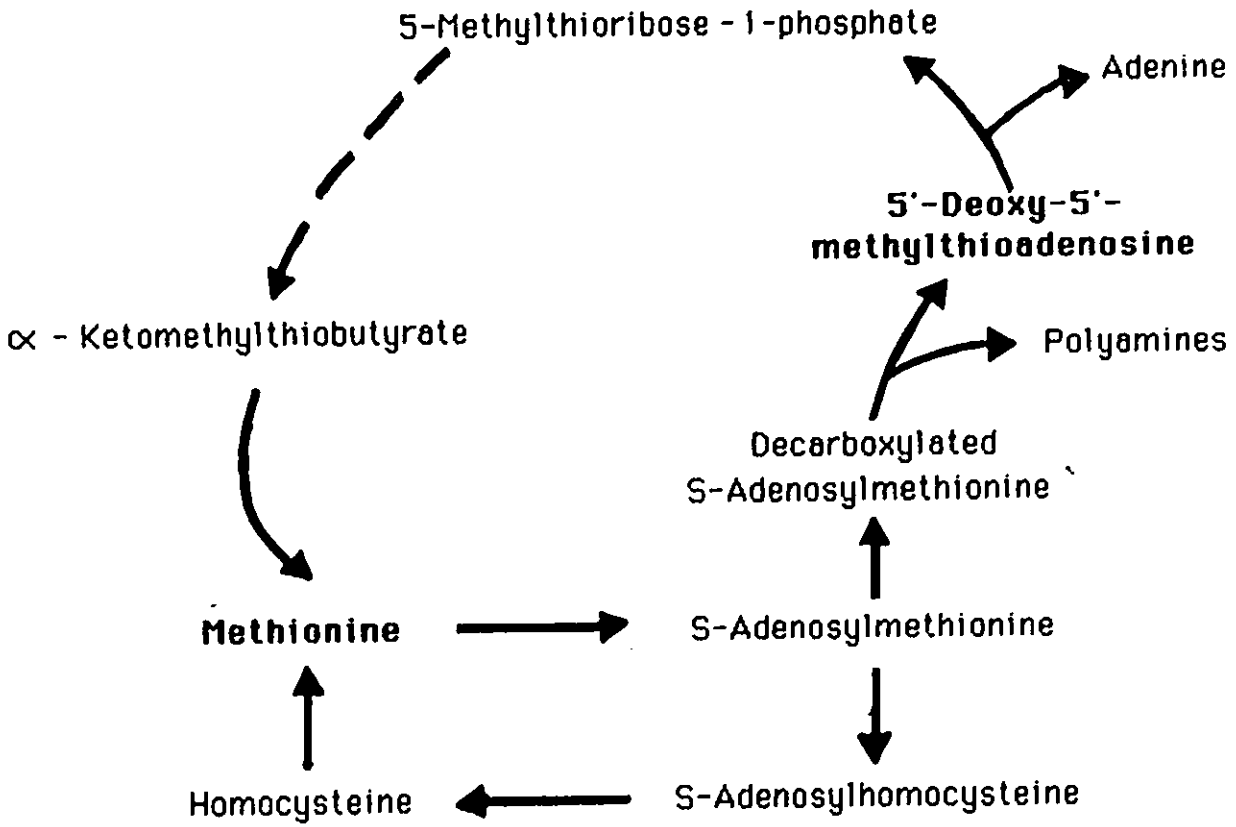
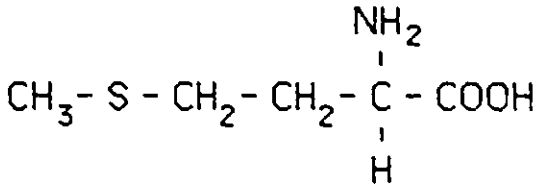
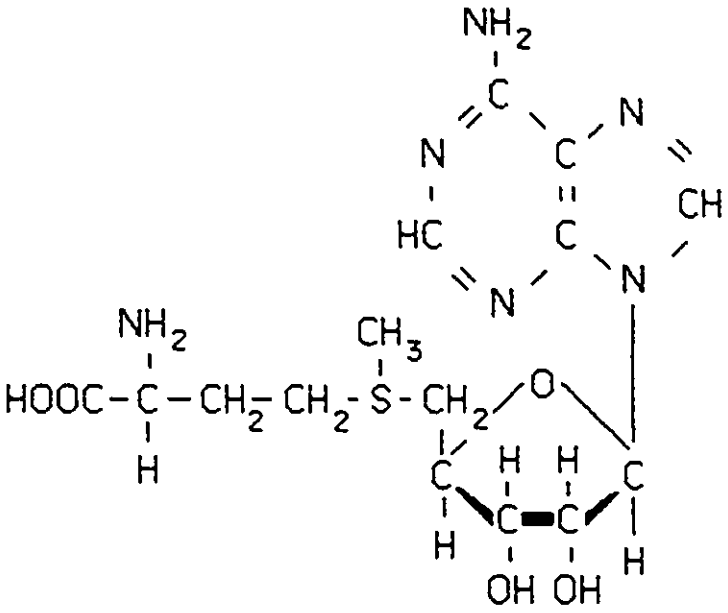


FIGURE 1

Structures of Methionine and S-Adenosylmethionine



Methionine



S-Adenosylmethionine

FIGURE 2

synthesized from methionine and is then either converted to AdoHcy upon the transfer of its methyl group to DNA, RNA, phospholipid or protein, or is decarboxylated to form S-methyl-adenosylhomocysteamine (or decarboxylated AdoMet). In the transmethylation pathway, the AdoHcy formed from AdoMet is metabolized to adenosine and homocysteine (Hcy), and Hcy can be methylated to produce methionine (Hoffman, 1984). In the second pathway, decarboxylated AdoMet serves as the aminopropyl donor in polyamine synthesis, reacting with either putrescine or spermidine (Spd), to form Spd and spermine (Spm) respectively. As a by-product of these reactions, 1 mole of 5'-deoxy-5'-methylthioadenosine (MeSAdo) per mole of Spd and 2 moles of MeSAdo per mole of Spm are produced (Pegg & Williams-Ashman, 1969). MeSAdo (FIGURE 3) is metabolized by MeSAdo phosphorylase to yield adenine and methylthioribose-1-phosphate (MTR-1-P). The adenine produced serves in the synthesis of RNA and/or DNA via its conversion to AMP and subsequently ATP or dATP. MTR-1-P is converted, by a series of not as yet unequivocally determined steps, to ketomethylthiobutyrate (KMTB), which following transamination produces methionine (Trackman & Abeles, 1983). Studies in bacteria have determined that MTR-1-P is converted to 5-methylthioribulose-1-phosphate prior to subsequent metabolism (Furfine & Abeles, 1988). The same intermediate

Structure of 5'-Deoxy-5'-methylthioadenosine

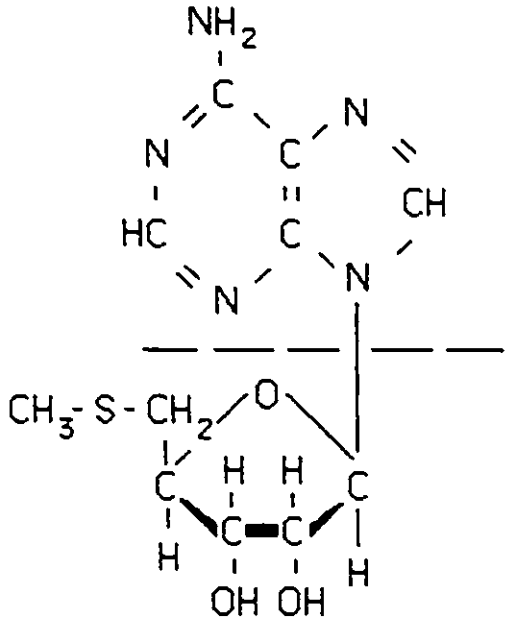


FIGURE 3

has been postulated to exist in mammalian cells, however, others remain as yet unidentified (Trackman & Abeles, 1983; Furfine & Abeles, 1988).

The metabolism of methionine is central to transmethylation reactions. Since DNA methylation is important in gene regulation and 5-mC levels are reduced in tumor cells, a number of studies have determined whether methionine metabolism is altered within cancer cells. All 'normal' untransformed human cells were found to be capable of proliferating in medium containing Hcy, vitamin B_{12} and folic acid in place of methionine (Hoffman, 1985). Most, but not all, transformed and malignant cells are not capable of proliferation under the same conditions (Halpern, Clark, Hardy, Halpern & Smith, 1974; Hoffman, 1982; Mecham, Rowitch, Wallace, Stern & Hoffman, 1983; Stern, Wallace & Hoffman, 1984; Hoffman, 1985; Liteplo, 1989). Hoffman (1982) described cells having no or a reduced ability to proliferate when methionine is replaced by its immediate precursor Hcy in culture medium as "methionine-dependent". The term "methionine-independent" was used to refer to cells which could proliferate in this medium (Hoffman, 1982). Hoffman's description of methionine-dependence led to the characterization of a diverse set of tumor cell lines that were all defective in at least one aspect of methionine metabolism. Since methionine-dependence occurs with high

frequency in tumor cells and is not observed in normal cells, it was suggested that it may be involved in oncogenic transformation and that a general metabolic defect may exist in cancer that is related to the metabolism of methionine (Mecham et al., 1983; Stern et al., 1984; Tisdale 1984). The inability of cells to proliferate in a methionine-free, Hcy-containing medium is not due to a lack of methionine synthesis, but has been suggested to be due to a higher methionine requirement of tumor cells (Tisdale, 1980). It was shown that many methionine-dependent cells synthesize a normal amount of methionine from Hcy, but are deficient in using this methionine for AdoMet synthesis, while exogenous methionine can be used for AdoMet synthesis (Coalson, Mecham, Stern & Hoffman, 1982; Stern et al., 1984). Therefore, the endogenously synthesized methionine appears not to be used in transmethylation reactions or polyamine synthesis.

The second pathway of conversion of AdoMet to methionine which involves MeSAdo as an intermediate, may be important in methionine conservation in cells which synthesize large amounts of polyamines (Hoffman, 1984). Fewer studies on the relationship between the pathway involving this nucleoside and alterations in methionine metabolism have been carried out. MeSAdo at high concentrations can inhibit the DNA methyltransferase and

perturb the pathways of methionine synthesis by various means, and therefore may be linked to the control of transmethylation reactions (Hoffman, 1984).

MeSAdo (FIGURE 3) was isolated in 1912 and its structure was proposed twelve years later (Williams-Ashman, Seidenfeld & Galletti, 1982). Various biochemical pathways for the formation of MeSAdo were proposed, in which either AdoMet or decarboxylated AdoMet served as an immediate precursor. Generally, these pathways are considered to be functional in order to produce biomolecules other than MeSAdo, but it is possible that under certain conditions, the production of MeSAdo may have a functional significance, although it does not normally accumulate within the cell (Williams-Ashman et al., 1982). MeSAdo is rapidly degraded to adenine and MTR-1-P and has been shown to have a variety of regulatory effects on normal cellular metabolism (Williams-Ashman et al., 1982). As many as five independent pathways for the synthesis of MeSAdo have been identified, however, the two major pathways found in higher organisms are: (1) the cleavage of AdoMet (by AdoMet hydrolase) into MeSAdo and homoserine lactone and (2) the pathway involving polyamine synthesis which produces MeSAdo as a by-product (Ferro, 1979).

The carbons from the ribose portion of the MeSAdo molecule, the methyl group and the sulfur atom are

incorporated into methionine (FIGURE 3; Backlund & Smith, 1981). Backlund and Smith (1982) showed that there was significant salvage of methionine from MeSAdo in mammalian cells. These observations led to studies similar to those carried out with Hcy, in which the ability of cells to grow in medium devoid of methionine, but containing MeSAdo was determined. The results indicated that the phenomenon of methionine-dependence could be described not only for Hcy, but also for MeSAdo (Carson, Willis & Kamatani, 1983; Christa, Kersual, Perignon & Cartier, 1986a; Christa, Kersual, Auge & Perignon, 1986b). Christa et al. (1986b) suggested that methionine-dependence could not be considered as the inability of the cell to grow on endogenously synthesized methionine, but that it depended on the precursor used and the possible toxic effect of the precursor in the absence of methionine. Because methionine-dependence could be observed with both Hcy and MeSAdo, it was necessary to introduce new terminology that would describe the precursor involved in the phenomenon of methionine auxotrophy. Liteplo (1990) suggested the term "Hcy-responsive" be used to describe cells that were able to proliferate in methionine-free medium containing Hcy, vitamin B_{12} and folic acid, and "Hcy-nonresponsive" to describe those cells which could not grow under these conditions. Similarly, "MeSAdo-responsive" cells were those

which could proliferate in methionine-free medium containing MeSAdo, while "MeSAdo-nonresponsive" cells could not proliferate under these conditions.

The production of MeSAdo by the pathway involving AdoMet and decarboxylated AdoMet may have a functional significance apart from its involvement in methionine synthesis (Williams-Ashman, et al., 1982). Several enzyme regulatory functions have been suggested for MeSAdo. Concentrations of MeSAdo in the micromolar range have been shown to inhibit spermine synthesis (via the inhibition of spermine synthase); higher concentrations can interfere with transmethylation reactions either directly or via the inhibition of AdoHcy hydrolase (resulting in an accumulation of AdoHcy which inhibits transmethylation reactions); MeSAdo can regulate the activities of ornithine decarboxylase and AdoMet decarboxylase (the principal enzymes of polyamine synthesis) and can inhibit c-AMP phosphodiesterase (Carson, Kajander, Carrera, Yamanaka, Iizasa, Kubota, Willis & Montgomery, 1986; Porter & Sufrin, 1986). Ferro (1979) suggested that the intracellular metabolism of MeSAdo may play a role in the control of gene expression by decreasing the rate of transcription, perhaps by altering DNA methylation. As well, mammalian cells have been shown to generate adenine at a considerable rate only via the polyamine-MeSAdo phosphorylase pathway (Carson et al.,

1986), with most *de novo* adenine synthesis occuring via cleavage of MeSAdo (Schlenk, 1983; Kamatani & Carson, 1981).

The rapid degradation of MeSAdo within the cell would provide an advantage to it since the methylthio-group could be salvaged for methionine synthesis instead of being trapped in a dead-end accumulation product (Schlenk, 1983). Despite this, Kamatani and Carson (1980) and Carson, Nobori, Kajander, Carrera, Kubota and Yamanaka (1988) found that some tumor cell lines lacked MeSAdo phosphorylase activity, while normal human tissues have been found to contain the enzyme (Zappia, Oliva, Cacciapuoti, Galletti, Mignicci & Carteni-Farina, 1978; Cacciapuoti, Oliva & Zappia, 1978; Kar & Pearson, 1980; Sahota, Webster, Potter, Simmonds, Rodgers & Gibson, 1983). The first observation that certain cultured malignant cells had no detectable MeSAdo phosphorylase was made by Toohey (1977, 1978) in murine malignant hematopoietic cells. Using mouse-human somatic cell hybrids, the gene for the human enzyme was localized to chromosome 9 in the region 9pter --> 9q12 (Carrera, Willis, Chilcote, Kubota & Carson, 1986). Cells lacking MeSAdo phosphorylase secrete large amounts of the nucleoside and although most of the MeSAdo is removed from the cell, low concentrations are still maintained within the cell (Kamatani & Carson, 1980). Low concentrations of MeSAdo are capable of stimulating putrescine production which may be

beneficial to the cell. Elevated putrescine levels can have a growth promoting effect on mammalian cells and therefore the lack of MeSAdo phosphorylase activity could provide a selective advantage to malignant cells by increasing intracellular putrescine concentrations and encouraging cellular proliferation (Kamatani & Carson, 1980).

MeSAdo phosphorylases have been purified from both normal human tissues such as the prostate (Zappia et al., 1978), placenta (Cacciapuoti et al., 1978) muscle (Kar & Pearson, 1980) and erythrocytes (Sahota et al., 1983) and various rat tissues (Pegg & Williams-Ashman, 1969; Garbers, 1978; Ferro, Wrobel & Nicolette, 1979). It has also been purified from neoplastic mammalian tissues such as ascites cells (Savarese, Crabtree & Parks, 1981) and breast (Smaaland, Schanche, Kvinnsland, Hostmark & Ueland, 1987). The enzyme has an absolute requirement for inorganic orthophosphate, an optimal pH of approximately 7.5 and no requirement for metal ions or other cofactors (Pegg & Williams-Ashman, 1969; Williams-Ashman et al., 1982). The reaction mechanism of MeSAdo phosphorylase is an equilibrium ordered process with the reaction product adenine serving as a competitive inhibitor of the enzyme (Garbers, 1978). MeSAdo phosphorylase has been found to be almost exclusively cytosolic and no isoenzyme variants have been identified (Williams-Ashman et al., 1982).

