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5-Amino-4-Imidazolecarboxamide Ribotide And Metabolites: Accumulation During Methotrexate Treatment And Metabolic And Cytotoxic Effects.

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5-AMINO-4-IMIDAZOLECARBOXAMIDE RIBOTIDE AND METABOLITES: ACCUMULATION DURING METHOTREXATE TREATMENT AND METABOLIC AND CYTOTOXIC EFFECTS

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title 5-Amino-4-imidazolecarboxamide Ribotide and Metabolites: Accumulation During Methotrexate Treatment and Metabolic and Cytotoxic Effects

An intermediate of purine de novo biosynthesis, 5-amino-4-imidazolecarboxamide ribotide (ZMP), and its metabolites (Z-metabolites) accumulate during methotrexate (MTX) treatment in vivo and in vitro. We have evaluated whether the immunosuppressive effect of "low-dose" MTX treatment is mediated by these metabolites. In vitro effect of Z-metabolites and MTX was tested on the inhibition of cell growth and the activities of two enzymes; S-adenosyl-L-homocysteine (SAH) hydrolase and adenosine deaminase (ADA).

SAH hydrolase and ADA were competitively inhibited by 5-amino-4-imidazolecarboxamide riboside (Z-riboside), and both Z-riboside and ZMP irreversibly inactivated SAH hydrolase. However, MTX did not inhibit SAH hydrolase and ADA in a cell-free system.

When cultured human T-lymphoblasts (MOLT-4) and B-lymphoblasts (Ramos) were exposed to Z-riboside, cell growth and SAH hydrolase activity were decreased in a dose-dependent fashion. These inhibitory effects were greater in

MOLT-4 than in Ramos cells. Inhibition of ADA by Z-riboside was not detected in cell culture.

When MOLT-4 and Ramos cells were exposed to "low-dose" MTX (0.01 or 0.02 μM), the growth of MOLT-4 cells, but not of Ramos cells, was inhibited. Z-riboside potentiated the toxic effect of "low-dose" MTX in cultured MOLT-4 cells.

When MOLT-4 cells were exposed to "low-dose" MTX, ZMP accumulated, and the activities of ZMP transformylase and SAH hydrolase were inhibited, but these effects were not observed in Ramos cells. When MOLT-4 cells were treated with "high-dose" MTX (0.1 μM), ZMP did not accumulate, and inhibition of SAH hydrolase was not detected. In contrast to MOLT-4 cells, "high-dose" MTX induced ZMP accumulation and inhibition of SAH hydrolase in Ramos cells.

Our results indicate that MTX is more toxic to T-lymphoblasts than to B-lymphoblasts. Our data suggest that the accumulation of ZMP by MTX treatment is responsible for the inhibition of SAH hydrolase in T-lymphoblasts and may be one of the immunosuppressive mechanisms producing efficacy of "low-dose" MTX in treating autoimmune/inflammatory diseases.

Abstract Approved by: Committee Chairman Joseph E Baggott

Program Director

Date _____ Dean of Graduate School W. A. Sibley

DEDICATION

This dissertation is dedicated to the memory of my parents,

Yong-Moo Hyun and Kyung-Sook Kim.

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LIST OF ABBREVIATIONS

ADA	adenosine deaminase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
APRT	adenine phosphoribosyltransferase
ATP	adenosine triphosphate
BSA	bovine serum albumin
5,10-CH^+-H_4PteGlu	5,10-methenyltetrahydrofolic acid
EDTA	ethylenediaminetetraacetic acid
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
fGAR	5'-phosphoribosyl-N-formylglycinamide
fZMP	5-formamino-4-imidazolecarboxamide ribotide
GAR	5'-phosphoribosylglycinamide
GAR transformylase	5'-phosphoribosylglycinamide formyltransferase
GMP	guanosine monophosphate
5-HCO-H_4PteGlu	folinic acid
10-HCO-H_2PteGlu	10-formyldihydrofolic acid
10-HCO-H_4PteGlu	10-formyltetrahydrofolic acid
10-HCO-PteGlu	10-formylfolic acid
HGPRT	hypoxanthine guanine phosphoribosyltransferase

LIST OF ABBREVIATIONS (Continued)

HPLC	high performance liquid chromatography
H_2PteGlu	dihydrofolic acid
H_4PteGlu	tetrahydrofolic acid
IMP	inosine monophosphate
k	first order rate constant
K_i	inhibitor binding constant
K_{INACT}	binding constant for inactivation site
K_m	Michaelis constant
MTX	methotrexate
$MTXGlu_n$	methotrexate polyglutamates
pABG	p-aminobenzoyl glutamate
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PNP	purine nucleoside phosphorylase
PRPP	5-phosphoribosyl-1-pyrophosphate
PteGlu	folic acid
RA	rheumatoid arthritis
SAM	S-adenosyl-L-methionine
SAH	S-adenosyl-L-homocysteine
SCID	severe combined immunodeficiency disease
SEM	standard error of the mean
sZMP	5-amino-4-imidazole-N-succinocarboxamide ribotide
sZ-riboside	5-amino-4-imidazole-N-succinocarboxamide riboside

LIST OF ABBREVIATIONS (Continued)

Tris	tris(hydroxymethyl)-aminomethane
V_{Imax}	maximal rate of inactivation
V_{max}	maximum velocity
Z-base	5-amino-4-imidazolecarboxamide
ZDP	5-amino-4-imidazolecarboxamide riboside diphosphate
Z-metabolites	metabolites containing the structure of 5-amino-4-imidazolecarboxamide
ZMP	5-amino-4-imidazolecarboxamide ribotide
ZMP transformylase	5-amino-4-imidazolecarboxamide ribotide formyltransferase
Z-riboside	5-amino-4-imidazolecarboxamide riboside
ZTP	5-aminoimidazole-4-carboxamide riboside triphosphate

INTRODUCTION

Some compounds possessing immunosuppressive effects (e.g., azathioprine, methotrexate) or anti-inflammatory activity (e.g., sulfasalazine, sulindac, naproxen) have been found to inhibit 5-amino-4-imidazolecarboxamide ribotide formyltransferase (EC 2.1.2.3, ZMP transformylase), a folate-dependent enzyme in purine de novo biosynthesis (Baggott et al. 1986; Ha et al. 1990; Baggott et al. 1992b). These findings suggest that a common property of the agents which are useful in the treatment of autoimmune/ inflammatory diseases is their ability to inhibit this enzyme. Inhibition of ZMP transformylase causes accumulation of ZMP and other metabolites containing the structure of 5-amino-4-imidazolecarboxamide (Z-metabolites), and these metabolites have been shown to accumulate as a result of methotrexate (MTX) treatment (tables 1 and 2). The antifolate, MTX, in "low-dose" (5-25 mg/week) has been used for the treatment of a variety of autoimmune/inflammatory diseases, such as rheumatoid arthritis (RA), psoriasis, and inflammatory bowel disease (for reviews: Hanno et al. 1980; Kremer 1990; Morgan and Baggott 1991). However, the mecha-nism(s) by which "low-dose" MTX affects immune function is not well understood.