The present study involved the cell lines MeWo and MeWo-LC1. The pigmented human melanoma cell line MeWo originated from a lymph node metastasis of a patient with malignant melanoma (Kerbel, Man & Dexter, 1984). MeWo is poorly metastatic in athymic nude mice (Kerbel et al., 1984). MeWo-LC1, a highly metastatic cell line, was derived from MeWo (Kerbel et al., 1984). Previous studies had established that the poorly metastatic MeWo cell line was both Hcy- and MeSAdo-responsive (Liteplo, 1989). In contrast, the highly metastatic MeWo-LC1 cell line was found to be Hcy- and MeSAdo-nonresponsive (Liteplo, 1989). Initially the focus of the present study was to determine the biochemical basis for this difference in methionine auxotrophy between these two closely related cell lines. Perhaps the difference in the metastatic potential of MeWo and MeWo-LC1 cells was related to differences in their methionine metabolism. These biochemical studies focused only on the MeSAdo pathway of methionine re-synthesis or salvage.

The approach taken to determine the basis of the difference in methionine auxotrophy between MeWo and MeWo-LC1 cells involved the characterization of these two cell lines. The existence of MeSAdo phosphorylase deficient tumor cell lines suggested that the difference between these two cell lines may have simply been due to the absence of

the enzyme in MeWo-LC1. The *in vitro* enzyme assay showed that both MeWo and MeWo-LC1 cells contained MeSAdo phosphorylase activity. However, while MeWo cells were capable of growth in methionine-free medium containing dialyzed equine serum (dES) and low concentrations of MeSAdo, MeWo-LC1 cells could not grow under these conditions. Interestingly, when MeWo-LC1 cells were grown in the absence of dES but with transferrin, they were MeSAdo-responsive, that is, they grew as well in methionine-free medium containing MeSAdo as they did in the presence of methionine. It appeared that the addition of dES to the medium was affecting the cells' ability to respond to MeSAdo in the absence of methionine. This observation led to reformulation of the question posed at the beginning of the investigation. The focus of the project became determining the mechanism by which serum affected the MeSAdo-responsiveness of MeWo-LC1 cells.

It was found that MeSAdo could have both positive and negative effects on cell growth. Under conditions where serum is present, the negative effects of the nucleoside appear to predominate. Christa, Kersual, Auge and Perignon (1988) showed that in methionine-dependent cells, methionine could be synthesized from MeSAdo, but that the cells could not proliferate in methionine-free medium supplemented with the nucleoside. The results presented here suggest a

possible explanation for the problem of the inability of cells to grow in MeSAdo. A factor(s) present in serum potentiates the negative effect of MeSAdo on cell proliferation, both in the presence and absence of methionine. The observed cytostatic effect of MeSAdo appears to be due to the nucleoside itself and is enhanced in the presence of serum. Therefore, although cells cultured in MeSAdo do not grow, this is not likely a result of inadequate methionine synthesis, but due to a direct effect of the nucleoside itself in the presence of serum. It appears that, in serum, MeSAdo can act as both a methylthio-source and as an inhibitor of cell growth.

MATERIALS AND METHODS

A. Chemicals and Biochemicals:

[methyl-^{3}H]Thymidine (25 Ci/mmol) was purchased from Amersham Canada Limited (Oakville, ONT). 5'-Deoxy-5'-[8-^{14}C]methylthioadenosine (56 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA). HPLC was carried out using a Whatman Partisil ODS-3 reverse phase column obtained from Chromatographic Specialties (Brockville, ONT).

Methionine and RPMI-1640 medium were from GIBCO/BRL (Burlington, ONT). MeSAdo, ketomethylthiobutyrate, adenine, azaserine, spermine, spermidine, transferrin, 2'-deoxyadenosine, periodate oxidized adenosine, DL-dithiothreitol and equine serum albumin were purchased from the Sigma Chemical Company (St. Louis, MO). 2'-Deoxycoformycin was provided by the National Products Branch, Division of Cancer Treatment, National Cancer Institute.

Fetal bovine (FBS) and equine (ES) serum were obtained from Flow Laboratories (Mississauga, ONT).

B. Methods:

CELL LINES AND CULTURE CONDITIONS

The highly metastatic MeWo-LC1 cell line was derived from the pigmented human melanoma cell line MeWo (Kerbel,et al., 1984). MeWo originated from a lymph node metastasis of a patient with malignant melanoma (Kerbel et al., 1984). Cells were routinely cultured in RPMI-1640 medium containing 7% (v/v) FBS in a 5% CO_2/95% air atmosphere at 37°C. Both FBS and ES were heat-inactivated at 56°C for 30 min, and for metabolic studies, routinely dialyzed to remove endogenous methionine (Liteplo, 1989). The cells were screened and found to be free of *Mycoplasma* contamination (Schneider, Stanbridge & Epstein, 1974). Metabolic studies were carried out in medium containing either 5% (v/v) dES (dES-medium) or 10 µg/ml transferrin (SFT-medium). Methionine-free medium (Met$^-$-medium) containing dES was supplemented with either 100 µM methionine (Met$^+$(dES)-medium) or 25 µM MeSAdo (MeSAdo$^+$(dES)-medium). Similarly, Met$^+$(SFT)-medium and MeSAdo$^+$(SFT)-medium were supplemented with either 100 µM methionine or 25 µM MeSAdo respectively. Cell viability was determined by trypan blue exclusion and only cells with a viability greater than 95% were used for experiments.

QUANTITATION OF DNA SYNTHESIS

The ability of MeWo or MeWo-LCl cells to synthesize DNA in the various methionine and MeSAdo-containing media was determined by measuring the incorporation of [methyl-^{3}H] thymidine into cellular DNA (Liteplo, 1989; Sauvaigo, Fretts, Riopelle & Lagarde, 1986; Kerbel & Man, 1984). Tumor cells (10^4) were plated into each well of a 24-well tissue culture plate in regular culture medium. The following day, the cells were washed twice with isotonic saline and cultured in the appropriate Met$^-$- or MeSAdo$^-$-medium. Four days later, the cells were incubated for 24 hours with [methyl-^{3}H]thymidine (1 µCi/well). The cells were then washed twice with isotonic saline and solubilized with either 0.25 N NaOH or 1% (w/v) SDS (1 ml). The DNA was precipitated by adding 250 µl of 50% (w/v) trichloroacetic acid (TCA) to 800 µl of the cell lysate (containing 50 µg carrier DNA). The lysates were then incubated for 1 hour at 0°C. The lysates were spun at 12,000 x g for 5 min and the pellet washed once with 5% TCA. The pellets were resolubilized by the addition of 500 µl of 0.25 N NaOH and incubated at 37°C for 30 min. The radioactivity incorporated into 400 µl of the resolubilized DNA was determined by liquid scintillation counting in a LKB Wallac 1219 Rackbeta Liquid Scintillation Counter (5 min). The results were usually expressed as a percentage of the

[methyl-^{3}H]thymidine incorporated into DNA relative to the incorporation in cells grown in the corresponding Met$^+$-medium. Blanks (where [methyl-^{3}H]thymidine was added just prior to harvest) were subtracted from all values. [methyl-^{3}H]Thymidine incorporation in the appropriate Met$^-$-medium was usually less than 3% of that in the corresponding Met$^+$-medium.

QUANTITATION OF [8-^{14}C]MeSAdo INCORPORATION INTO CELLULAR MACROMOLECULES

MeWo-LC1 cells (10^4) were plated into each well of a 24-well tissue culture plate in regular culture medium. The following day the cells were washed twice with serum-free Met$^+$-medium and cultured in Met$^+$-medium containing either dES or SFT. After 3 days, the cells were provided with fresh medium and incubated with 0.5 µCi/ml [8-^{14}C]MeSAdo for up to 24 hours. The incorporated radioactivity was determined as described above.

QUANTITATION OF CELLULAR [8-^{14}C]MeSAdo UPTAKE

MeWo-LC1 cells (4×10^4) were plated into each well of a 24-well tissue culture plate in regular culture medium. The cells were then cultured in either Met$^+$(dES)-medium or Met$^+$(SFT)-medium for 3 days. [8-^{14}C]MeSAdo was added (0.5 µCi/ml, 9 µM) and the cells incubated for up to 3 hours at

37°C in a 5% CO_2/95% air atmosphere. The cells were washed twice with isotonic saline and lysed by the addition of 1 ml of 1% (w/v) SDS. Total cell-associated radioactivity was determined by liquid scintillation counting of 800 µl aliquots of cell lysate. Blanks (where the [8-^{14}C]MeSAdo was added just prior to the analysis) were subtracted from all experimental values. The results were expressed as cpm/10^5 cells. The number of cells per well was determined by trypsinizing the cells and counting.

DETERMINATION OF MeSAdo PHOSPHORYLASE ACTIVITY

MeSAdo phosphorylase activity was determined essentially as described by Sunkara, Chang and Lachman (1985). MeWo and MeWo-LC1 cells were cultured for 4 days in either Met$^-$(dES)- or Met$^-$(SFT)-medium. Cells were then trypsinized and pelleted. The cell pellets were resuspended in extraction buffer (2.5 mM Tris, pH 7.5; 0.1 mM EDTA and 2.5 mM DTT) at a density of 5x10^6 cells/ml. The cells were freeze-thawed 3 times in liquid nitrogen, and the lysate centrifuged at 12,000 x g for 20 min at 4°C, the supernatant removed and assayed for protein content and enzyme activity. MeSAdo phosphorylase activity was determined by monitoring the conversion of [8-^{14}C]MeSAdo to [8-^{14}C]adenine. The standard reaction mixture contained 100 mM phosphate buffer, pH 7.2, 1.5 mM DTT, 0.1 mM MeSAdo (3.63 µCi/mmol) and 40 µg

of protein in a total volume of 0.45 ml. The reaction was started by the addition of [8-^{14}C]MeSAdo and incubated at 37°C for 30 min. The reaction was stopped by the addition of 50 µl of 4 N perchloric acid, kept on ice for 30 min and samples neutralized by the addition of 500 µl of 0.8 N potassium hydroxide. The resulting salt was allowed to precipitate, 5 µl of standard adenine (2 mg/ml) was added to each sample which was then centrifuged for 5 min at 12,000 x g. The supernatant was filtered and 750 µl was injected into a Whatman Partisil ODS-3 column. Samples where the reaction was stopped immediately following the addition of substrate (time zero controls) were treated in the same manner. The [8-^{14}C]adenine and [8-^{14}C]MeSAdo were separated by reverse-phase high performance liquid chromatography using gradient elution at a flow rate of 1.0 ml/min. The chromatographic system consisted of an LKB Bromma 2150 solvent delivery system, an LKB Bromma 2151 variable wavelength monitor and an LKB Bromma 2220 recording integrator. Buffer A contained 10 mM KH_2PO_4 in 5% MeOH pH 6.0 and buffer B contained 10 mM KH_2PO_4 in 80% MeOH pH 6.0. The column was equilibrated with buffer A (1.0 ml/min,30 min). The gradient was programmed as follows: 0-30 min, a linear gradient of 0-50% buffer B. The column was washed for 10 min with 100% buffer B and re-equilibrated with buffer A for 15 min. Elution of [8-^{14}C]adenine and

[8-^{14}C]MeSAdo were compared to that of known standards and elution times were 6 and 20 min respectively. The 1 ml column fractions were collected directly into scintillation vials and the radioactivity determined by liquid scintillation counting. The results were expressed as nmol adenine produced/mg protein/30 min.

PROTEIN ASSAY

The protein content of samples was determined by the modified method of Lowry (Markwell, Haas, Bieber and Tolbert, 1978), using bovine serum albumin as the standard.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Student *t* test with differences being assessed at $p < 0.01$.

RESULTS

GROWTH PATTERNS OF MeWo AND MeWo-LC1 CELLS

Previous studies of the highly metastatic human tumor cell line MeWo-LC1 revealed that it exhibited an absolute requirement for methionine for growth, that is, the cells were unable to proliferate in methionine-free medium supplemented with either homocysteine (Hcy) or MeSAdo (Liteplo, 1989). In contrast, the less metastatic parental cell line MeWo, from which MeWo-LC1 was derived, was able to proliferate in methionine-free medium supplemented with either Hcy or MeSAdo (Liteplo, 1989). The initial focus of the project was to determine the biochemical basis for this difference in methionine auxotrophy between these two cell lines, focusing on the response of the cells to MeSAdo.

The MeWo-LC1 cell line represents a heterogeneous population of cells. It was therefore possible that their reduced ability to proliferate in methionine-free medium supplemented with MeSAdo (MeSAdo$^+$(dES)-medium) represented an average of cells that proliferated well in MeSAdo and those that did not. Therefore, clonal cell lines of MeWo-LC1 (chosen randomly) were tested for their ability to synthesize DNA in MeSAdo$^+$(dES)-medium. The results are shown in TABLE 1. All clones showed a [^{3}H]thymidine incorporation into their DNA that was similar to that for MeWo-LC1. The similarity in methionine auxotrophy between

TABLE 1

Cell Line	% Relative [^{3}H]Thymidine Incorporation	
	(Hcy$^-$)[a]	(MeSAdo$^-$)[b]
MeWo-LC1	1.1 ± 0.4	25.9 ± 11.0
MeWo-LC1-1	1.3 ± 0.4	28.2 ± 6.2
MeWo-LC1-3	1.1 ± 0.1	21.8 ± 8.4
MeWo-LC1-5	1.1 ± 0.5	28.9 ± 11.2
MeWo-LC1-9	1.0 ± 0.1	18.9 ± 1.5
MeWo-LC1-10	0.7 ± 0.1	22.6 ± 5.8
MeWo-LC1-12	0.7 ± 0.1	17.8 ± 6.8

a-Relative DNA synthesis in methionine-free medium containing Hcy.

b-Relative DNA synthesis in methionine-free medium containing MeSAdo.

METHIONINE AUXOTROPHY OF MeWo-LC1 CLONES- The ability of clones of MeWo-LC1 to incorporate [^{3}H]thymidine into their DNA in methionine-free medium containing either 25 µM MeSAdo and 5% (v/v) dialyzed equine serum (dES) or 200 µM Hcy, 1.5 µM vitamin B_{12}, 100 µM folic acid and 7% dialyzed fetal bovine serum (dFBS) relative to the incorporation in the appropriate methionine-containing medium was determined as described in the Methods. The results are an average ± S.D. of 1-4 determinations carried out in triplicate.

the clones and the parental line indicated that further studies could be carried out using MeWo-LC1 itself. The MeWo cell line has not been cloned and therefore was not tested in the same manner. MeWo has been found to show 50% DNA synthesis in MeSAdo^{+}(dES)-medium relative to that in methionine medium and 43% DNA synthesis in medium containing Hcy in place of methionine (Liteplo, 1989).

Little is unequivocally known about the pathway that involves the conversion of MeSAdo to methionine. What is known, is that as a final step KMTB is transaminated to produce methionine (Christa, Thullier, Munier & Perignon, 1984). In order to determine if the defect in the pathway between MeSAdo and methionine was between MeSAdo and KMTB or between KMTB and methionine, MeWo and MeWo-LC1 cells were grown in varying amounts of MeSAdo or KMTB in methionine-free medium. If the cells could synthesize DNA in KMTB and not MeSAdo, then the defect might lay somewhere between the conversion of MeSAdo to KMTB. As shown in FIGURE 4, both cell lines were able to synthesize DNA in KMTB as well as they did in methionine-containing medium. The amount of [^{3}H]thymidine incorporated in methionine-containing medium was similar for both cell lines (MeWo 1.4 x 10^{5} cpm/well; MeWo-LC1 1.25 x 10^{5} cpm/well). However, in the absence of methionine but in the presence of MeSAdo, [^{3}H]thymidine incorporation was much lower in MeWo-LC1 cells (MeWo 9.5 x 10^{4} cpm/well; MeWo-LC1 2.5 x 10^{4} cpm/well). In

Figure 4

Growth of MeWo and MeWo-LC1 in MeSAdo and KMTB. MeWo (●, ■) and MeWo-LC1 cells (▲, ◆) were cultured in methionine-free medium containing 5% (v/v) dES and supplemented with either MeSAdo (●, ▲) or KMTB (■, ◆). The amount of [^{3}H]thymidine incorporated into DNA was determined as described in the Methods and was expressed as the relative percent of [^{3}H]thymidine incorporated into DNA in supplemented methionine-free medium relative to the incorporation in the corresponding methionine-containing medium. The results are an average ± S.D. of 3 determinations each performed in triplicate.