Table 1. Effect of MTX on ZMP accumulation in vitro

Cell type	MTX treatment		Increase in ZMP accumulation	References
	Conc. (μM)	Duration		
MTX-resistant E.coli	40	35 min	14 fold	Tomisek et al. (1958a)
	100	35 min	95 fold	
S.typhimurium	800	50 min	19 fold	Bochner and Ames (1982)
Human T-lymphoblasts	0.02	24 hr	18 fold	Bokkerink et al. (1986)
	0.2	24 hr	*	
Human B-lymphoblasts	0.02	24 hr	5 fold	Bokkerink et al. (1988)
	0.2	24 hr	*	
Human breast cancer cell	10	5 hr	2.3 fold	Allegra et al. (1987)
Mouse leukemia cells	25	2 hr	2 fold	Lyons and Christopherson (1991)

* : No increase in ZMP accumulation

Table 2. Effect of MTX on Z-base excretion

Subjects	MTX treatment	Increase in Z-base excretion [a]	References
Acute leukemia patients	2.5 mg/day	2-5 fold	Luhby and Cooperman (1962)
Acute leukemia patients	(not described)	3-4 fold	Lulenski et al. (1970)
Healthy rats	1.5-5 mg/kg [b]	2-7 fold	McGeer and McGeer (1963)
	12 mg/kg [c]	*	

a : Increase above normal base line levels
b : Single dose
c : Cumulative dose for 3 days
* : No increase in Z-base excretion

Baggott et al. (1993) proposed a hypothesis that the effectiveness of "low-dose" MTX is mediated through the accumulation of Z-metabolites which produce an immunosuppressed condition similar to that found in adenosine deaminase (EC 3.5.4.4, ADA) deficiency. ADA deficiency also causes secondary inhibition of S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1, SAH hydrolase) by relatively high levels of adenosine and deoxyadenosine (Kredich and Hershfield 1989) (figure 1).

The purpose of this study is to investigate the effects of MTX on accumulation of Z-metabolites and activity of ZMP transformylase, and the effects of Z-metabolites and MTX on the activities of SAH hydrolase and ADA in both a cell-free system and a cell-culture system.

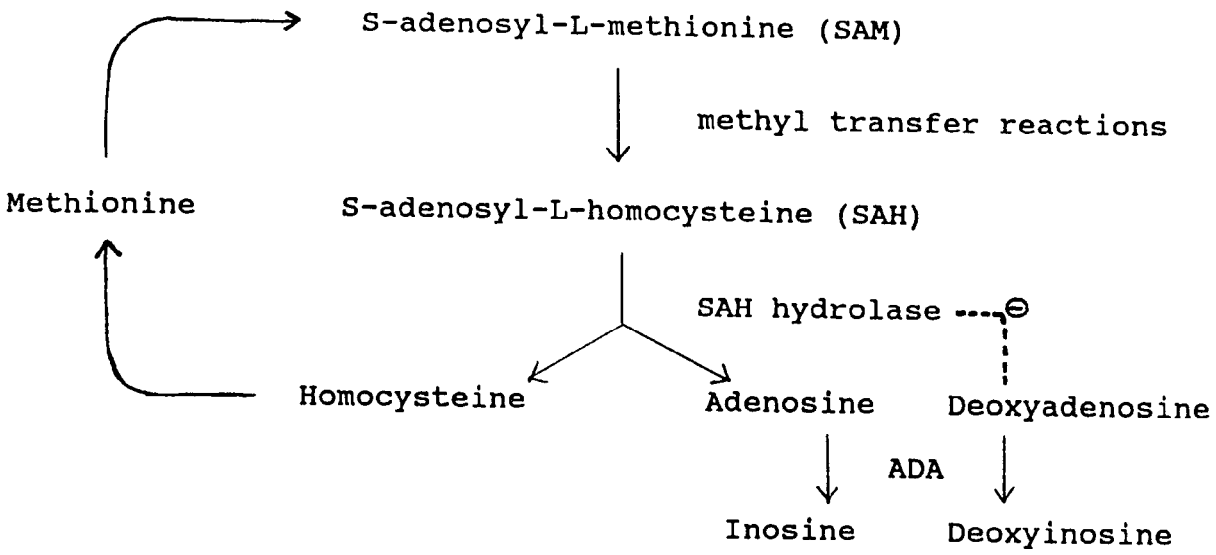


Figure 1. Secondary inhibition of SAH hydrolase in ADA deficiency.

REVIEW OF LITERATURE

Purine metabolism in lymphocytes

Genetic deficiencies of the purine metabolizing enzymes, adenosine deaminase (ADA) and purine nucleosides phosphorylase (PNP), are associated with defects in immune functions (Giblett et al. 1972; Giblett et al. 1975). These discoveries have established the importance of normal purine metabolism in immune functions.

Purine nucleotides are synthesized by the de novo pathway from low-molecular-weight precursors or by the salvage pathways from purine bases or purine nucleosides. The de novo pathway consists of a stepwise addition of individual atoms to phosphoribosyl pyrophosphate (PRPP) to synthesize the purine ring skeleton (figure 2). There are two folate-dependent transformylases in this pathway. Phosphoribosylglycinamide formyltransferase (EC 2.1.2.2, GAR transformylase) catalyzes the incorporation of a formyl group from 10-formyltetrahydrofolic acid (10-HCO-H_4PteGlu) into position 8 of the purine ring, and 5-amino-4-imidazolecarboxamide ribotide formyltransferase (EC 2.1.2.3, ZMP transformylase) catalyzes a similar incorporation into position 2 (figure 2).