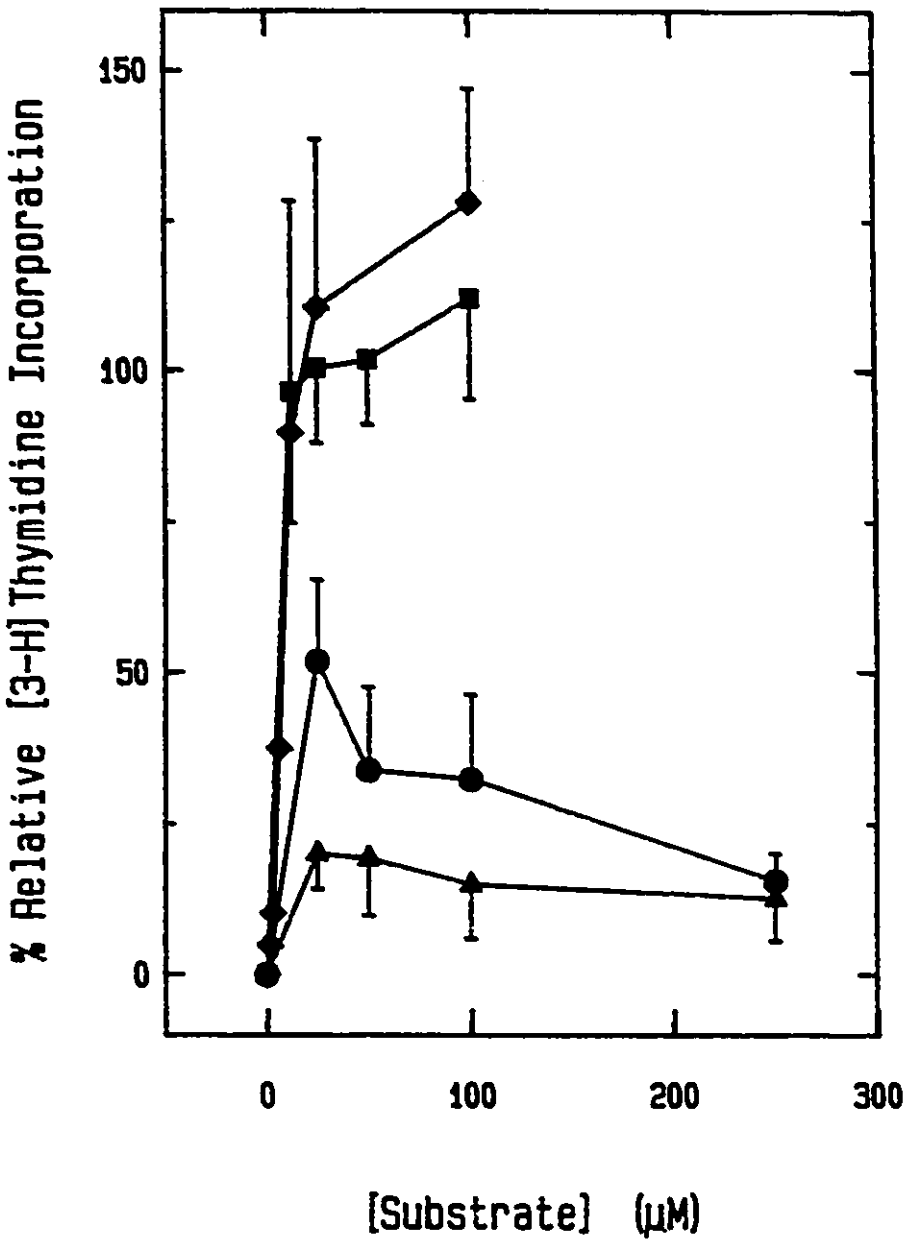

% Relative [3-H] Thymidine Incorporation
150
100
50
0
0
100
200
300
[Substrate] (μM)

FIGURE 4

contrast to previous experiments (Liteplo, 1989), MeWo showed only a 50% DNA synthesis in MeSAdo$^+$(dES)-medium relative to that in methionine medium. Nevertheless, this was 2-fold higher than the amount of DNA synthesized by MeWo-LC1 in the same medium. The lowered DNA synthesis observed with MeWo cells may simply have been due to differences in the lots of serum used. The differences may also be attributed to the fact that these experiments were carried out in the presence of 5% (v/v) dES while Dr. Liteplo's experiments were carried out in 10% (v/v) dES.

These experiments as well as those done by others have all been carried out in medium containing dialyzed equine serum (dES-medium) since it is known to contain little or no endogenous MeSAdo phosphorylase activity (Riscoe & Ferro, 1984). When cells were grown in methionine-free medium supplemented with MeSAdo in the presence of dFBS, they showed greater DNA synthesis than in dES-medium (TABLE 2). It was possible that this increase in DNA synthesis was a result of an increased availability of metabolites as a consequence of degradation of MeSAdo within the medium by MeSAdo phosphorylase known to be present in dFBS (Riscoe & Ferro, 1984). However, the degradation of MeSAdo within the medium would also render it unavailable to the cell, suggesting that DNA synthesis should go down. It seemed that in both dES and dFBS DNA synthesis was decreased in MeSAdo$^+$-medium relative to methionine-containing medium.

TABLE 2

Culture Conditions	% Relative [^{3}H]Thymidine Incorporation	
	Hcy$^+$	MeSAdo$^+$
7% dFBS	1.1 ± 0.4	56.3 ± 9.3
5% dFBS	N.D.	42.8 ± 22.5
5% dES	N.D.	25.9 ± 11.0
SFT	1.1 ± 1.2	106.0 ± 18.0
SFT+5% dFBS	N.D.	45.8 ± 21.4
SFT+5% dES	N.D.	36.8 ± 9.2

EFFECT OF THE MEDIUM ON METHIONINE AUXOTROPHY OF THE MeWo-LC1 CELL LINE- Cells were cultured in methionine-free medium supplemented with either Hcy (200 μM), vitamin B_{12} (1.5 μM) and folic acid (100 μM; Hcy$^+$) or MeSAdo (25μM; MeSAdo$^+$) and containing either dialyzed fetal bovine serum (dFBS), dialyzed equine serum (dES) or 10 μg/ml transferrin (SFT). N.D. indicates that DNA synthesis was not determined under those conditions. The results are an average ± S.D. of 2-3 determinations (each performed in triplicate) and are expressed as the relative percent of [^{3}H]thymidine incorporated into DNA in methionine-free supplemented medium relative to the incorporation in the corresponding methionine-containing medium.

The results obtained in dES-medium supported the notion that the failure of MeWo-LC1 cells to grow in MeSAdo$^+$(dES)-medium was due to a defect in the conversion of MeSAdo to KMTB perhaps due to a deficiency in MeSAdo phosphorylase activity or a failure to synthesize DNA because the MeSAdo was not getting into the cell. Perhaps the exogenous MeSAdo added to the cells was being bound by the serum components, rendering it unavailable to the cell. If this was the case, then one would expect that MeWo cells would also be unable to synthesize DNA in MeSAdo$^+$(dES)-medium, however these cells have been shown to be capable of DNA synthesis in this medium. It was possible that the extent to which serum bound up the MeSAdo was not enough to affect MeWo cells, but was enough to affect MeWo-LC1 cells. Thus, these studies focused on MeWo-LC1 cells and a further assessment of MeWo cells was not carried out.

When MeWo-LC1 cells were grown in methionine-free, dES-medium (Met$^-$(dES)-medium) with varying amounts of MeSAdo, up to 1.0 mM, there was no increased synthesis of DNA at higher MeSAdo concentrations (FIGURE 5). This result indicated that MeSAdo was not likely being bound up by serum components and supported the notion that the inability of MeWo-LC1 cells to synthesize DNA in MeSAdo$^+$(dES)-medium was due to some biochemical defect in the conversion of MeSAdo to KMTB that was present in MeWo-LC1, but absent in MeWo.

Another possible explanation for the inability of MeWo-

Figure 5

Effect of the Medium on the Synthesis of DNA by MeWo-LC1 in MeSAdo. MeWo-LC1 cells were cultured in methionine-free medium containing 5% (v/v) dES (●) or 10 µg/ml transferrin (■) and supplemented with varying concentrations of MeSAdo (up to 1.0 mM). The incorporation of [^{3}H]thymidine into the DNA of cells in methionine-free supplemented medium relative to the incorporation in methionine-containing medium was determined as described in the Methods. The results are an average ± S.D. of 3 determinations each performed in triplicate.

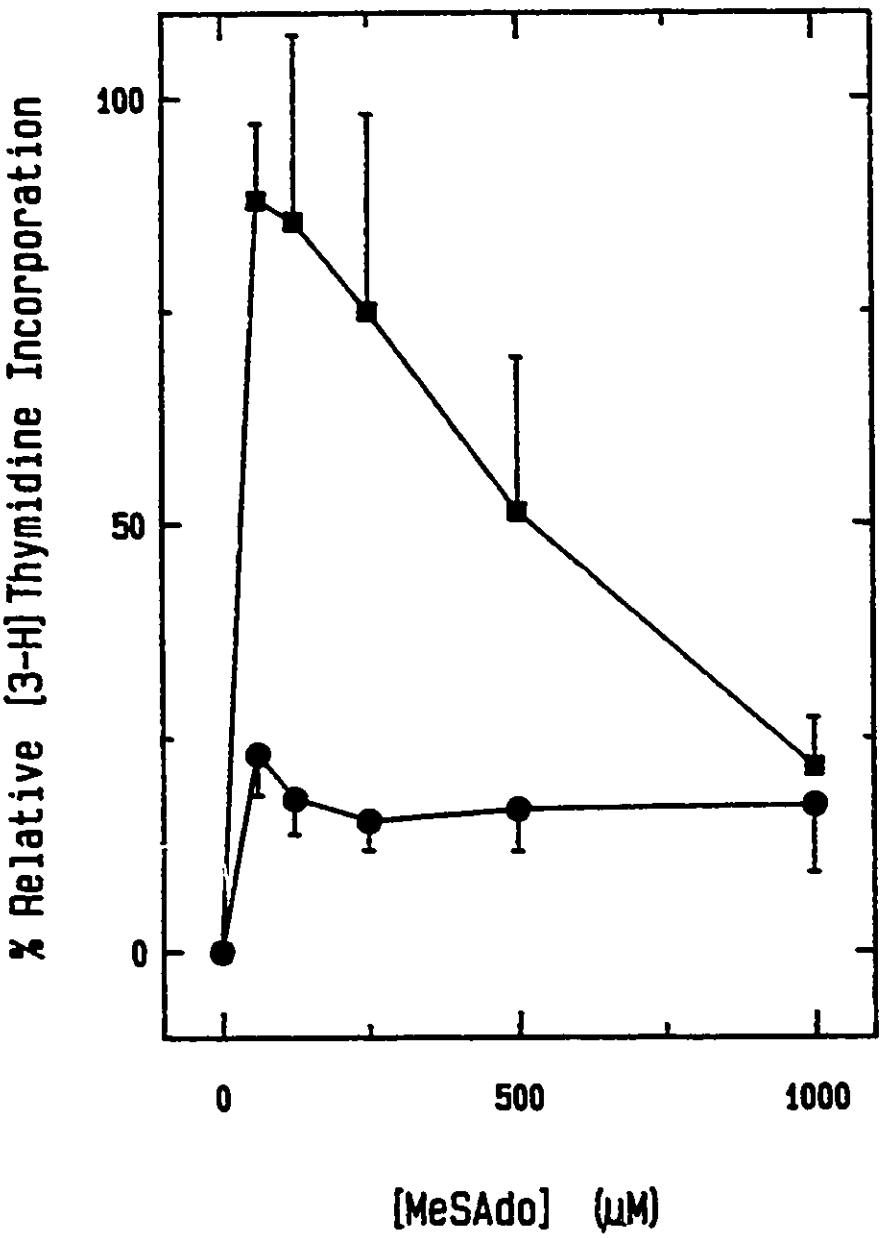

% Relative [3-H] Thymidine Incorporation
100
50
0
0
500
1000
[MeSAdo] (μM)

FIGURE 5

LC1 cells to synthesize DNA in MeSAdo$^+$(dES)-medium was that the dES was somehow degrading the MeSAdo thereby converting it to something that could not be used by MeWo-LC1 cells or was toxic to them. Sauvaigo et al. (1986) demonstrated that MeWo-LC1 exhibits a high degree of growth factor autonomy and is able to proliferate in serum-free medium supplemented with transferrin. To eliminate complications due to the presence of dES, cells were grown without methionine in serum-free medium supplemented with transferrin (SFT-medium). Surprisingly, MeWo-LC1 cells synthesized DNA as well as or better in medium supplemented with MeSAdo (MeSAdo$^+$(SFT)-medium) as they did with methionine supplemented medium (Met$^+$(SFT)-medium) (TABLE 2), indicating that the absence of dES resulted in the ability to synthesize DNA in MeSAdo$^+$-medium. Similarly, in experiments where dFBS was added to SFT-medium, DNA synthesis was decreased although not to the same extent as in dES-medium (TABLE 2). It appeared that serum (dES or dFBS) was affecting the MeSAdo-dependent synthesis of DNA in MeWo-LC1 cells.

Previous studies on the ability of Hcy to replace methionine had also been carried out in the presence of serum (Liteplo, 1989). MeWo-LC1 was shown to be unable to use Hcy as a methylthio-source (Liteplo, 1989). In light of the above results, these experiments were repeated in SFT-medium. TABLE 2 indicates that MeWo-LC1 cannot

synthesize DNA in methionine-free medium supplemented with Hcy in either dES- of SFT-medium. Thus, it appeared that the differential effect between dES- and SFT-medium was restricted to the MeSAdo arm of methionine recycling within the cell and that, in the absence of dES, MeWo-LC1 could use MeSAdo in place of methionine, but that in its presence MeSAdo could not replace methionine or had an inhibitory effect on DNA synthesis.

As a result of these findings, the focus of the project became determining the mechanism by which dES affected the ability of MeWo-LC1 to synthesize DNA in MeSAdo$^+$-medium.

MeSAdo PHOSPHORYLASE STUDIES

The simplest explanation for the difference in methionine auxotrophy between the MeWo and MeWo-LC1 cell lines was that the level of the enzyme responsible for the metabolism of MeSAdo, namely MeSAdo phosphorylase, was high in MeWo, while it was either absent or present in lower concentrations in MeWo-LC1. Therefore, *in vitro* MeSAdo phosphorylase activity was measured in both MeWo and MeWo-LC1 cells. The basis of the assay was monitoring the conversion of [^{14}C]MeSAdo to [^{14}C]adenine. Standard [^{3}H]adenine and [^{14}C]MeSAdo were shown to be clearly separable by HPLC under the conditions described in the Methods (FIGURE 6). Therefore, it was possible to quantitate the level of MeSAdo phosphorylase activity within

Figure 6

Separation of Adenine and MeSAdo by HPLC. Elution profiles of [^{3}H]adenine (——) and [^{14}C]MeSAdo (- - -) were obtained using a reverse phase column. Samples were eluted using gradient elution with a 10 mM KH_2PO_4 buffer containing increasing amounts of methanol. Samples were collected and radioactivity determined by liquid scintillation counting.