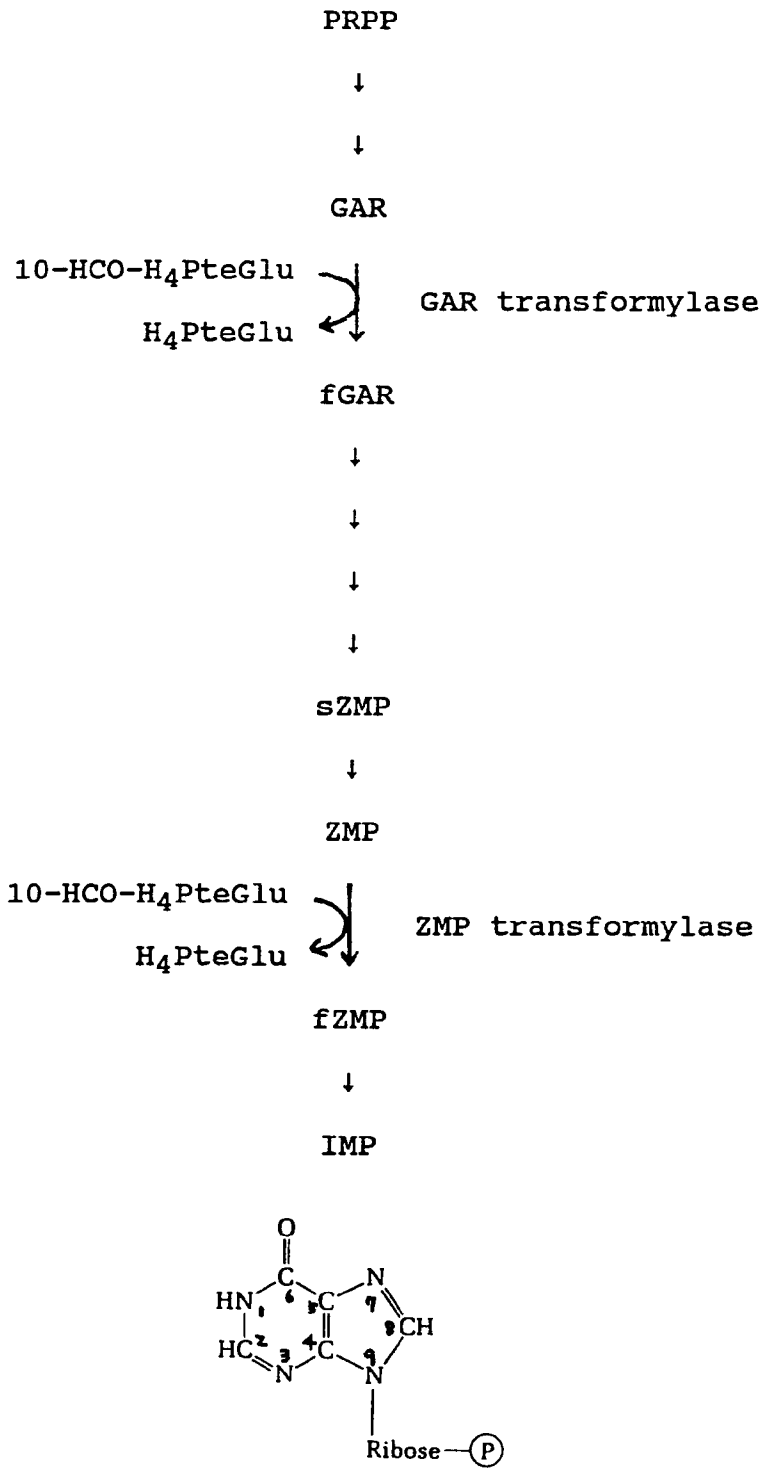


Figure 2. Purine de novo biosynthetic pathway and purine numbering system.

The salvage pathways from purine bases are catalyzed by two phosphoribosyl transferases: adenine phosphoribosyltransferase (APRT; reaction 1) and hypoxanthine guanine phosphoribosyltransferase(HGPRT; reactions 2 and 3).

$$\text{Adenine} + \text{PRPP} \rightarrow \text{AMP} + \text{PPi} \quad (1)$$

$$\text{Guanine} + \text{PRPP} \rightarrow \text{GMP} + \text{PPi} \quad (2)$$

$$\text{Hypoxanthine} + \text{PRPP} \rightarrow \text{IMP} + \text{PPi} \quad (3)$$

The second type of salvage reaction utilizing purine nucleosides is catalyzed by adenosine kinase (reaction 4).

$$\text{Adenosine} + \text{ATP} \rightarrow \text{AMP} + \text{ADP} \quad (4)$$

The relative contributions to purine synthesis by the de novo and salvage pathways differ in various cell types. In an earlier study, Lajtha and Vane (1958) suggested that bone marrow cells are dependent upon the salvage pathways by showing no incorporation of [^{14}C] formate into a purine base (adenine) of bone marrow cells when the liver, a major site of de novo purine synthesis, was removed in rabbits. However, more recently, Deacon et al. (1985) demonstrated that the specific activities of two transformylases in de novo purine biosynthesis are several-fold greater in rat bone marrow than in liver, suggesting that bone marrow is an active site of de novo biosynthesis. This observation has been confirmed by other investigators who have demonstrated that de novo biosynthesis is active in human lymphoid cells (Brosh et al. 1976; Hovi et al. 1977; Huisman et al. 1979; Nishida et al. 1980; Gordon et al. 1982; King et al. 1983; McCairns et al. 1983). Allison et al. (1975) reported that

the de novo pathway is important for normal functioning of human lymphocytes. Several aspects of normal T-lymphocyte function, such as delayed hypersensitivity, peripheral blood T-lymphocyte counts, and lymphocyte responses to phytohemagglutinin, were normal in patients with Lesch-Nyhan syndrome, an inborn error of metabolism in which the purine salvage pathway is not functioning due to HGPRT deficiency. In contrast, abnormal B-lymphocyte functions, such as low peripheral blood B-lymphocyte counts, low serum IgG levels, and subnormal responses to pokeweed mitogen, were demonstrated in these patients. These results suggest that the salvage pathway catalyzed by HGPRT is not required for the proliferation and functions of human T-lymphocytes, but is essential for the normal function of human B-lymphocytes. Allison et al. (1975) also demonstrated that azaserine, an inhibitor of the de novo pathway of purine biosynthesis, suppressed the response of T- and B-lymphocytes isolated from normal people to phytohemagglutinin and pokeweed mitogen, respectively. This observation indicates that the de novo pathway is required for normal function of both T- and B-cells.

A number of pathways for metabolism of purine nucleosides exist in lymphocytes (table 3). Purine nucleosides are formed from the corresponding nucleotides by 5'-nucleotidase or by nonspecific phosphatase (Newby 1980). Adenosine and deoxyadenosine are deaminated by ADA to inosine and deoxyinosine, respectively. PNP catalyzes the

Table 3. Enzymes for metabolizing purines

Adenosine deaminase (EC 3.5.4.4; ADA)

Adenosine	→	Inosine
Deoxyadenosine	→	Deoxyinosine

Purine nucleoside phosphorylase (EC 2.4.2.1; PNP)

Inosine	→	Hypoxanthine
Deoxyinosine	→	Hypoxanthine
Guanosine	→	Guanine
Deoxyguanosine	→	Guanine

Hypoxanthine guanine phosphoribosyltransferase (EC 2.4.2.8; HGPRT)

Hypoxanthine	→	IMP
Guanine	→	GMP

Adenine phosphoribosyltransferase (EC 2.4.2.7; APRT)

Adenine	→	AMP

Adenosine kinase (EC 2.7.1.20)

Adenosine	→	AMP

catabolism of inosine and deoxyinosine to the nucleobase, hypoxanthine. In human lymphocytes, the specific activity of PNP is higher than that of ADA (Van der Weyden and Bailey 1978; Nishida et al. 1980; North et al. 1980; Peters et al. 1981). Adenosine can also be phosphorylated by adenosine kinase in lymphocytes. Although the K_m (Michaelis constant) of adenosine kinase is lower than that of ADA, ADA has a substantially higher V_{max} (maximum velocity) (Seegmiller 1985). This indicates that ADA is needed for the rapid removal of excess adenosine. Deoxyadenosine is predominantly deaminated, since the kinase has a relatively low V_{max} and a high K_m for deoxyadenosine (Peters et al. 1981).

In summary, normal purine metabolism is essential for immune functions. Genetic deficiency of ADA impairs the function of both T- and B-lymphocytes whereas genetic deficiency of the next enzyme in the pathway, PNP, results in a defect in T-lymphocyte function with either normal or hyperactive B-lymphocyte function (Kredich and Hershfield 1989). However, the mechanisms by which these enzyme deficiencies lead to immunodeficiency diseases are not clearly understood.

Adenosine deaminase (ADA) deficiency

ADA is widely distributed in most human tissues with the highest activity in the thymus and other lymphoid tissues, such as spleen and circulating lymphocytes (Van der Weyden and Kelley 1976; Adams and Harkness 1976). A relationship between ADA deficiency and severe combined

immunodeficiency disease (SCID) was first identified by Giblett et al. (1972) who proposed the importance of ADA in immune functions. Approximately 85% of children with ADA deficiency demonstrate symptoms of SCID including recurrent bacterial, viral, and fungal infections, and diarrhea. These children have small tonsils and lymph nodes, and decreased numbers of circulating lymphocytes (Carson and Carrera 1990).

In ADA deficiency, the plasma concentrations of adenosine and deoxyadenosine, as well as the urinary excretion of deoxyadenosine, are elevated (Kredich and Hershfield 1989). In in vitro studies, adenosine and deoxyadenosine inhibit growth of a variety of cell types, including lymphoid cells (Henderson and Scott 1980; Henderson et al. 1980). One mechanism proposed to explain the growth inhibitory effects of these metabolites is that a secondary inhibition of S-adenosyl-L-homocysteine (SAH) hydrolase occurs when adenosine and deoxyadenosine accumulate (Kredich and Hershfield 1989).

SAH hydrolase catalyzes the reversible hydrolysis of SAH to adenosine and homocysteine (figure 1). Although the reaction favors synthesis of SAH in vitro, the metabolic flow is in the hydrolytic direction in vivo due to rapid metabolism of adenosine and homocysteine (De la Haba and Cantoni 1959). In ADA deficiency, excess adenosine drives the reaction catalyzed by SAH hydrolase to the direction of SAH formation (Kredich and Martin 1977). In addition,

deoxyadenosine irreversibly inactivates SAH hydrolase (Hershfield 1979). Therefore, the activity of SAH hydrolase appears to be regulated by levels of adenosine and deoxyadenosine which are controlled by ADA. This relationship has been confirmed in vivo by Hershfield et al. (1979) who demonstrated that SAH hydrolase activity of erythrocytes of ADA-deficient children was less than 2% of controls.

In eukaryotes, SAH hydrolase is the only enzyme that uses SAH as a substrate, therefore, inhibition of SAH hydrolase essentially traps SAH (Walker and Duerre 1975). SAH is a potent product inhibitor of a number of S-adenosyl-L-methionine (SAM)-dependent transmethylations (Ueland 1982a). SAM is a methyl donor in a large variety of methylation reactions including synthesis of DNA (Brown and Attardi 1965), mRNA (Perry and Kelley 1974), tRNA (Kerr and Borek 1972), rRNA (Greenberg and Penman 1966), proteins (Paik and Kim 1975), and a variety of small molecules (Greenberg 1963). Since SAH inhibits SAM-dependent transmethylases, Deguchi and Barchas (1971) suggested that changes in the intracellular concentrations of SAH and the activity of SAH hydrolase might play a key role in controlling transmethylations. Inhibition of methylation reactions and subsequent changes in biological phenomena have been observed by altering the intracellular concentrations of SAH, or the ratio of SAM/SAH (Cantoni 1985).

Specific ADA inhibitors such as deoxycoformycin and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) have been used

to investigate the mechanisms by which ADA deficiency leads to immunodeficiency. Hershfield et al. (1983) showed that deoxycoformycin inhibited SAH hydrolase, elevated the levels of SAH, and inhibited RNA methylation in the lymphoblasts of patients. Treatment of lymphoid cell lines with EHNA and adenosine caused sufficient accumulation of SAH to inhibit methylation of RNA and DNA (Kredich and Martin 1977; Kredich and Hershfield 1979). Addition of adenosine to EHNA-treated phytohemagglutinin-stimulated human peripheral lymphocytes also resulted in SAH accumulation and inhibition of DNA methylation (Johnston and Kredich 1979). Treatment of isolated human monocytes and polymorphonuclear leukocytes with EHNA plus adenosine and L-homocysteine increased SAH levels, and subsequently decreased protein carboxy O-methylation and chemotactic responsiveness (Pike et al. 1978; Snyderman et al. 1980).

An inhibitor of SAH hydrolase, 3-deazaadenosine, also suppresses several immune functions in vitro, including chemotaxis (Aksamit et al. 1983), phagocytosis (Leonard et al. 1978; Sung and Silverstein 1985), antibody-dependent cytotoxicity (Medzihradsky 1984), lymphocyte-mediated cytolysis (Zimmerman et al. 1978), and interleukin-1 synthesis (Schmidt et al. 1990). However, as Schmidt et al. (1990) stated, these alterations in immune functions may not be due solely to changes in transmethylation reactions. Recently, Wolos et al. (1993) demonstrated that an irreversible

inhibitor of SAH hydrolase, MDL 28,842, inhibited proliferation of human mononuclear cells treated with concanavalin A and pokeweed mitogen in vitro and suppressed antibody response to a T-cell-dependent agent in vivo. In addition, the intracellular accumulation of SAH inhibits the growth of cultured human lymphoblasts (Kredich and Hershfield 1979; Palella et al. 1982). Considering all the findings mentioned above, it is reasonable to assume that the immune suppression associated with inhibition of SAH hydrolase results from the inhibition of SAM-mediated methylation reactions.