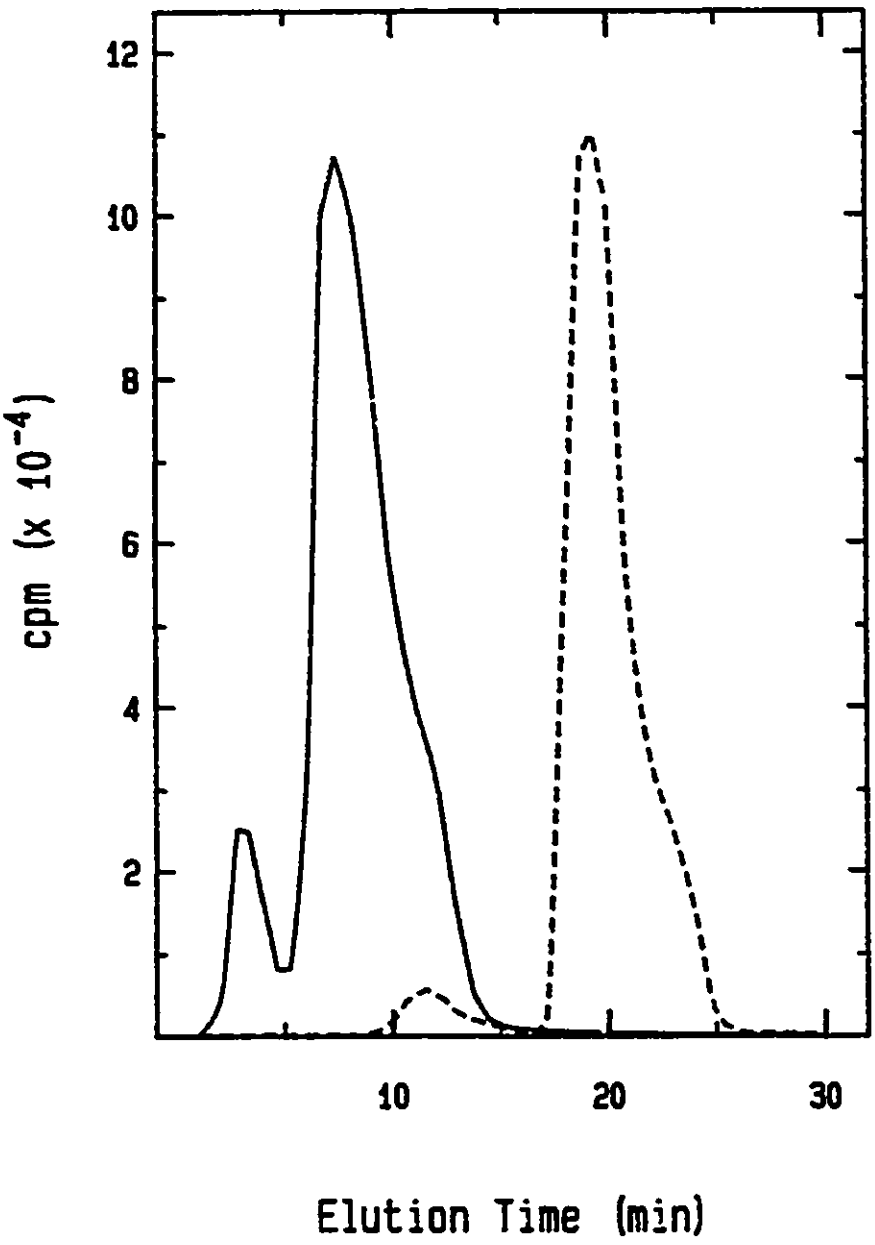


FIGURE 6

a cell by this method. Prior to the analysis of both cell types, the optimal assay conditions within our system had to be established. It was confirmed for both cell lines that the concentration of protein present in the system was in the linear range of activity (FIGURE 7); that the amount of substrate ([^{14}C]MeSAdo) was saturating (FIGURE 8) and that the assay was in the linear range of activity for the duration of the reaction time chosen (FIGURE 9). After establishing the conditions for the assay, MeSAdo phosphorylase activity was determined using MeWo and MeWo-LC1 cells that had been cultured in methionine-containing medium supplemented with dES (i.e. Met^{+}(dES)-medium). As shown in TABLE 3, MeWo contained 1.5 times the specific activity found in MeWo-LC1. When the total activity of the enzyme was considered (nmol adenine produced/30 min/5x10^{6} cells), MeWo contained twice the activity of MeSAdo phosphorylase than MeWo-LC1 in dES-medium. A higher enzyme activity in the MeWo cell line would allow it to metabolize a greater amount of MeSAdo than could MeWo-LC1 in the same amount of time. MeSAdo has been reported to have a cytostatic effect on cells at higher concentrations (Williams-Ashman et al., 1982), and the removal of the nucleoside by its metabolism could prevent this effect from being observed. Perhaps MeWo was able to metabolize enough of the MeSAdo to prevent its effects while MeWo-LC1 could not and therefore showed a lower synthesis of

Figure 7

Effect of Protein Concentration on MeSAdo Phosphorylase Activity. The *in vitro* MeSAdo phosphorylase assay was carried out using varying amounts of protein extracted from cell lysates as described in the Methods. The amount of adenine produced (nmol/30 min) was determined after separation of the product from the substrate by HPLC and is expressed as an average ± S.D. of 1 determination performed in triplicate.

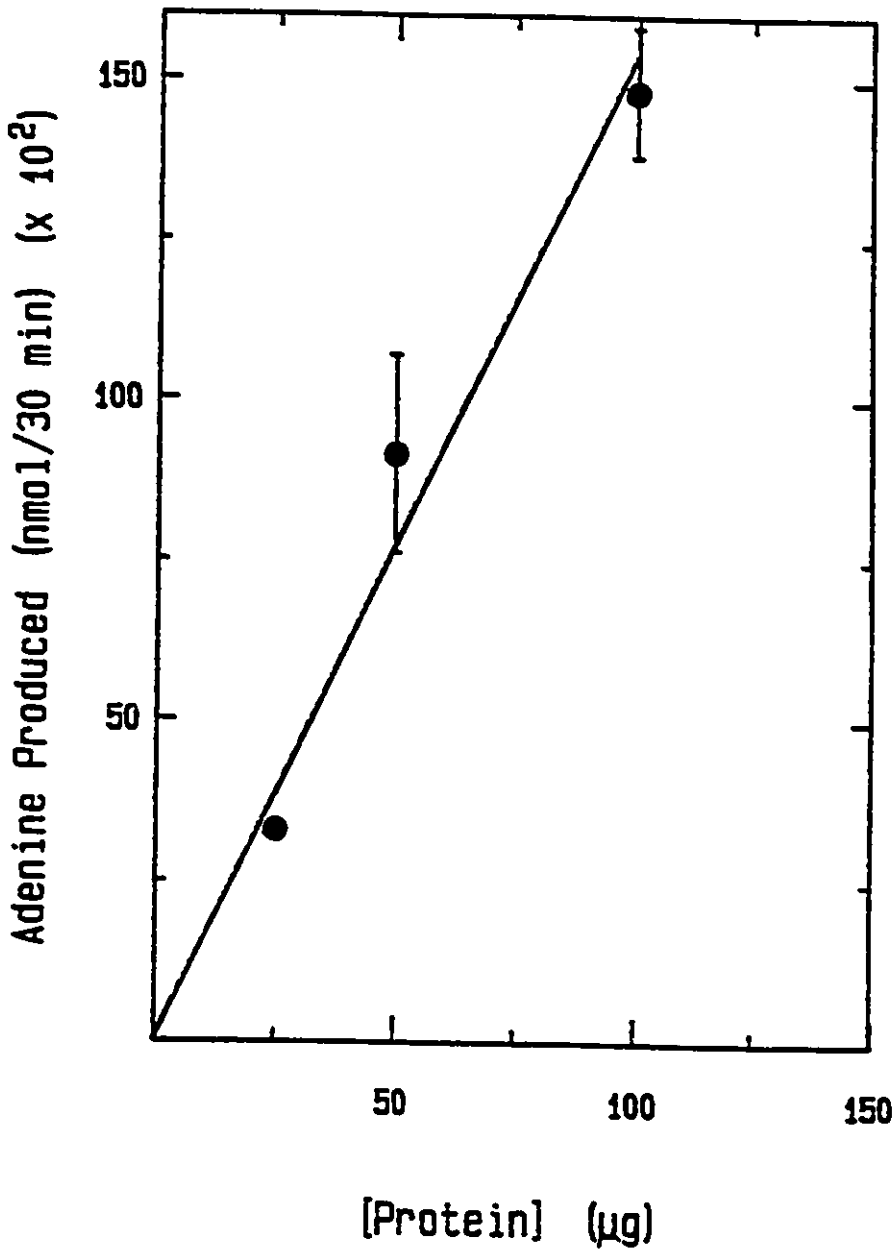

Adenine Produced (nmol/30 min) (x 10²)
150
100
50
50
100
150
[Protein] (µg)

FIGURE 7

Figure 8

Effect of Substrate Concentration Upon MeSAdo Phosphorylase Activity. The *in vitro* assay of MeSAdo phosphorylase was carried out as described in the Methods with varying concentrations of [^{14}C]MeSAdo. The amount of adenine produced (nmol/mg protein/60 min) was determined after separation of the product from the substrate by HPLC. The results are expressed as an average ± S.D. of 1 determination performed in triplicate.

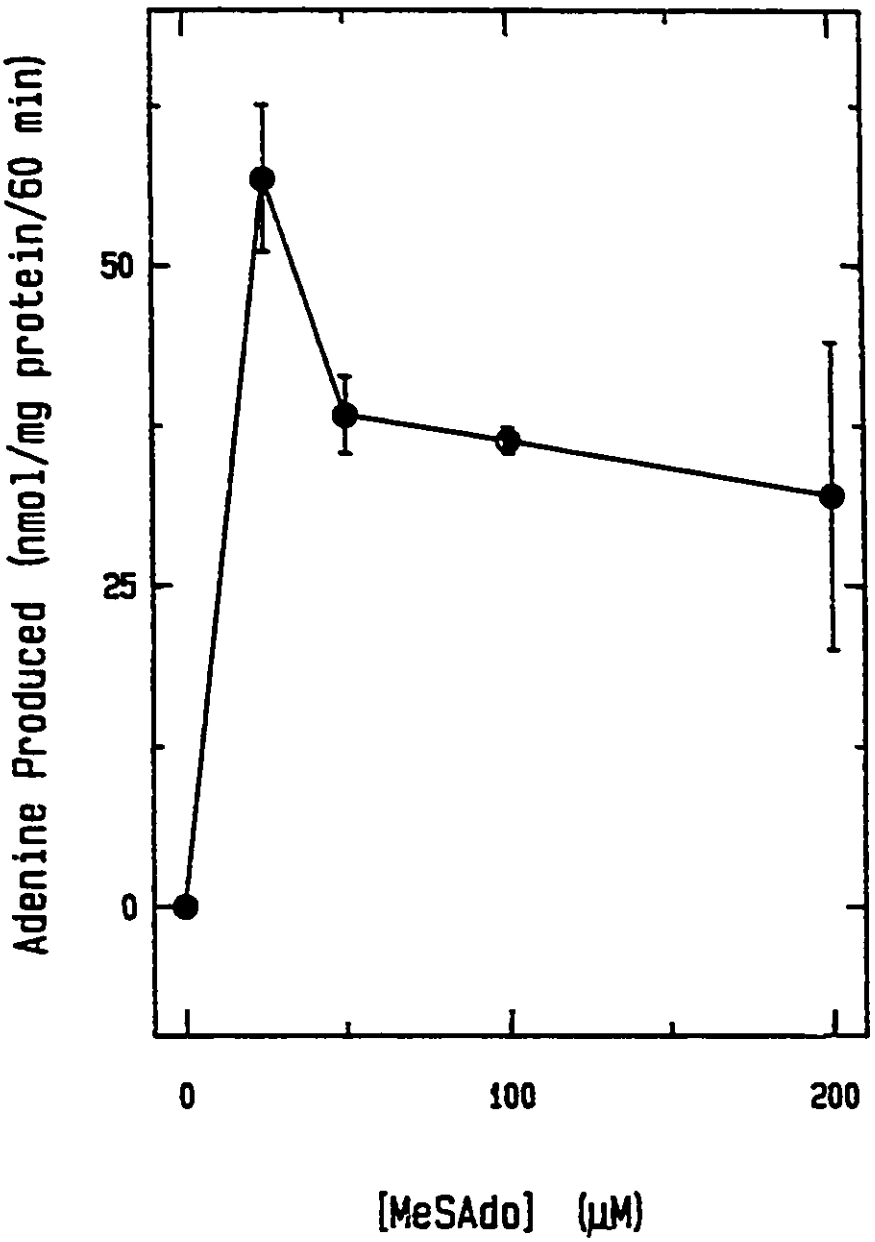

Adenine Produced (nmol/mg protein/60 min)
50
25
0
0
100
200
[MeSAdo] (μM)

FIGURE 8

Figure 9

Determination of Reaction Time for the Assay of MeSAdo Phosphorylase. The *in vitro* assay of MeSAdo phosphorylase was carried out as described in the Methods. The reaction mixtures were incubated for varying periods of time and the amount of adenine produced (nmol/mg protein) was determined after separation of the product from the substrate by HPLC. The results are expressed as an average ± S.D. of 1 determination performed in triplicate.

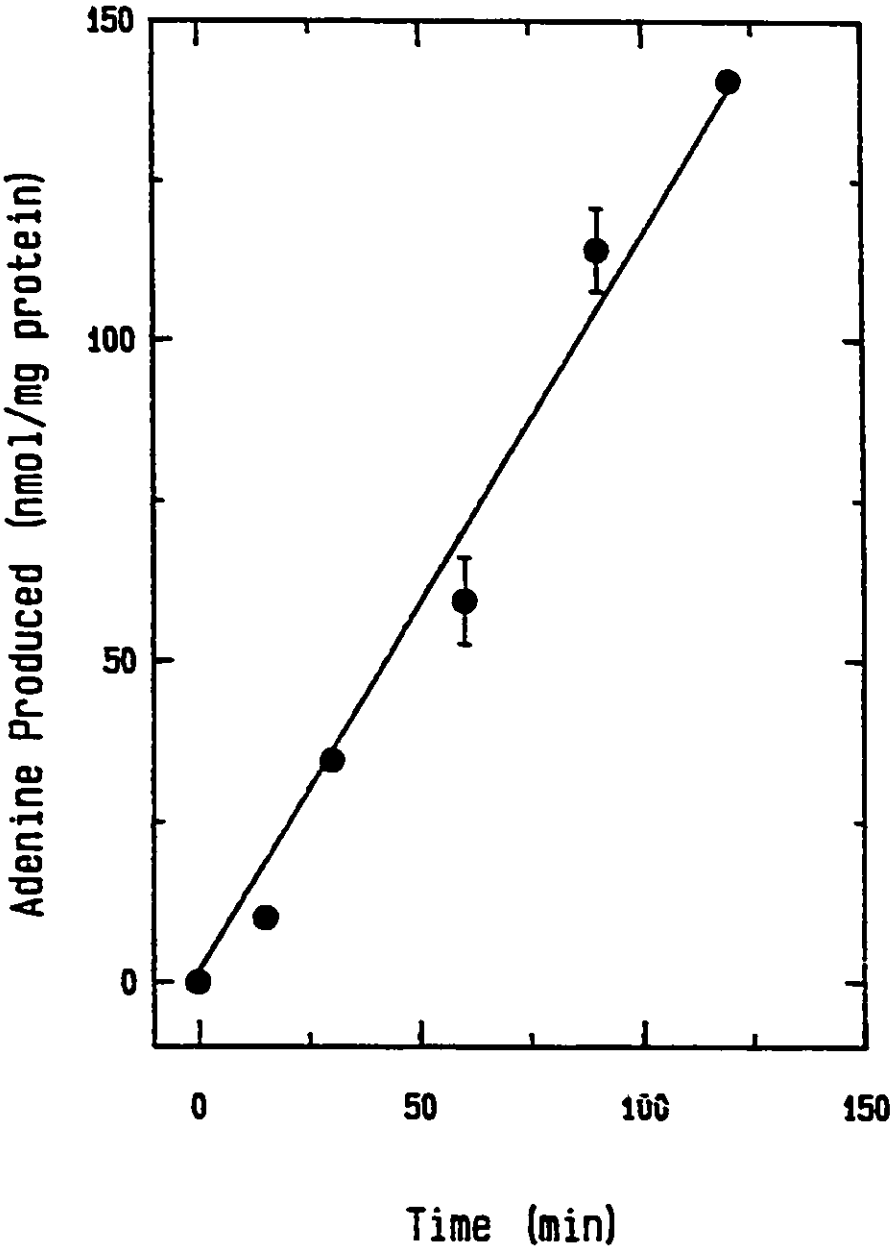

150
100
50
0
Adenine Produced (nmol/mg protein)
0
50
100
150
Time (min)

FIGURE 9

TABLE 3

Cell Line	Enzyme Activity (nmol/mg protein/30 min)	
	dES	SFT
MeWo	43.7 ± 5.2	35.0 ± 10.0
MeWo-LC1	28.0 ± 11.2	21.0 ± 12.2

MeSAdo PHOSPHORYLASE ACTIVITY IN MeWo AND MeWo-LC1 CELLS- Cells were cultured for 4 days in medium containing methionine and either 5% (v/v) dES or 10 µg/ml transferrin (SFT) and were assayed for *in vitro* enzyme activity. Activity is expressed as nmol adenine produced/mg protein/30 min and is an average ± S.D. of 1 determination performed in triplicate.

DNA in MeSAdo$^+$(dES)-medium. However, it was possible that the level of MeSAdo phosphorylase activity within MeWo-LC1 cells was still more than enough to metabolize the MeSAdo present inside the cell and that the failure to synthesize DNA in MeSAdo$^+$(dES)-medium was due to some other biochemical defect.

The observation that MeWo-LC1 cells could synthesize DNA in MeSAdo$^+$(SFT)-medium suggested that the level of MeSAdo phosphorylase within the cell was adequate. It was possible that the presence of dES in the medium was in some way affecting the intracellular level of MeSAdo phosphorylase enzyme. Therefore, the effect of culture conditions on *in vitro* MeSAdo phosphorylase levels was determined (TABLE 3). MeWo and MeWo-LC1 cells were grown under optimal conditions, i.e. in the presence of methionine, in either SFT- or dES-medium. It was necessary to use these conditions since MeSAdo-phosphorylase is cell cycle dependent, showing its highest levels during cell proliferation (Sunkara et al., 1985). Therefore, since MeWo-LC1 cells cannot proliferate in the presence of MeSAdo in dES-medium, a decrease in MeSAdo phosphorylase activity could either be due to dES in the medium or to changes in the cell cycle. MeWo-LC1 cells cultured in Met$^+$(SFT)-medium for 4 days prior to analysis exhibited a slightly lower phosphorylase activity than cells grown in Met$^+$(dES)-medium. However, this difference was not significant at $p<0.01$ and

MeWo-LC1 cells were able to readily synthesize DNA in MeSAdo$^+$(SFT)-medium while DNA synthesis was reduced in MeSAdo$^+$(dES)-medium. Since enzyme activity was not reduced in the presence of serum, it was unlikely that the dES was exerting its effect via inhibition of the phosphorylase enzyme, but that it was acting by some other mechanism.