5-amino-4-imidazolecarboxamide ribotide (ZMP) accumulation during methotrexate (MTX) treatment

The antifolate, methotrexate (MTX), has been widely used in the treatment of neoplastic and non-neoplastic diseases (Morgan and Baggott 1991). The primary action of MTX is to inhibit dihydrofolate reductase which catalyzes the reductions of folic acid (PteGlu) to dihydrofolic acid (H_2PteGlu) and H_2PteGlu to tetrahydrofolic acid (H_4PteGlu) (Osborn et al. 1958). The function of H_4PteGlu coenzyme is to transfer one carbon units in the biosynthesis of pyrimidines, purines, and some amino acids. As a result of the reaction catalyzed by thymidylate synthase, H_4PteGlu is converted to H_2PteGlu, which must be converted back to H_4PteGlu to regain its ability to transfer one carbon units. Therefore, inhibition of dihydrofolate reductase by MTX results in a depletion of H_4PteGlu and a subsequent reduction of DNA synthesis.

MTX undergoes intracellular polyglutamylation (Baugh et al. 1973). In contrast to the monoglutamyl form of MTX, which rapidly leaves the cell after the extracellular concentration of MTX decreases, MTX polyglutamates ($MTXGlu_n$) remain within the cell (Jolivet et al. 1983). Retention of $MTXGlu_n$ in the cell is influenced by polyglutamate chain lengths. The chain lengths are dependent on both the concentration of MTX and the duration of MTX exposure (Jolivet et al. 1983). $MTXGlu_n$ not only inhibit dihydrofolate reductase (Jacobs et al. 1975), but also inhibit other folate-dependent enzymes including thymidylate synthase (Allegra et al. 1985a), ZMP transformylase (Allegra et al. 1985b; Baggott et al. 1986) and GAR transformylase (Allegra et al. 1985c). Since ZMP transformylase has a lower K_i (inhibitor binding constant) for $MTXGlu_n$ than GAR transformylase (Allegra et al. 1985c), ZMP accumulates more readily than GAR after MTX treatment (Tomisek et al. 1958a). Polyglutamates of $H_2PteGlu$, which accumulate in MTX-treated cells (White and Goldman 1976), also inhibit ZMP transformylase (Allegra et al. 1985b; Baggott et al. 1986).

Several studies showing that MTX causes ZMP accumulation are summarized in tables 1 and 2. Tomisek et al. (1958a) reported that ZMP accumulated in an MTX-resistant mutant of E.coli exposed to 40 μM MTX, a concentration sufficient to inhibit the growth of this mutant. However, formyl GAR production was not inhibited at this concentration and was only partially inhibited at 800 μM MTX. The

same group of investigators also showed that ZMP accumulated in the intestine of MTX-treated leukemic mice (Tomisek et al. 1958b). Bochner and Ames (1982) found that ZMP, 5-amino-4-imidazolecarboxamide riboside triphosphate (ZTP), and 5-amino-4-imidazole-N-succinocarboxamide ribotide (sZMP) accumulated when Salmonella typhimurium was exposed to 800 μM MTX for 50 min.

Bokkerink et al. (1986; 1988) demonstrated that 0.02 μM MTX increased intracellular ZMP levels after 24 hr in cultured human T- and B-lymphoblasts, while incubation with 0.2 μM MTX did not increase ZMP levels. These results suggest that this higher concentration of MTX completely inhibits GAR transformylase, hence preventing ZMP synthesis. Allegra et al. (1987) observed an accumulation of ZMP during a 5-hr incubation in cultured human breast cancer cells exposed to 10 μM MTX. Similarly, Lyons and Christopherson (1991) showed that 25 μM MTX induced an accumulation of ZMP during a 2-hr incubation in mouse leukemic cells.

Daily urinary excretion of 5-amino-4-imidazolecarboxamide (Z-base), a product of ZMP degradation, is constant in humans (McGeer et al. 1961). Luhby and Cooperman (1962) reported that urinary excretion of Z-base increased after administration of a daily dose of 2.5 mg MTX to acute leukemia patients. Lulenski et al. (1970) also demonstrated that urinary Z-base excretion was elevated in acute leukemia patients treated with MTX, while other antileukemic drugs had no such effect. McGeer and McGeer (1963) showed

increased excretion of Z-base after a single dose of MTX (1.5-5 mg/kg body weight) in rats. When rats were given 2.5 mg/kg for two days followed by 7 mg/kg on the third day, excretion of Z-base increased for the first two days only, suggesting that the higher cumulative dose of MTX inhibits GAR transformylase, preventing ZMP accumulation.

"Low-dose" MTX: relationship between dose and efficacy

Administration of "low-dose" MTX (5-25 mg/week) has been used for the treatment of rheumatoid arthritis (RA) (Kremer 1990). However, only a few investigators have evaluated the efficacy of various doses of MTX within this range (Thompson et al. 1984; Furst et al. 1989). Furst et al. (1989) showed a dose-dependent therapeutic effect of MTX at 5 mg/m^2/week versus 10 mg/m^2/week (about 8 mg/week versus 15 mg/week). However, Thompson et al. (1984) failed to demonstrate an improvement of efficacy of 25 mg/week compared with 10 mg/week of MTX in RA patients. In this study, 10 mg/week were more effective than 25 mg/week for the improvement of some clinical variables tested.

Baggott et al. (1992a) found that a dose of 25 mg/kg/week of MTX is better than 100 mg/kg/week in suppressing indices of bone and cartilage erosion in MRL/lpr mice, which are considered to be a suitable model of human RA. Galivan et al. (1986) demonstrated that 150 μg/kg/week of MTX suppressed inflammation associated with adjuvant arthritis in rats, but increasing the dose to 600 μg/kg/week reduced therapeutic effects. Khan et al. (1991) also showed

that when various doses of MTX were given to donor rats 24 hr before cardiac transplantation, recipient rats survived significantly longer after 100 mg/kg body weight of MTX was given rather than 400 or 1,000 mg/kg body weight. These results, therefore, suggest that there is an optimal dose of MTX for the treatment of various diseases and that effectiveness actually decreases when the dose exceeds this optimal level.

The findings above are consistent with the hypothesis that an optimal dose of MTX causes accumulation of Z-metabolites which play a role in the action of MTX. Since Z-metabolites have a chemical structure which resembles the structure of adenine-metabolites, it is possible that they act as adenosine antagonists possibly by interfering with adenosine metabolizing enzymes, SAH hydrolase and ADA.

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Accumulation of 5-Amino-4-imidazolecarboxamide ribotide (ZMP) and 5-amino-4-imidazolecarboxamide riboside (Z-riboside) during "low-dose" methotrexate (MTX) treatment causes inhibition of S-adenosyl-L-homocysteine (SAH) hydrolase and adenosine deaminase (ADA) in cultured human T- and B-lymphoblasts.

Specific aims

In order to test this hypothesis, the following studies were performed:

1. Z-riboside was tested as an inhibitor of SAH hydrolase and ADA in a cell-free system.
2. MTX was tested as an inhibitor of SAH hydrolase and ADA in a cell-free system.
3. Z-riboside and ZMP were tested as irreversible inactivators of SAH hydrolase.
4. Z-riboside was tested for its effect on the growth of human T- and B-lymphoblasts and its ability to potentiate MTX toxicity in a cell-culture system.
5. MTX was tested for its ability to cause accumulation of Z-riboside and ZMP in cultured human T- and B-lymphoblasts.

6. MTX was tested for its ability to inhibit ZMP transformylase activity in cultured human T- and B-lymphoblasts. ZMP transformylase activity was measured using a newly developed colorimetric assay.

7. Z-riboside and MTX were tested for their abilities to inhibit SAH hydrolase of human T- and B-lymphoblasts in cell culture.

8. Z-riboside and MTX were tested for their abilities to inhibit ADA of human T- and B-lymphoblasts in cell culture.

MATERIALS AND METHODS

Materials

Two cell lines, MOLT-4 (T-cell line; ATCC #1582) and Ramos (B-cell line; ATCC #1596), which had been purchased from the American Type Culture Collection (Bethesda, MD), were obtained from Dr. Peter D. Burrow's laboratory (Department of Microbiology, University of Alabama at Birmingham). The MOLT-4 cell line was originally derived from a patient with acute lymphoblastic leukemia, and the Ramos cell line was from a patient with Burkitt lymphoma.

RPMI 1640 medium, fetal bovine serum, penicillin, streptomycin were purchased from GIBCO BRL (Gaitherburg, MD). Methotrexate (MTX), 5-amino-4-imidazolecarboxamide riboside (Z-riboside), and S-adenosyl-L-homocysteine (SAH) were obtained from Sigma Chemical Co. (St. Louis, MO), and 5-amino-4-imidazolecarboxamide ribotide (ZMP) was from Boehringer Manheim (Manheim, Germany). L-5-formyltetrahydrofolic acid (L-5-HCO-H_4PteGlu) was a gift from Lederle Laboratories (Pearl River, NY).

SAH hydrolase purified from rabbit erythrocytes and [8-^{3}H] adenosine (20 Ci/mmol) and [8-^{14}C] adenosine (0.36 mCi/mmol) were purchased from Sigma Chemical Co. Deoxycoformycin (pentostatin) was obtained from Parke-Davis

(Ann Arbor, MI). All other chemicals used in this study were reagent grade or higher.

Cell culture

MOLT-4 and Ramos cells were grown in RPMI 1640 media containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM mercaptoethanol, and 2 mM glutamine. The cultures were incubated at 37°C in a controlled atmosphere containing 5% CO_2/95% air.

The protocol used for cell growth studies is shown in figure 3. Cells were suspended in fresh media at a density of 0.3 X 10^6 cells/ml, and 3 ml of cell suspension were transferred to each well of 24-well plates (Corning, Houston, TX). After a 24-hr incubation, MTX was added to final concentrations of 0, 0.01, or 0.02 μM, and 8 hr later, Z-riboside was added to final concentrations of 0, 100, or 200 μM. Both MTX and Z-riboside were dissolved in media and added to the cell culture media in a small volume (1/100 fraction). After 24 and 48 hr of MTX exposure, the number of viable cells, determined by trypan blue exclusion, and total cells were counted with a hemocytometer after appropriate dilution. The cell number in each treatment was calculated as percent of control (i.e., without MTX and Z-riboside in the media).

In order to test the effects of Z-riboside and MTX on ZMP accumulation and enzyme activities, cells were suspended in fresh media at a density of approximately 1.5 X 10^6 cells/ml, and 100 ml of cell suspension were transferred to

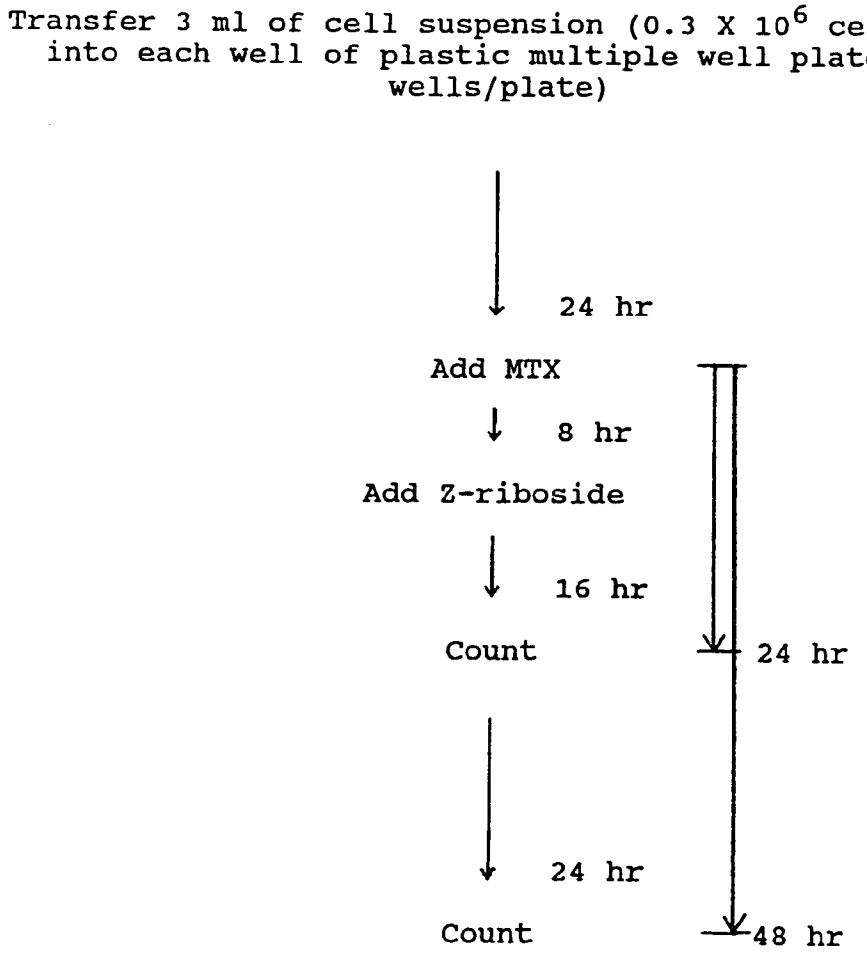


Figure 3. Protocol for measuring the effects of MTX and Z-riboside on the growth of MOLT-4 and Ramos cells in culture.