The *in vitro* assay of MeSAdo phosphorylase activity cannot serve to indicate if the enzyme is indeed functional *in vivo*. The nature of the procedure for isolation of the enzyme could affect the natural conformation of the enzyme thereby affecting its activity. Further, measurements of enzyme activity in crude extracts can only provide indirect information concerning metabolic rates within intact cells. It was possible that the MeSAdo phosphorylase within MeWo-LC1 cells was functional and that the inability of the cells to synthesize DNA in MeSAdo$^+$(dES)-medium was due to some other biochemical defect. Perhaps the exogenously added MeSAdo could not enter the cell or if it could, once inside was inhibiting some cellular function. It was therefore necessary to assay the *in vivo* activity of the enzyme.

Azaserine inhibits the *de novo* synthesis of adenine by blocking the transfer of ammonia from glutamine to 5'-phosphoribosyl-1-pyrophosphate during its conversion to adenine. The result is an accumulation of the pyrophosphate and a lack of adenine and, as a consequence, DNA synthesis

is reduced and a cessation of cell growth follows (Lehninger, 1982). In order to prevent the cytostatic effect of azaserine from being observed, adenine must be supplied exogenously. Since one of the products of MeSAdo phosphorylase activity is adenine, the addition of MeSAdo to cells containing an active MeSAdo phosphorylase enzyme should prevent the cytostatic effect of azaserine from being observed. In contrast, if the MeSAdo phosphorylase activity is too low or totally absent, then adenine but not MeSAdo should prevent the cytostatic effect of azaserine from being observed. When MeWo-LC1 cells were grown in methionine-containing dES-medium with azaserine, the addition of both adenine or MeSAdo prevented the cytostatic action of azaserine from being observed (TABLE 4). This indicated that the MeSAdo phosphorylase present within MeWo-LC1 cells was indeed functional, even under conditions where MeSAdo cannot support DNA synthesis. In SFT-medium (TABLE 4), both adenine and MeSAdo were effective in preventing the cytostatic effect of azaserine from being observed. Similar experiments with MeWo cells showed that both adenine and MeSAdo were effective in preventing the inhibitory effect of azaserine on DNA synthesis from being observed (TABLE 4).

METABOLISM OF MeSAdo IN EQUINE SERUM

Equine serum has been shown to contain no endogenous

TABLE 4

Cell Line	Additions To Culture Medium	% Relative [^{3}H]Thymidine Incorporation	
		dES+AzaS	SFT+AzaS
MeWo	None	17.9 ± 12.6	49.7 ± 10.5
	50 μM Ade	109.0 ± 19.0	159.8 ± 27.9
	25 μM MeSAdo	94.6 ± 31.9	156.2 ± 13.0
MeWo-LC1	None	22.3 ± 1.6	23.1 ± 4.0
	50 μM Ade	95.4 ± 10.0	120.7 ± 31.2
	25 μM MeSAdo	67.4 ± 4.5	106.4 ± 29.2
	50 μM MeSAdo	74.8 ± 10.6	97.2 ± 31.5
	100μM MeSAdo	67.5 ± 2.7	92.3 ± 19.9

ABILITY OF MeSAdo TO RESTORE THE PROLIFERATION OF CELLS GROWTH-ARRESTED IN AZASERINE- Cells were cultured in methionine-containing medium supplemented with either 5% (v/v) dES and 5 μM azaserine (AzaS) or 10 μg/ml transferrin (SFT) and 5 μM azaserine. Cells were given either adenine (Ade) or MeSAdo to restore their growth. The results are the average ± S.D. of 1-3 determinations (performed in triplicate) and are expressed as the percent of [^{3}H]thymidine incorporated into DNA in the test medium relative to the incorporation into DNA in methionine-containing medium in the absence of azaserine.

MeSAdo phosphorylase activity (Riscoe & Ferro, 1984) and was shown above (FIGURE 5) not to sequester MeSAdo thereby rendering it unavailable to the cell. It was possible that the dES was degrading the MeSAdo by some other mechanism. Although mammalian adenosine deaminase is not known to have an appreciative activity towards MeSAdo (Williams-Ashman, et al., 1982), it has been reported to be present in low levels in dES (Daddona, Frohman & Kelley, 1980). Therefore, it was determined if adenosine deaminase activity in dES was responsible for the reduced ability of MeWo-LC1 cells to synthesize DNA in MeSAdo$^+$(dES)-medium relative to the synthesis in Met$^+$(dES)-medium.

2'-Deoxycoformycin (dCF) is a potent inhibitor of adenosine deaminase (Helland & Ueland, 1983; Hershfield, Kredich, Koller, Mitchell, Kurtzberg, Kinney & Falletta, 1983), and was added to the dES-medium to inhibit the enzyme. To confirm that the inhibitor was effective under the culture conditions employed, the effect of 2'-deoxyadenosine (2dA) on the synthesis of DNA in MeWo-LC1 cells was determined in the presence and absence of dCF. 2dA has a cytostatic effect on cells and is readily metabolized by adenosine deaminase (Helland & Ueland, 1983; Hershfield et al., 1983). If dCF was inhibiting both intra- and extracellular adenosine deaminase, the toxicity of 2dA should be enhanced in its presence. FIGURE 10 illustrates that in the presence of dCF, a markedly lower concentration

Figure 10

Determination of the Effectiveness of Adenosine Deaminase Inhibition by 2'-Deoxycoformycin. MeWo-LC1 cells were incubated in methionine-containing medium with increasing amounts of 2'-deoxyadenosine in the presence (■) and absence (●) of 10 μM 2'-deoxycoformycin. The relative incorporation of [^{3}H]thymidine into DNA was determined as described in the Methods and is an average ± S.D. of 3 determinations (performed in triplicate).

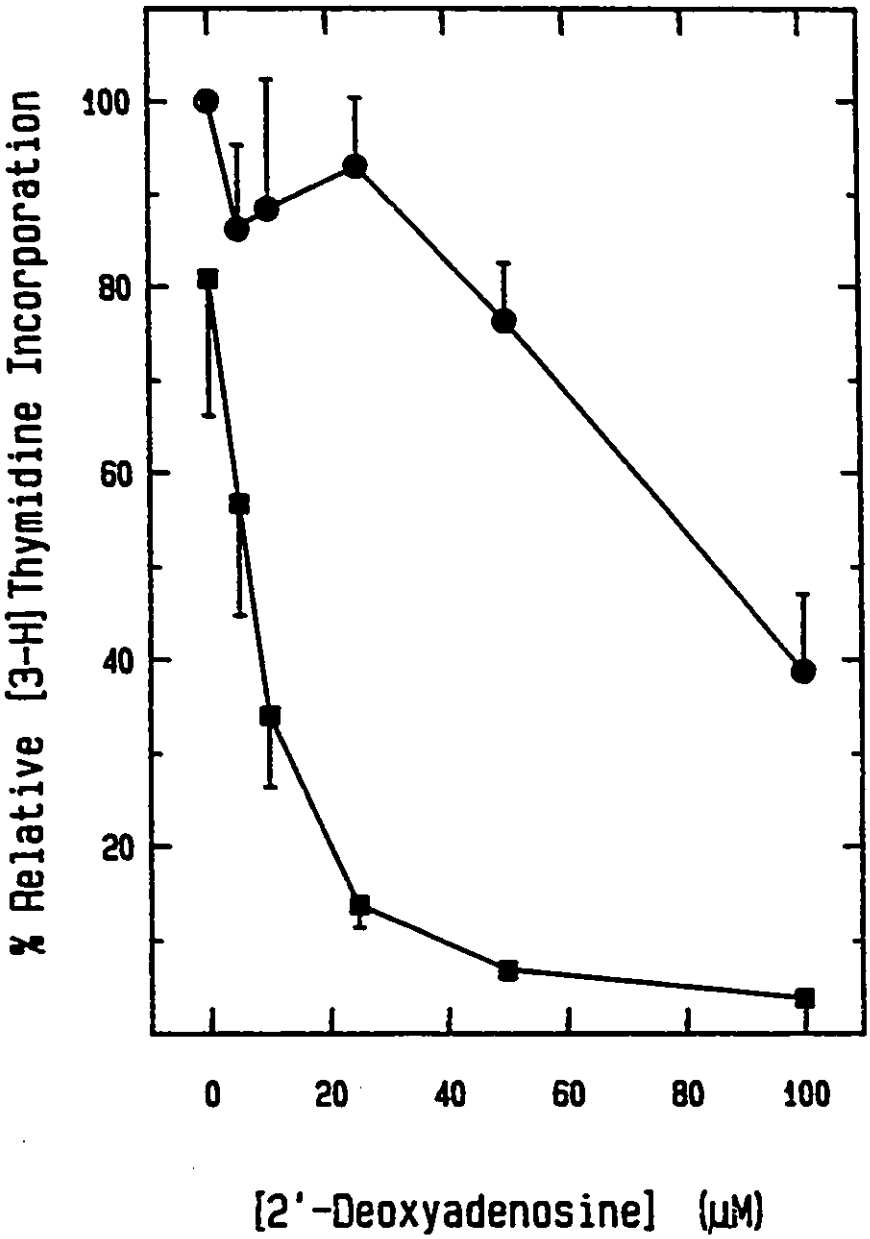

% Relative [3-H] Thymidine Incorporation
100
80
60
40
20
0
20
40
60
80
100
[2'-Deoxyadenosine] (μM)

FIGURE 10

of 2dA was required to inhibit DNA synthesis by 50%, indicating that dCF was actively inhibiting adenosine deaminase under the culture conditions employed. The addition of increasing concentrations of dCF (up to 50 uM) to cells growing in dES medium had no effect on the relative MeSAdo-dependent synthesis of DNA in MeWo-LC1 cells growing in medium supplemented with dES (FIGURE 11). Therefore, it was unlikely that any metabolism of MeSAdo by adenosine deaminase in dES was responsible for the inhibitory effect of the serum on the MeSAdo-dependent DNA synthesis in MeWo-LC1 cells.

The metabolic experiments were routinely carried out in medium containing 5% (v/v) dES which contains 3.2 mg protein per ml of culture medium or 1.6 mg serum albumin per ml of culture medium. Since half of the protein present in 5% (v/v) dES was albumin, the possibility existed that it was the albumin that was responsible for the reduction in MeSAdo-dependent DNA synthesis in dES-medium, but as shown before it was not likely that the MeSAdo was simply bound up. To determine if the effect of serum on MeSAdo-dependent DNA synthesis was due to a non-specific effect of equine serum albumin (ESA), MeWo-LC1 cells were grown in serum-free medium supplemented with transferrin and containing increasing concentrations of either dES or ESA, to determine if the same decrease in MeSAdo-dependent DNA synthesis was observed with both sources of protein (FIGURE 12). When

Figure 11

Effect of the Inhibition of Adenosine Deaminase Activity on Methionine Auxotrophy of MeWo-LC1. MeWo-LC1 cells were cultured in methionine-free medium containing 5% (v/v) dES and either 100 μM methionine or 25 μM MeSAdo. Varying amounts of the adenosine deaminase inhibitor 2'-deoxycoformycin were added to the medium and the incorporation of [^{3}H]thymidine into DNA in the methionine-free test medium supplemented with MeSAdo was determined relative to the incorporation in the corresponding methionine-containing medium (as described in the Methods). The results are an average ± S.D. of 3 determinations each performed in triplicate.

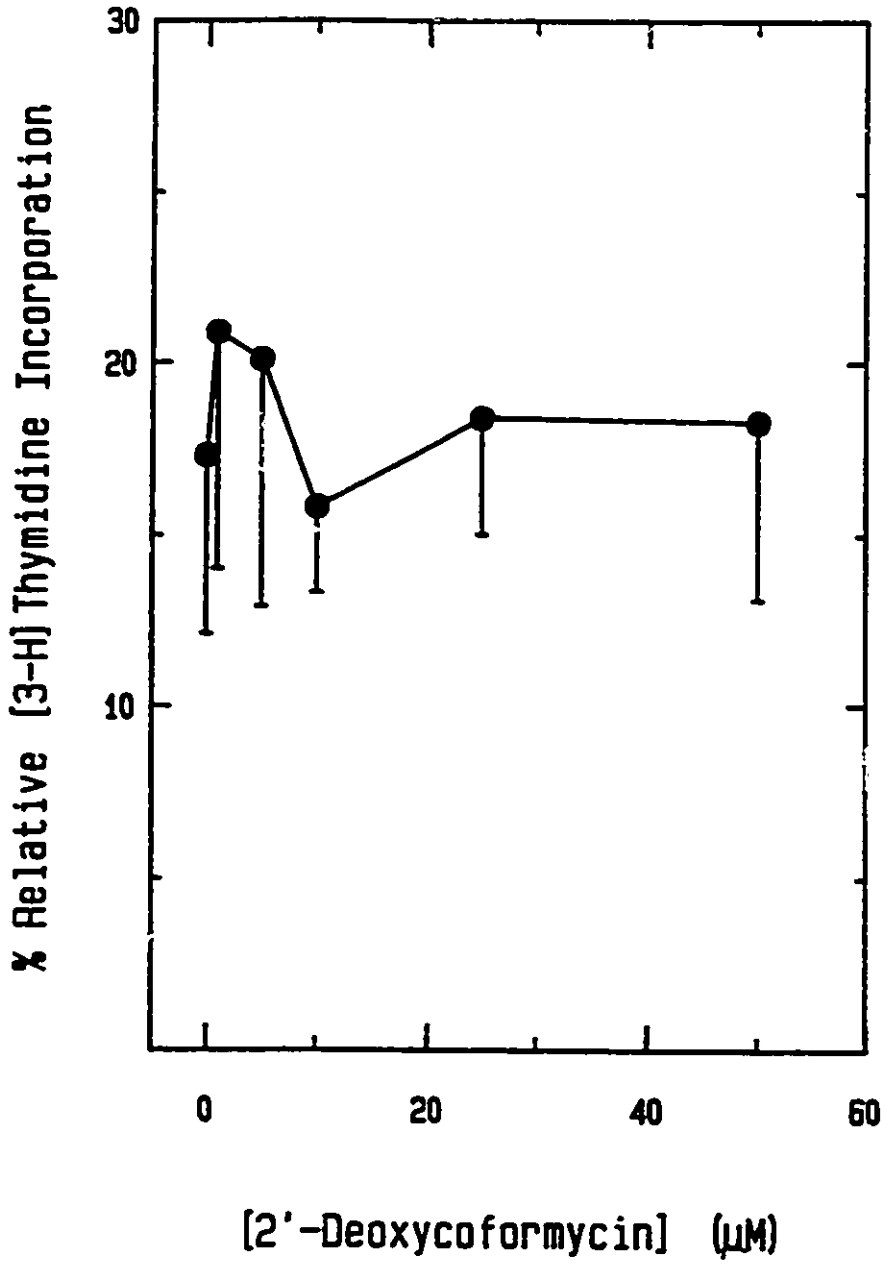

30
20
10
% Relative [3-H] Thymidine Incorporation
0
20
40
60
[2'-Deoxycoformycin] (μM)

FIGURE 11

Figure 12

Effect of Protein Concentration on DNA Synthesis in MeSAdo. MeWo-LC1 cells were cultured in methionine-free medium supplemented with transferrin (SFT-medium) and either 100 µM methionine or 25 µM MeSAdo. Varying amounts of dES (●) or ESA (■) were added to the SFT-medium and the [^{3}H]thymidine incorporation into DNA in MeSAdo-containing medium relative to the incorporation in the corresponding methionine-containing medium was determined as described in the Methods. The results are an average ± S.D. of 3 determinations each performed in triplicate.