each cell culture flask (figure 4). After 24 hr, Z-riboside was added to final concentrations of 0, 30, 100, 200 μM or MTX to final concentrations of 0, 0.01, 0.02, 0.1 μM. After a 24-hr incubation, these cells were processed as described below.

Determination of intracellular ZMP and Z-riboside levels

Sample preparation The procedure for preparing cell extracts for high performance liquid chromatography (HPLC) is summarized in figure 4. MOLT-4 or Ramos cells, incubated with various concentrations of MTX or Z-riboside as described above, were harvested by centrifugation (1,200 X g) for 10 min at 4°C. After washing three times with phosphate-buffered saline (PBS), the cells were suspended in PBS, disrupted using an ultrasonic cell disrupter (Model XL 2005, Heat Systems Incorporated, Farmingdale, NY) for 15 seconds on ice, and centrifuged (1,200 X g) for 10 min at 4°C to remove cell debris. An aliquot of the supernatant was removed for protein analysis, and the remaining supernatant was transferred to an Amicon Centrifree Micropartitioning Cartridge (No. 4104, Amicon Inc., Beverly, MA) and centrifuged (2,000 X g) to remove proteins with molecular weights above 30,000. The ultrafiltrate was stored at -70°C for future analyses.

Determination of intracellular ZMP levels using HPLC

Concentrations of ZMP were determined using an HPLC method described by Sabina et al. (1982) with slight modifications. HPLC was performed with an Isopure LC system (Perkin-Elmer

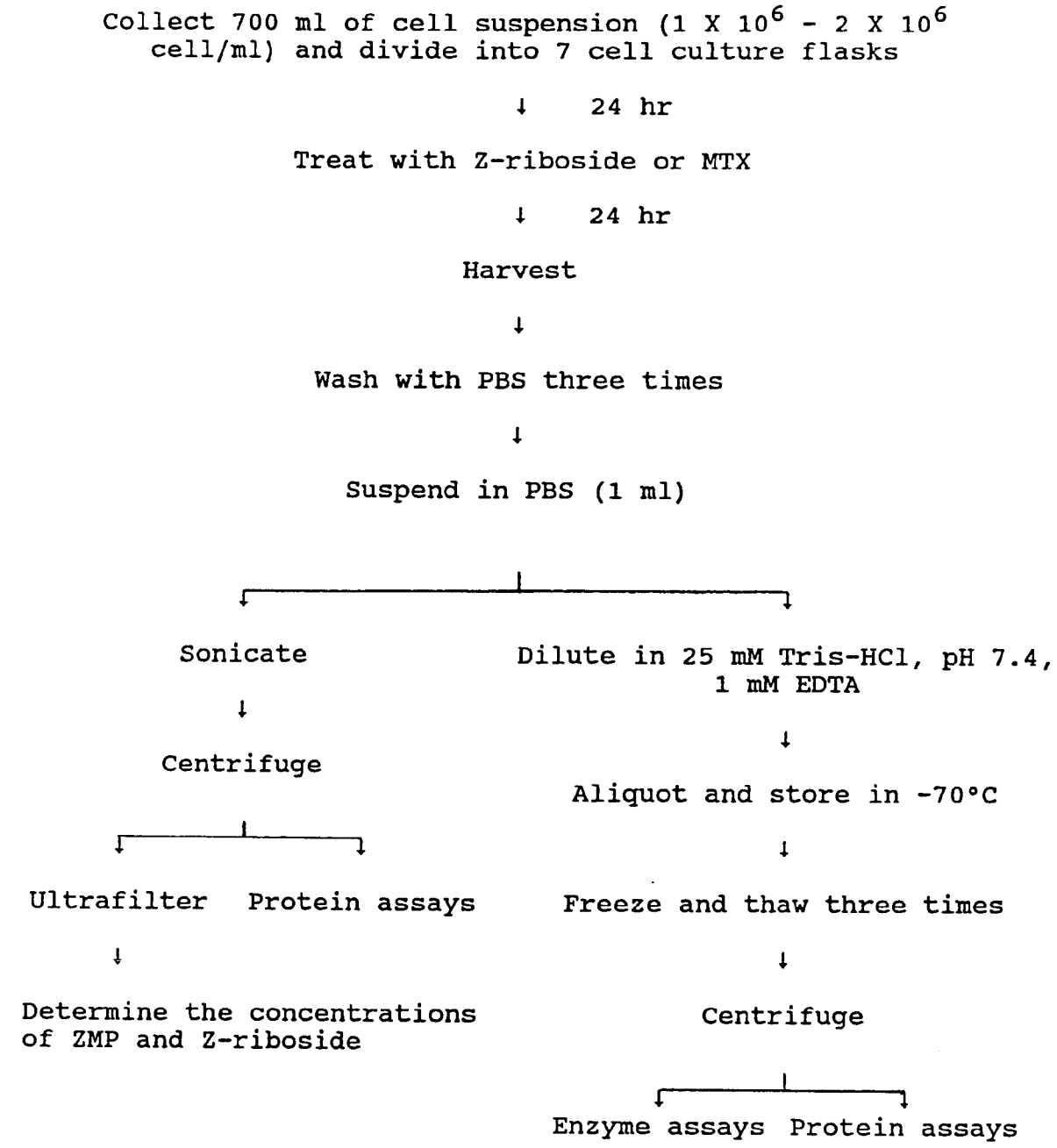


Figure 4. Procedure for making MOLT-4 and Ramos cell extracts.

Corporation, Norwalk, CT). Eluting compounds were monitored at 267, 254 and 280 nm with a Waters 490 Programmable Multiwavelength Detector (Millipore Corporation, Milford, MA). A computer program (Maxima 820; Millipore Corporation) was used for peak integration and to monitor retention time. In order to measure the peak area, peaks at 267 nm were cut out and weighed. Concentrations were calculated from peak area using a calibration curve.