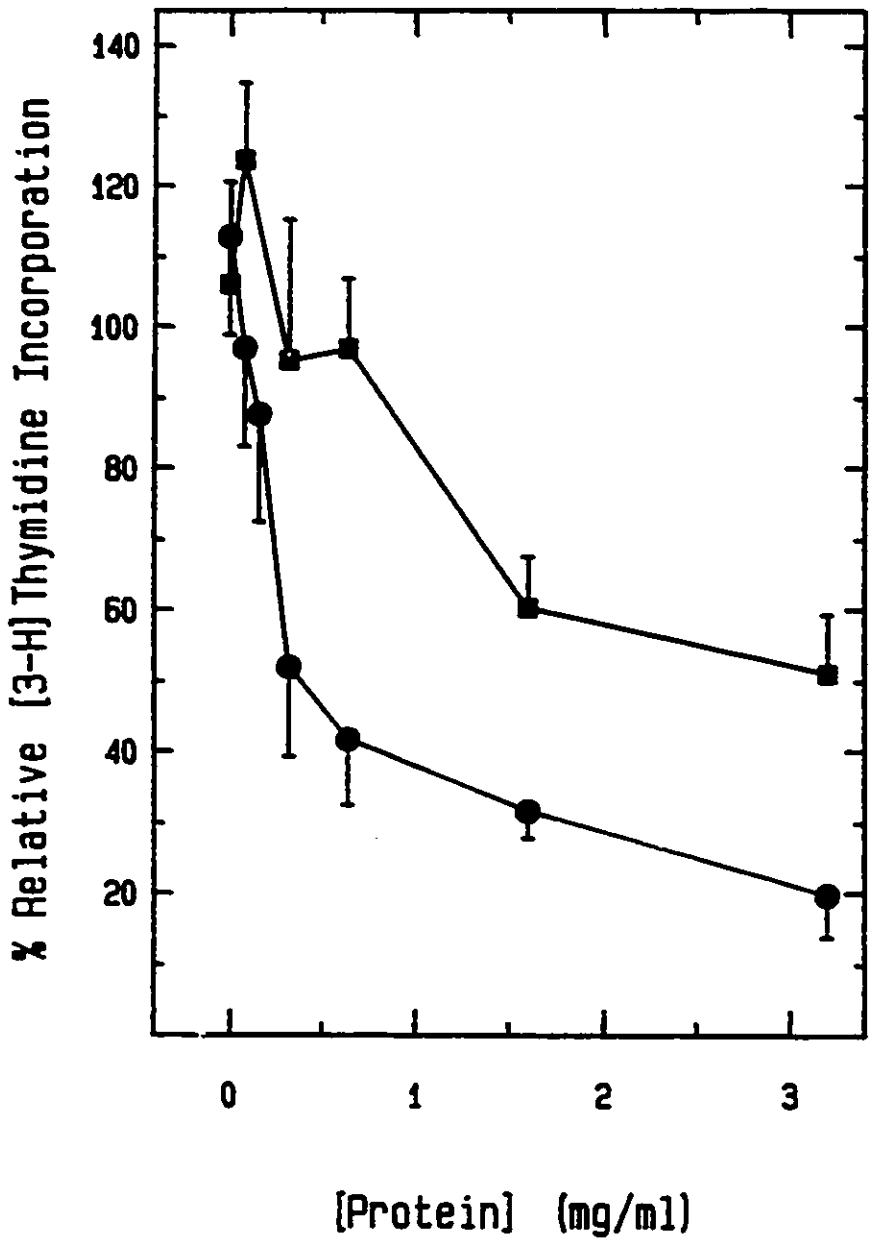

% Relative [3-H] Thymidine Incorporation
140
120
100
80
60
40
20
0
1
2
3
[Protein] (mg/ml)

FIGURE 12

MeWo-LC1 cells were incubated in SFT-medium with increasing concentrations of dES (at a constant concentration of MeSAdo), DNA synthesis decreased gradually, achieving a 50% inhibition at a concentration of 0.32 mg dES protein/ml culture medium. In the case of ESA, DNA synthesis was largely unaffected up to a concentration of 1.6 mg ESA/ml culture medium and was inhibited by 50% at 3.2 mg ESA/ml culture medium (twice the concentration of albumin present in 5% (v/v) dES-medium). It therefore seemed unlikely that the reduction in MeSAdo-dependent DNA synthesis in dES was due totally to a non-specific protein effect of ESA. The addition of dES or ESA to SFT-medium containing methionine resulted in an increase in [^{3}H]thymidine incorporation. However, in the presence of MeSAdo, there was a 5-fold decrease in [^{3}H]thymidine incorporation in the presence of dES and only a 2-fold decrease in the presence of ESA. The partial decrease in DNA synthesis observed with ESA alone was not likely due to the sequestering of MeSAdo by the protein rendering it unavailable to the cell. To ensure that this was not the case, MeWo-LC1 cells were incubated with varying concentrations of MeSAdo in the presence of ESA at 1.6 and 3.2 mg/ml of culture medium. As shown in FIGURE 13, there was no increase in DNA synthesis at higher MeSAdo concentrations. A concentration of 1.6 mg ESA/ml culture medium was equivalent to the amount of the protein present in 5% (v/v) dES. In FIGURE 4, the relative DNA synthesis

Figure 13

Effect of MeSAdo Concentration on DNA Synthesis in the Presence of ESA. MeWo-LCl cells were cultured in methionine-free medium supplemented with transferrin, containing ESA at a concentration of 1.6 (●) or 3.2 mg/ml (■) and supplemented with varying amounts of MeSAdo. The [^{3}H]thymidine incorporation into DNA was determined relative to the incorporation in the corresponding methionine-containing medium as described in the Methods. The results are an average ± S.D. of 3 determinations each performed in triplicate.

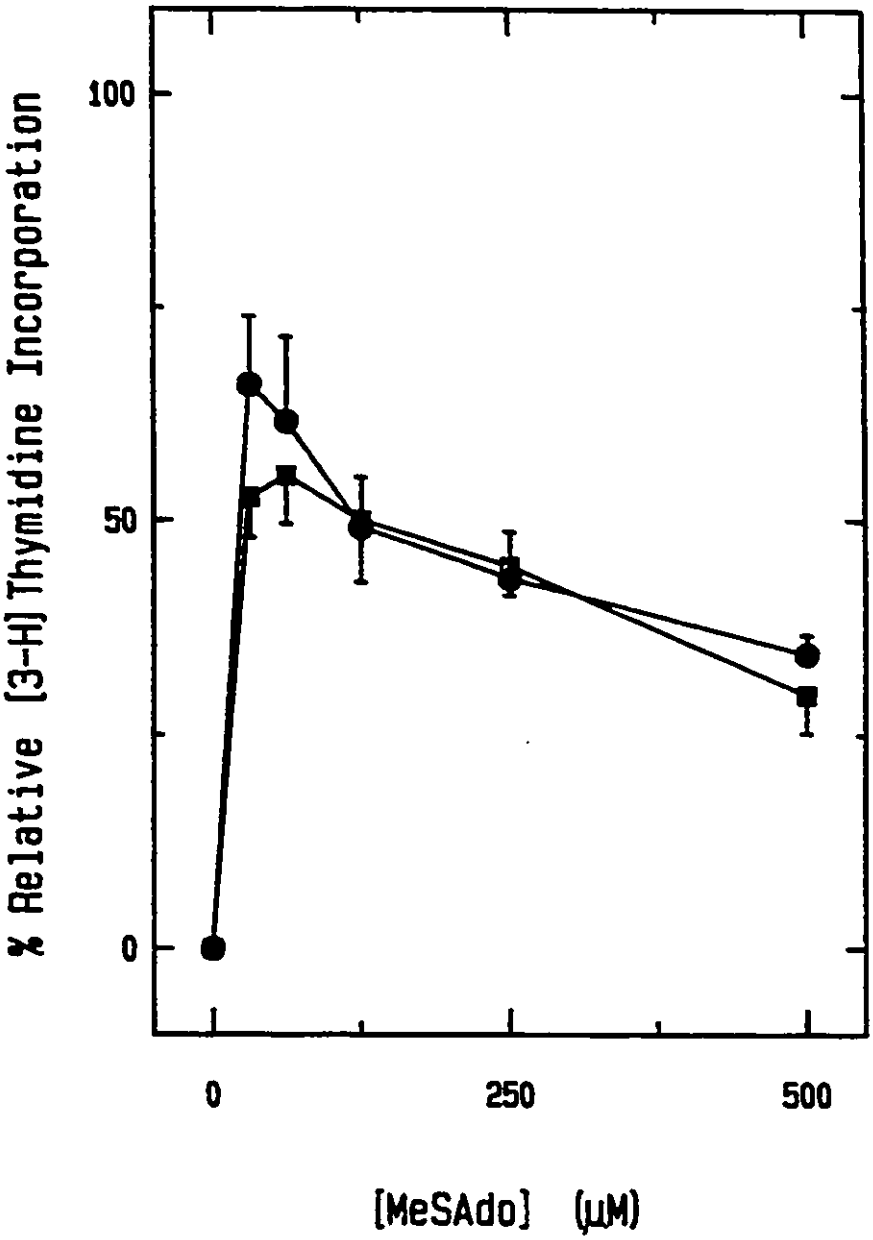

% Relative [3-H] Thymidine Incorporation
100
50
0
0
250
500
[MeSAdo] (µM)

FIGURE 13

remained in the range of 15-20% in 5% (v/v) dES, whereas in medium containing only ESA at 1.6 mg/ml culture medium, the relative DNA synthesis was between 36 and 60%. Even under conditions where the amount of ESA present in the medium was equivalent to the total amount of protein present in 5% (v/v) dES (3.2 mg/ml culture medium), the inhibition of DNA synthesis in ESA was not as great as with dES. Therefore, there was probably some other factor that was involved in the inhibition of DNA synthesis by dES. Indeed at high concentrations of MeSAdo, the inhibition of DNA synthesis was increased indicating that the nucleoside begins to have an inhibitory effect on the cells. The same experiments were not carried out using the MeWo cell line since it is not capable of proliferating in serum-free medium. Further, at this point in the study the emphasis had been shifted from differences in the methionine metabolism between MeWo and MeWo-LC1 cells to the determination of the mechanism by which dES altered the MeSAdo-dependent synthesis of DNA in MeWo-LC1 cells.

MeSAdo has been reported to have a cytostatic effect on cell growth (Williams-Ashman et al.,1982). When MeWo-LC1 cells were grown under optimal conditions, i.e. in the presence of methionine, with varying concentrations of MeSAdo in dES-medium, a MeSAdo concentration of 0.1 mM decreased DNA synthesis by 50%, while a concentration of 0.75 mM was required to achieve the same inhibition in

SFT-medium (FIGURE 14A). There was a significant difference (at $p<0.01$) in the amount of DNA synthesis between these two media at a MeSAdo concentration of 0.125 mM. Therefore, using the amount of DNA synthesis as an index of cell growth, MeSAdo was having an inhibitory effect on MeWo-LC1 cells and the effect was greater in the presence of dES than in SFT-medium. When the same experiment was carried out in the presence of ESA (FIGURE 14B), a 50% inhibition of DNA synthesis was observed at a MeSAdo concentration 7 to 7.5-fold higher than that required to achieve the same inhibition in dES, suggesting that it was not the albumin alone that was responsible for the enhancement of the inhibitory effect of MeSAdo in the presence of dES. In fact, when the same experiment was carried out in the presence of dFBS (FIGURE 14A), the result was similar to that found with ESA in the medium, suggesting that the inhibition of DNA synthesis in MeSAdo$^+$-medium was restricted to medium containing dES. It was possible that the effect of MeSAdo on DNA synthesis was not as pronounced in dFBS-containing medium due to its degradation by MeSAdo phosphorylase. As a result, the actual concentration of the nucleoside outside the cell would be lower and therefore, less effective.

In order to determine if the inhibitory effect of MeSAdo in Met$^+$(dES)-medium was due to a greater accumulation of the nucleoside within the cell, the incorporation of

Figure 14

Cytostatic Effect of MeSAdo in Various Media. MeWo-LC1 cells were incubated in methionine-containing medium supplemented with varying amounts of MeSAdo and containing A: either 5% (v/v) dFBS (▲), 5% (v/v) dES (●) or 10 µg/ml transferrin (■) or B: either 1.6 mg/ml ESA (▼), 3.2 mg/ml ESA (◆) or 10 µg/ml transferrin (■). The results are expressed as the ratio of the incorporation of [^{3}H]thymidine into DNA in MeSAdo-containing medium to the incorporation in the corresponding medium without MeSAdo x 100. The results are an average ± S.D. of 3-5 determinations each performed in triplicate.

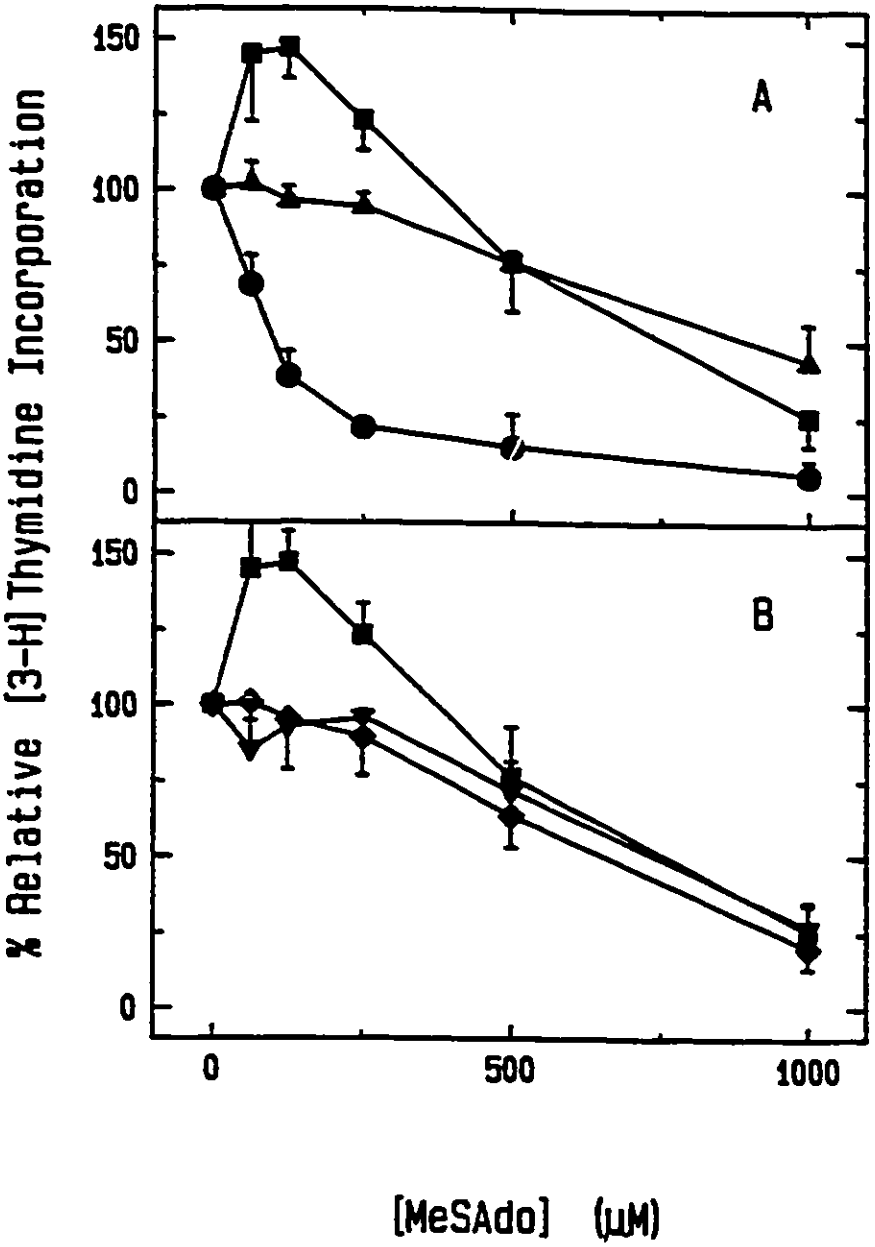

% Relative [3-H] Thymidine Incorporation
150
100
50
0
A
B
0
500
1000
[MeSAdo] (μM)

FIGURE 14

[^{14}C]MeSAdo into cellular macromolecules (nucleic acids) in MeWo-LC1 cells was determined when cells were cultured in either Met$^+$(dES)-medium and Met$^+$(SFT)-medium. [^{14}C]MeSAdo is converted to [^{14}C]adenine and methylthioribose-1-phosphate by MeSAdo phosphorylase. The [^{14}C]adenine is then converted to [^{14}C]AMP by the addition of ribose phosphate. The [^{14}C]AMP is subsequently phosphorylated to [^{14}C]ATP which can be used in RNA synthesis or finally to [^{14}C]dATP which is used in the synthesis of DNA (Lehninger, 1982). FIGURE 15 illustrates that although at some intermediate time points there were small differences in the amount of radioactivity incorporated, these differences were not significant (at $p<0.01$) and after 24 hours the level of incorporation was the same in both media. This suggested that there was no increased metabolism of MeSAdo within MeWo-LC1 cells in one medium when compared to the other. Even though the incorporation of MeSAdo into cellular macromolecules was similar in both media, the possibility remained that the accumulation of the nucleoside within the cell was different as a result of variations in the uptake of MeSAdo. Consequently, the uptake of [^{14}C]MeSAdo was determined over a 3 hour period with cells cultured for 3 days in either Met$^+$(dES)- or Met$^+$(SFT)-medium (FIGURE 16). The total accumulation of [^{14}C]MeSAdo and its metabolites was identical after 1 hour for MeWo-LC1 cells grown in either medium, after 3 hours, an approximately 2-fold higher amount

Figure 15

Incorporation of [^{14}C]MeSAdo into Cellular Macromolecules. MeWo-LC1 cells were incubated in methionine-containing medium supplemented with either 5% (v/v) dES (●) or 10 µg/ml transferrin (■). Cells were incubated with [^{14}C]MeSAdo for various times (up to 24 hours) and the amount of radioactivity incorporated into nucleic acids (cpm/mg protein) was determined as described in the Methods. The results are an average ± S.D. of 1 determination performed in duplicate.

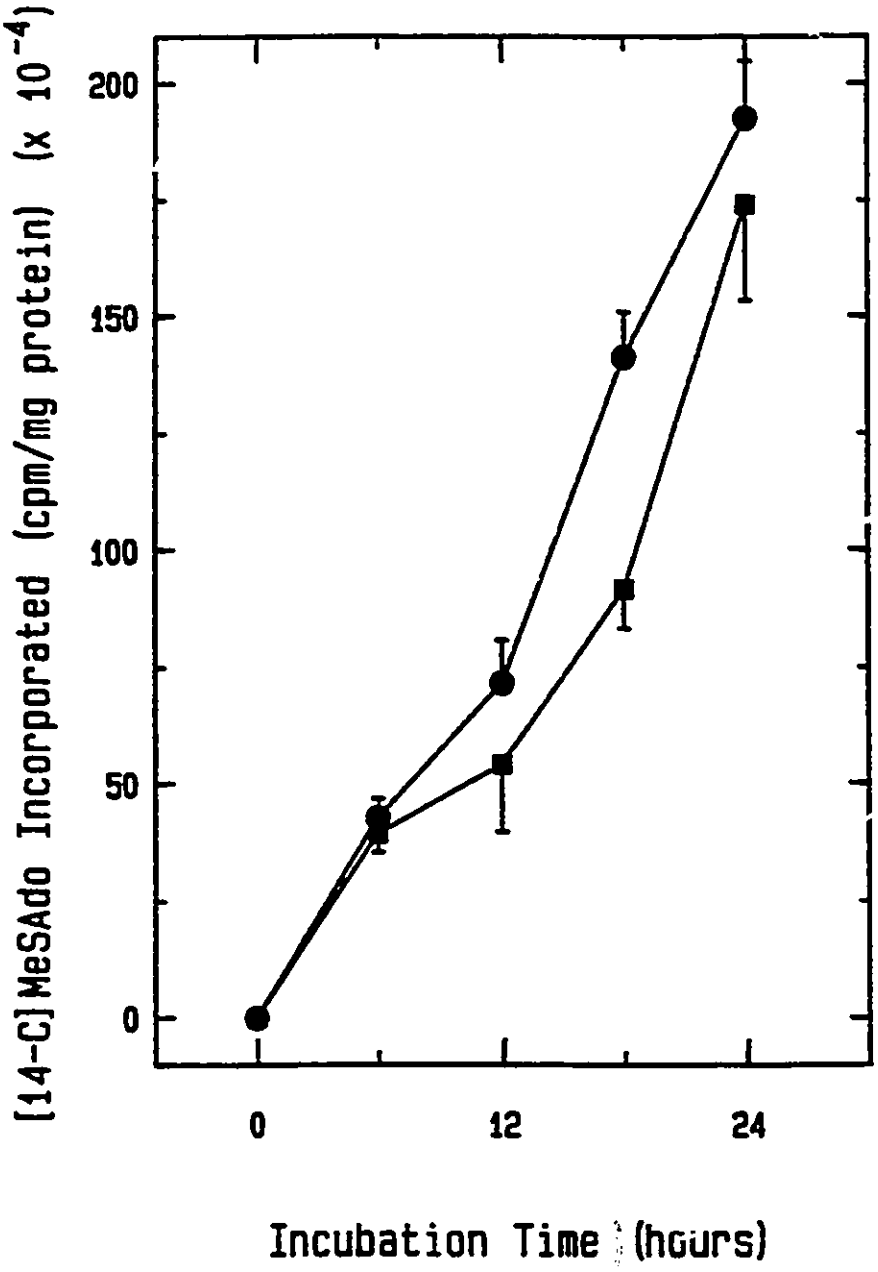

[14-C]MeSAdo Incorporated (cpm/mg protein) (x 10^{-4})
200
150
100
50
0
0
12
24
Incubation Time (hours)

FIGURE 15

Figure 16

Cellular Uptake of [^{14}C]MeSAdo. MeWo-LC1 cells were cultured for 3 days in methionine-containing medium supplemented with either 5% (v/v) dES (●) or 10 µg/ml transferrin (■). Cells were incubated with [^{14}C]MeSAdo for up to 3 hours. Cells were lysed, an aliquot of the cell lysate was counted and the total cellular radioactivity per 10^5 cells was determined as described in the Methods. The results are an average ± S.D. of 2 determinations each performed in triplicate.

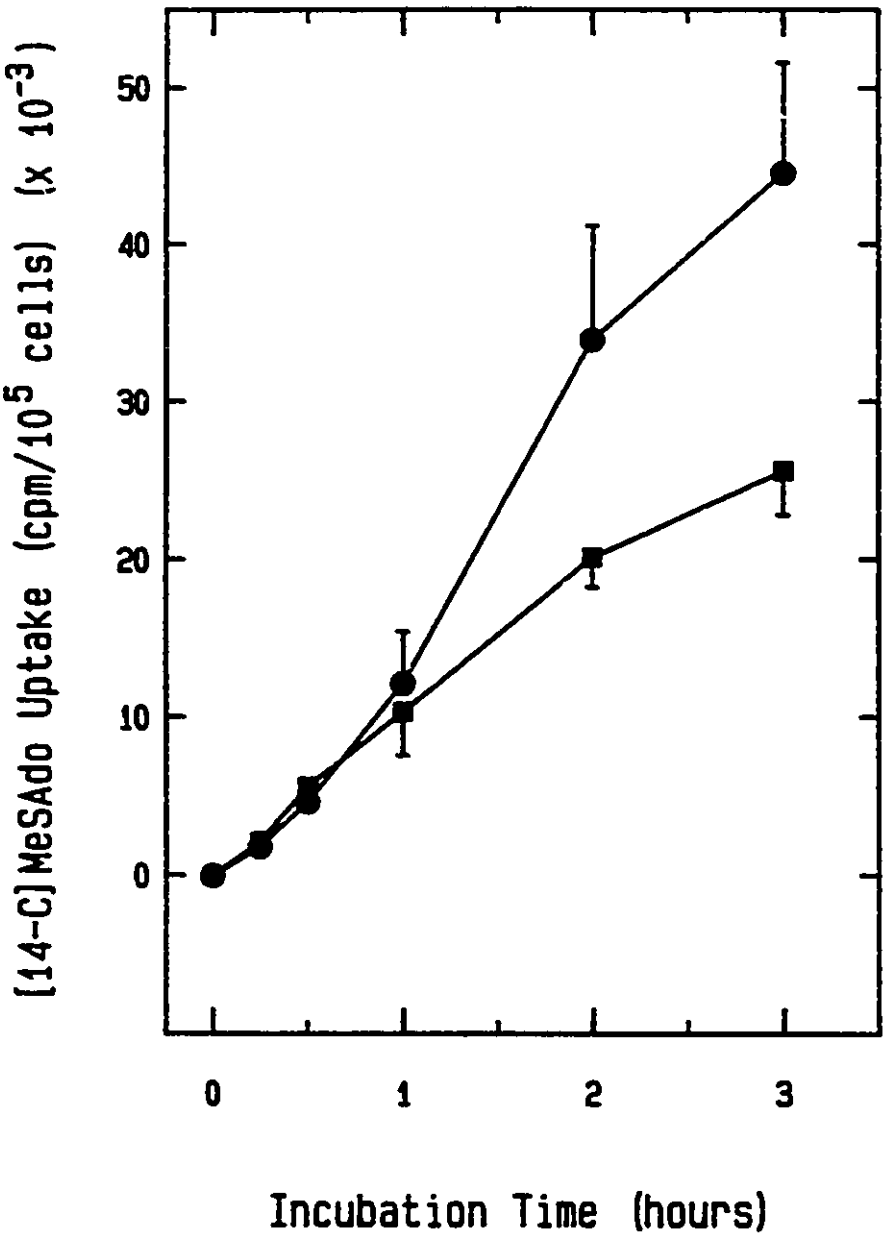

[14-C]MeSAdo Uptake (cpm/10^5 cells) (x 10^-3)
50
40
30
20
10
0
0
1
2
3
Incubation Time (hours)

FIGURE 16

had accumulated in the cells grown in Met$^+$(dES)-medium, compared to cells grown in Met$^+$(SFT)-medium.

MECHANISM OF THE INHIBITORY EFFECT OF MeSAdo

The above results showed that at low MeSAdo concentrations, in the presence of dES, DNA synthesis was reduced in MeWo-LCl cells. Under conditions where methionine was not limiting, high concentrations of MeSAdo had an inhibitory effect, especially in the presence of dES. The mechanism by which MeSAdo exerts its effects is as yet not completely defined, although some possibilities have been suggested. MeSAdo has been reported to be a potent *in vitro* inhibitor of the spermidine (Spd) and spermine (Spm) synthases as well as S-adenosylhomocysteine (AdoHcy) hydrolase (Kamatani & Carson, 1980; Williams-Ashman et al., 1982). It was possible that since more MeSAdo was accumulating within the cells, it was exerting its cytostatic effect via one of these two enzyme systems. It has been suggested that the cytostatic effects of MeSAdo could be due to the depletion of polyamines (Riscoe, Schwamborn, Ferro, Olson & Fitchen, 1987). Therefore if the polyamine synthases were affected, the addition of exogenous polyamines should have resulted in a relief of the cytostatic effect. Upon the addition of Spd, Spm or both together to cells cultured in MeSAdo$^+$(dES)-medium, no increase in DNA synthesis was observed (TABLE 5). This

TABLE 5

Culture Conditions	% Relative [^{3}H]Thymidine Incorporation	
	25μM MeSAdo[a]	250μM MeSAdo[b]
No additions	22.7 ± 3.6	19.2 ± 9.5
+1μM Spd	24.7 ± 2.0	20.4 ± 9.4
+5μM Spd	21.9 ± 4.0	15.3 ± 10.6
+1μM Spm	20.8 ± 6.9	21.0 ± 8.9
+5μM Spm	26.0 ± 8.2	13.2 ± 3.6
+1μM Spd+1μM Spm	20.0 ± 2.8	14.7 ± 4.9
+5μM Spd+5μM Spm	25.2 ± 1.6	13.2 ± 6.5

a- Cells were cultured in methionine-free medium supplemented with 5% (v/v) dES and either 25 μM MeSAdo or 100 μM methionine.

b- Cells were cultured in medium containing methionine supplemented with 5% (v/v) dES and 250 μM MeSAdo.

THE INABILITY OF EXOGENEOUS POLYAMINES TO RESCUE MeWo-LC1 CELLS GROWING IN MeSAdo*(dES)-MEDIUM- One or both polyamines spermidine (Spd) and spermine (Spm) were added to cells cultured in MeSAdo. The results are the average ± S.D. of 3-4 determinations (performed in triplicate). The values for cells in 25 μM MeSAdo are expressed as the percent [^{3}H]thymidine incorporated into DNA in the test medium (methionine-free) relative to that incorporated in the corresponding methionine-containing medium. Results for cells in 250 μM MeSAdo are expressed as the percent [^{3}H]thymidine incorporated into DNA in the test medium relative to that incorporated in methionine-containing medium without any polyamines or MeSAdo.

suggested that it was unlikely that MeSAdo was inhibiting the spermidine and spermine synthases resulting in a depletion of intracellular polyamines and that MeSAdo was not exerting its toxic effect via polyamine metabolism.

The inhibition of endogenous AdoHcy hydrolase activity would result in perturbation of methylation reactions within the cell and could affect c-AMP levels (Ferro, Vandenbark & MacDonald, 1981). To determine if MeSAdo acted via the AdoHcy hydrolase, MeWo-LC1 cells were grown under optimal conditions (i.e. in the presence of methionine), and periodate oxidized adenosine (Adox), a potent inhibitor of the hydrolase (Hoffman, 1979; Bartel & Borchardt,1984), was added to the medium. If MeSAdo was exerting its cytostatic effect via the AdoHcy hydrolase, then the addition of Adox to cells growing in dES in the presence of methionine should show a similar reduction in DNA synthesis as would have been observed if MeSAdo had been added to the medium. Further, cells growing in SFT-medium should be largely unaffected by the addition of Adox, since they are not by the addition of MeSAdo. As shown in FIGURE 17, no significant difference (at $p<0.01$) was observed in the effect of Adox on cells cultured in the two media. These results seem to indicate that the mechanism of MeSAdo toxicity does not involve the AdoHcy hydrolase enzyme nor polyamine metabolism.

Figure 17

Effect of the Inhibition of AdoHcy Hydrolase on DNA Synthesis. MeWo-LC1 cells were cultured under optimal conditions in both 5% (v/v) dES (●) and 10 µg/ml transferrin (■). The relative incorporation of [^{3}H]thymidine into DNA upon exposure of the cells to varying amounts of the AdoHcy hydrolase inhibitor Adox was determined as described in the Methods. The results are an average ± S.D. of 4 determinations each performed in triplicate.

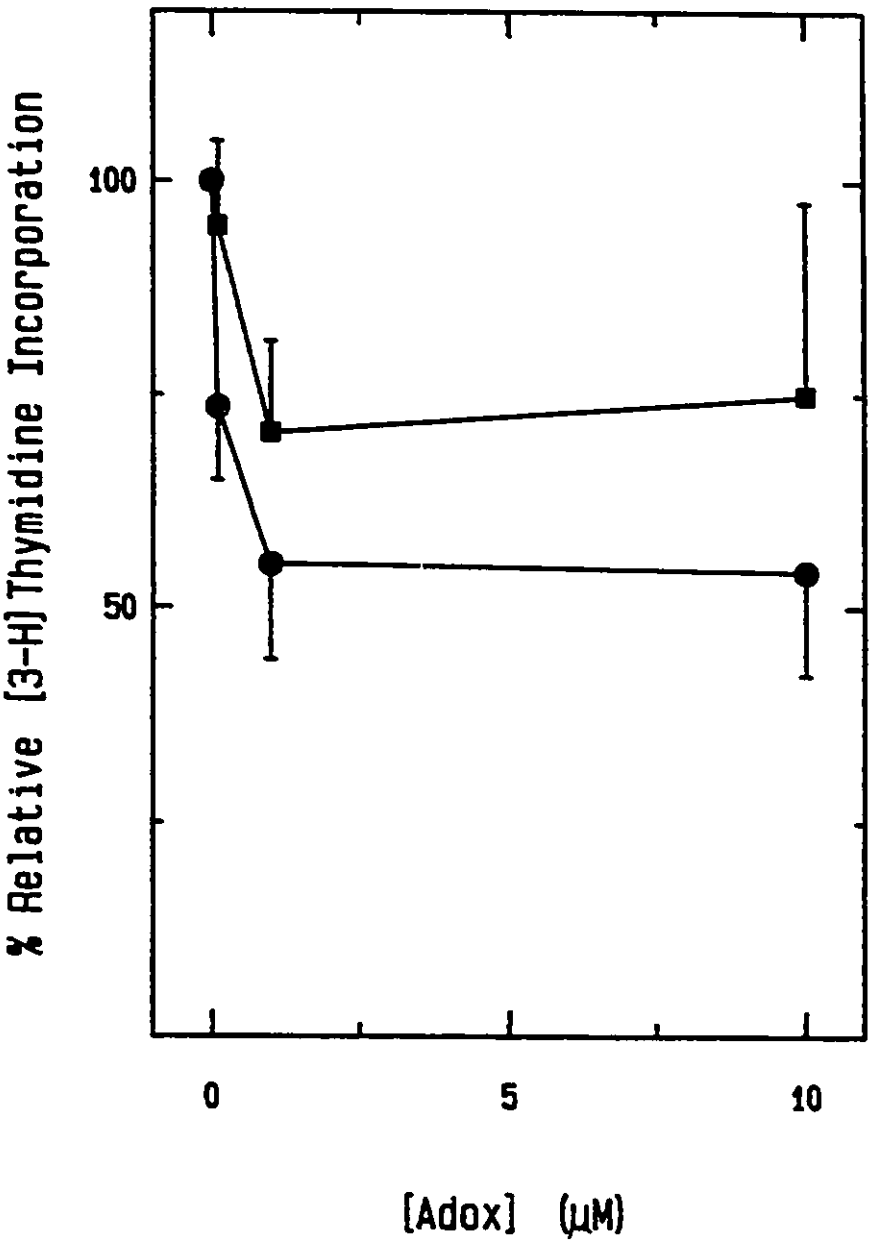


FIGURE 17

ISOLATION OF THE EQUINE SERUM FACTOR

Attempts to isolate the component(s) present in equine serum which accounted for the reduction in MeSAdo-dependent DNA synthesis in dES-medium were unsuccessful. Initially, serum was subjected to ammonium sulphate fractionation in order to determine if a protein was responsible for the observed inhibitory effect. No individual fraction was found to contain all the 'inhibitory' activity. Affinity chromatography was carried out in order to determine if a specific protein could be isolated that would account for the inhibitory activity of dES. Three types of columns were chosen: heparin, concanavalin A and Affi-gel Blue (Cibacron Blue F3GA dye). The heparin column was used since this glycosaminoglycan can bind a variety of proteins, especially coagulation factors and other plasma proteins and lipoproteins. Concanavalin A, a lectin, binds saccharides, glycopeptides and glycoproteins and Affi-gel Blue serves to remove albumin. Therefore, if the inhibitory factor in dES was a protein, it would likely have bound to one of these columns. The third column served to remove the albumin present in dES which had been shown not to be the source of the inhibition in earlier experiments. When dES was passed over the columns, there was no increase in the specific activity of the inhibitory factor, neither in the unbound fraction nor in the bound fraction (data not shown). Treatment of serum with acid or base for 2 hours had little

effect on DNA synthesis compared to that observed for untreated serum (data not shown). Finally, the lipids present in serum were extracted using butanol and di-isopropyl ether (Cham & Knowles, 1976). This method of lipid extraction avoids protein denaturation and the lipid is easily extracted into the organic phase. When MeWo-LC1 cells were grown in this delipidated serum, DNA synthesis was still reduced in MeSAdo$^+$-medium, suggesting that it was not a lipid that was responsible for the reduction in DNA synthesis in MeSAdo$^+$(dES)-medium (data not shown).

DISCUSSION

The question of what causes cancer, what metabolic differences exist that differentiate a cancer cell from a normal one and what makes one cancer cell more metastatic than another have plagued scientists for many years. The two human tumor cell lines MeWo and MeWo-LC1, one derived from the other, exhibit differences both in their metabolism of methionine and metastatic capacity. The poorly metastatic cell line MeWo is capable of proliferating in medium in which methionine has been replaced by its precursor(s), either Hcy or MeSAdo (Liteplo, 1989). MeWo-LC1, a highly metastatic variant cell line derived from MeWo (Kerbel & Man, 1984), cannot proliferate under these conditions (Liteplo, 1989). Although the MeWo-LC1 cell line represents a heterogeneous population of cells, the inability of these cells to proliferate in MeSAdo^{+}(dES)$^{-}$ medium was not an average of cells that can grow and those that cannot. The decrease in DNA synthesis reflected each cell since the determination of the methionine auxotrophy of a random sampling of MeWo-LC1 clones showed values similar to that of MeWo-LC1 cells (TABLE 1).

The incorporation of [^{3}H]thymidine into DNA was used as an indicator of cell proliferation. The values obtained by this method reflected those found in cell count experiments. That is, the value calculated for the relative growth of

cells under the various test conditions was the same when determined either by [^{3}H]thymidine incorporation or by cell counting.

The neoplastic transformation of cells has been associated with changes in the level of gene expression which plays an important role in cell development (Comings, 1973). Among the many factors influencing the expression of these genes is the extent to which their DNA is methylated (Cooper, 1983). As a consequence of its direct involvement in the methylation of DNA (as well as other macromolecules), alterations in methionine metabolism have been linked to the process of carcinogenesis. Studies have found that the level of 5-mC in tumor cells is, in general, lower than that found in normal cells (Diala et al., 1983; Liteplo & Kerbel, 1987). Therefore, it was of interest to establish whether tumor cells exhibit alterations in their methionine metabolism that are not present in normal cells. Various tumor cell lines, including MeWo-LC1, have been determined to exhibit an absolute requirement for methionine (Halpern et al., 1974; Hoffman, 1982; Liteplo, 1989). That is, these cells cannot grow in methionine-free medium that has been supplemented with one of its precursors, either Hcy or MeSAdo. Further, methyl-deficient diets have been found to contribute to the formation of hepatomas in rats and mice (Mikol et al., 1983; Wainfan et al., 1989). Thus, the link between changes in methionine metabolism and cellular

transformation was established.

The fact that two very closely related cell lines, MeWo and MeWo-LC1, exhibited differences in their methionine auxotrophy and at the same time in their metastatic capacity, was of great interest. Perhaps there was a link between alterations in methionine metabolism and the capacity of a cell to metastasize. The inability of MeWo-LC1 cells to proliferate in methionine-free medium supplemented with MeSAdo (and serum) could easily have been explained by a lack of MeSAdo phosphorylase, the enzyme necessary for the metabolism of the nucleoside. Previous studies had already established that a number of human and murine tumor cell lines are deficient in MeSAdo phosphorylase activity (Kamatani & Carson, 1980; Fitchen, Riscoe, Dana, Lawrence & Ferro, 1986; Carson et al., 1988). In contrast, MeWo cells which could proliferate in the same medium likely contained the enzyme. *In vivo* and *in vitro* assays of MeSAdo phosphorylase activity showed that both cell lines contained the enzyme and that it was indeed functional in intact cells (TABLES 3 & 4). Therefore, some other biochemical defect appeared to be responsible for the difference in methionine auxotrophy between the two cell lines.

Metabolic studies involving MeSAdo have routinely been carried out in medium supplemented with dES since, unlike dFBS, it contains little or no endogenous MeSAdo

phosphorylase activity (Riscoe & Ferro, 1984). To simplify the conditions further, MeWo-LC1 cells were grown in serum-free, methionine-free medium supplemented with transferrin and MeSAdo. It had been previously established by Sauvaigo et al. (1986) that the MeWo-LC1 cell line could readily proliferate in serum-free medium containing transferrin (SFT-medium). MeSAdo phosphorylase activity remained the same in MeWo and MeWo-LC1 cells in the presence and absence of dES (TABLE 3) and therefore, the serum did not appear to alter the *in vitro* activity of the enzyme. However, while MeWo-LC1 cells were MeSAdo-nonresponsive (i.e. they could not synthesize DNA when methionine was replaced by MeSAdo) in the presence of dES, they were MeSAdo-responsive (i.e. they were capable of DNA synthesis in methionine-free medium supplemented with MeSAdo) in SFT-medium. This differential response of MeWo-LC1 was restricted to the methionine precursor MeSAdo (i.e. the cells were Hcy-nonresponsive irregardless of the medium in which they were grown (TABLE 2)). Previous studies of various tumor cell lines had found many of them to be either Hcy- or MeSAdo-nonresponsive or both (Halpern et al., 1974; Hoffman, 1982; Liteplo, 1989) However, these studies did not observe a differential response of the cell lines since they were all carried out in the presence of serum. Therefore, although the cell lines tested in those studies appeared to be nonresponsive to the precursors of methionine, the observation may have

been due to the presence of serum and a similar result may not have been found in serum-free medium. In this study, the addition of serum appeared to be having a drastic effect on the MeSAdo-dependent synthesis of DNA in methionine-free medium, that is, the ability of MeWo-LC1 cells to synthesize DNA in methionine-free medium supplemented with MeSAdo appeared to depend on whether or not serum was present. The addition of serum to the medium caused a decrease in the synthesis of DNA and the inhibitory effect predominated (i.e. the addition of serum to SFT-medium resulted in decreased DNA synthesis (FIGURE 12, TABLE 2)). Therefore, it appeared that some component(s) in serum was altering the ability of MeWo-LC1 cells to respond to MeSAdo.

The decrease in DNA synthesis in MeSAdo^{+}(dES)-medium was not due to sequestering of the nucleoside by the components of serum, rendering it unavailable to the cell, and was not due to a non-specific protein effect of ESA. In experiments where increasing concentrations of MeSAdo were added to cells growing in the presence of either dES or ESA (at 1.6 and 3.2 mg/ml culture medium), DNA synthesis could not be restored to the level found in cells growing in methionine medium (FIGURES 5 & 13). In order for a marked decrease in DNA synthesis to be observed, dES itself had to be added to the medium (FIGURE 12), ESA alone and dFBS could only affect a partial decrease in DNA synthesis (FIGURE 12, TABLE 2). It was possible that the MeSAdo phosphorylase

enzyme present in dFBS was degrading the exogenous MeSAdo rendering it unavailable to the cell, thereby explaining the partial decrease in DNA synthesis observed under these conditions. At the same time, the degradation of MeSAdo could have provided the cell with the needed substrate, thereby allowing DNA synthesis to proceed. According to the model of Riscoe and Ferro (1984) for the growth of MeSAdo phosphorylase deficient cell lines in medium containing serum, MeSAdo is degraded to MTR-1-P by the endogenous MeSAdo phosphorylase in the serum. The MTR-1-P is subsequently converted to methylthioribose, which can then enter the cell and satisfy the methylthio requirement. The decrease in DNA synthesis caused by the presence of equine serum was not likely due to the exogenous metabolism of MeSAdo since the inhibition of adenosine deaminase by dCF had no effect on DNA synthesis in MeWo-LC1 cells growing in MeSAdo^{+}(dES)-medium.

A cytostatic effect of MeSAdo on cell growth has been previously described (Williams-Ashman et al., 1982) and had been attributed to the nucleoside itself and not its degradation products (Dante, Arnaud & Niveleau, 1983; Kubota, Kamatani & Carson, 1983). Since it had already been established that MeSAdo was not being metabolized or sequestered by serum components, it was possible that the decrease in DNA synthesis observed in the presence of serum was due to such a cytostatic effect of MeSAdo. Indeed, in

methionine-containing medium, MeSAdo had a greater inhibitory effect on DNA synthesis in the presence of dES than in SFT-, dFBS- or ESA-medium (FIGURE 14). In contrast to studies in methionine-free medium, under these conditions the cells were not dependent on the exogenous MeSAdo as a methylthio-source. Therefore, the inhibition of DNA synthesis was not due to a lack of methionine synthesis, but was due to a direct effect of MeSAdo. If MeSAdo was having an inhibitory effect on DNA synthesis in the presence of dES, then this would explain the failure of MeWo-LC1 cells to synthesize DNA in methionine-free medium supplemented with MeSAdo. It is probably due to an inhibitory effect of the nucleoside and not a lack of methionine synthesis. The uptake of MeSAdo by cells growing in the presence of dES was greater than that of cells grown in SFT-medium (FIGURE 16). However, the 2-fold higher uptake of MeSAdo could not account for the observation that a 7.5-fold lower concentration of MeSAdo in dES-medium than in SFT-medium, decreased DNA synthesis by 50%. Further, the amount of ^{14}C-MeSAdo incorporated into nucleic acids was the same for cells cultured in the two media, and therefore the metabolism of the nucleoside within the cell was likely the same under both conditions. The higher intracellular concentrations of MeSAdo in dES-medium may have contributed to the inhibitory effect of the nucleoside on DNA synthesis.

Several enzyme systems have been suggested to be

affected by MeSAdo, however the exact mechanism by which it inhibits cell growth has not yet been established. Riscoe et al. (1987) suggested that MeSAdo depleted the cells' polyamines by inhibiting the Spd and Spm synthases. It is not likely that MeSAdo was exerting its inhibitory effect via polyamine metabolism since, in agreement with previous studies (Riscoe et al., 1987; Christa et al., 1988), the addition of exogenous polyamines could not restore DNA synthesis in MeWo-LC1 cells cultured in MeSAdo (TABLE 5). Previous studies had found that the polyamines could inhibit their own synthesis (Kamatani & Carson, 1981) and this may explain the observation that there was actually a decrease in the synthesis of DNA upon their addition to the medium. In contrast, in studies using the inhibitor of polyamine synthesis, difluoromethylornithine, the re-addition of Spd and Spm to the medium restored cell growth (Mamont, Duchesne, Grove & Bey, 1978). Williams-Ashman, Coppoc, Schenone and Webber (1973) had gone so far as to suggest that the role of polyamine synthesis in the cell was to produce MeSAdo and not the polyamines themselves and that MeSAdo then serves as a regulator of various enzyme systems and as a precursor of physiologically important substances. However, this seems unlikely since studies have found that polyamines are essential for cell growth (Pegg & McCann, 1982). Further, it is unlikely that such a complicated synthetic pathway would exist within the cell if it was

unnecessary to it.

Although MeSAdo has been suggested to be an inhibitor of AdoHcy hydrolase (Williams-Ashman et al., 1982), its inhibitory effect on DNA synthesis does not appear to be via this enzyme in MeWo-LC1 cells. If MeSAdo was acting via this enzyme then the addition of Adox to the medium should cause the same decrease in DNA synthesis in the presence of dES that was observed with the addition of MeSAdo to the medium. The same decrease in DNA synthesis could not be achieved with the addition of Adox to the medium as was observed with the addition of MeSAdo (FIGURE 17). Further, the addition of Adox to SFT-medium did not cause a large decrease in the synthesis of DNA just as the addition of MeSAdo did not affect DNA synthesis in this medium.

Two alternative mechanisms for the inhibitory effect of MeSAdo on cell growth have recently been suggested. One involves the inhibition by MeSAdo of the synthesis of dipthamide, a post-translationally modified histidine which appears in eukaryotic elongation factor-2 (Carson et al., 1988; Yamanaka, Olavi, Kubota, Wasson, Willis & Carson, 1989). The second postulated mechanism involves the perturbation of histone acetylation (Yamanaka et al., 1989). Although MeSAdo can inhibit Spd and Spm synthases, it can activate ornithine decarboxylase and AdoMet decarboxylase (Yamanaka et al., 1989). As a result, decarboxylated AdoMet accumulates and competes with histone for acetylation

(Yamanaka et al., 1989). This modification of histone is an essential post-translational reaction in the rapid activation of genes (Perry & Chalkey, 1981). Further, decarboxylated AdoMet is a poor methyl donor and when its concentration exceeds that of AdoMet, it can begin to have an inhibitory effect on DNA methylation (Heby, Persson & Smith, 1988). MeSAdo has also been suggested to be a direct inhibitor of DNA transmethylase (Dante et al., 1983). However, a relatively high concentration of the nucleoside is required to achieve this inhibition and MeSAdo concentrations are normally low within the cell. Therefore, cellular DNA transmethylase is not likely the primary target of MeSAdo (Dante et al., 1983).

The factor in dES responsible for the inhibitory effect on DNA synthesis of MeSAdo remains undetermined. It is likely that it is either a protein or is associated with one, since the removal of serum lipids did not result in the abolition of the inhibitory effect of serum. Attempts to isolate and purify this protein using ammonium sulphate fractionation and affinity chromatography were unsuccessful. One could argue that it was not what is present in serum, but what is lacking that was responsible for the inhibition of MeSAdo-dependent DNA synthesis. Daddona et al. (1980) observed that fibroblast cell strains ceased to grow in medium with 10% (v/v) dES and suggested that it may lack essential growth factors. However, it was also possible

that a factor in dES was having an inhibitory effect on cell growth. Since MeWo-LC1 cells could grow in SFT-medium and did not require the presence of serum for growth, it is unlikely that what was lacking in dES was responsible for the effect, but that it was some factor present in dES that caused the inhibition of DNA synthesis.

MeSAdo appears to be having both positive and negative effects on cell growth. In the presence of dES, the ability of MeSAdo to inhibit DNA synthesis dominates over its ability to act as a methylthio-source. This may explain why others have found that, although cells cannot grow in methionine-free medium supplemented with MeSAdo, they can nevertheless synthesize methionine from MeSAdo (Christa et al., 1984). Christa et al. (1988) suggested that the activity of the metabolic pathway converting MeSAdo to methionine was dependent on cell proliferation. This study using MeWo-LC1 cells would suggest that the pathway is active, but that its activity is masked by an inhibitory effect of MeSAdo that predominates in the presence of dES.

This study has found that factors present in serum can alter the MeSAdo-dependent synthesis of DNA while Hcy-dependent DNA synthesis is unaffected. Cells which contain MeSAdo phosphorylase activity and likely make methionine from MeSAdo (as shown by Christa et al., 1984), but still cannot grow when methionine is replaced by the nucleoside in the medium, are likely subject to the inhibitory effect of

MeSAdo. Cells are capable of DNA synthesis in SFT-medium since the inhibitory effect of MeSAdo is not as pronounced in this medium, but is enhanced in the presence of serum. As a consequence, in the presence of serum, the ability of MeWo-LC1 cells to use MeSAdo as a methylthio-source appears to be masked by the inhibitory effect of the nucleoside, which predominates.

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APPENDIX

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