A Partisil SAX ion-exchange column (10 μm, 250 X 4.6 mm; Alltech Associates Inc., Deerfield, IL) was used to seperate ZMP. The mobile phase consisted of 5 mM $NH_4H_2PO_4$, pH 2.8 (Buffer A) and 750 mM $NH_4H_2PO_4$, pH 3.9 (Buffer B). Buffer pH was adjusted with phosphoric acid. The buffers were filtered through a 0.22 μm Millipore filter (Alltech Associates Inc.) under negative pressure, and degassed by continuous helium purging during chromatography. Following a 50 μl sample injection, Buffer A was pumped isocratically for 5 min, and a linear gradient was developed over 10 min from 0% Buffer B to 25% Buffer B. After completion of the gradient, the gradient was returned to 100% Buffer A for 5 min, and the column was washed with Buffer A for 20 min before injecting next sample. The flow rate was 2.0 ml/min.

The peaks of ZMP were confirmed by coelution with authentic ZMP and by the UV ratios (267 vs. 280 nm and 267 vs. 254 nm), which agreed with the authentic compound. The retention time for ZMP was 8.0 min. The recovery was 82% when authentic ZMP was added to a final concentration of 20

μM to 0.2 ml of the cell suspension prior to sonication of the cells. Figure 5 shows an example of the calibration curve of authentic ZMP. The day-to-day variation of the measurement of authentic ZMP was determined by repeating the measurement on 4 different days, and the coefficient of variation (CV) was 7.7%. The detection limit was 0.1 nmol/mg soluble protein.

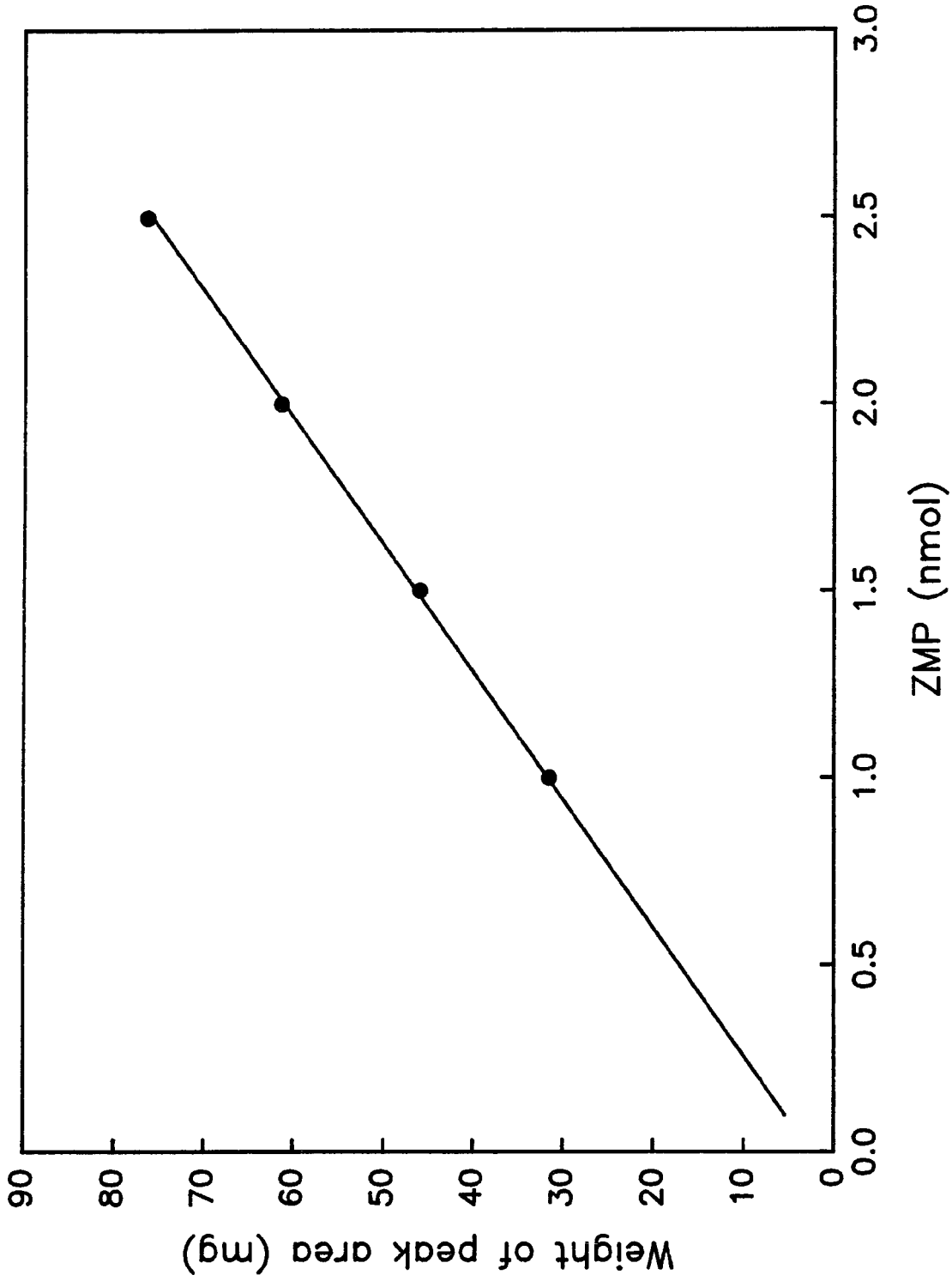
Determination of intracellular Z-riboside levels using HPLC

Concentrations of Z-riboside were determined using an HPLC method described by Sabina et al. (1985) with slight modifications. Reverse-phase HPLC was carried out with the same equipment described in the previous section except that a C_{18} column (5 μm, 250 X 4.6 mm; VYDAC, Hesperia, CA) was used. A 25 μl sample was injected on the column and was eluted isocratically with 50 mM potassium phosphate buffer, pH 6.35, at a flow rate of 1.0 ml/min. The retention time for Z-riboside was 8.5 min. When authentic Z-riboside was added to a final concentration of 10 μM to 0.36 ml of the cell suspension prior to sonication of the cells, the recovery was 105%. The detection limit was 0.1 nmol/mg soluble protein.

Another method described by Dixon et al. (1989) was tried to confirm the results. Reverse-phase HPLC using a C_{18} column (5 μm, 250 X 4.6 mm; Alltech Associates Inc., Deerfield, IL) was carried out with 112 Solvent Delivery Module (Beckman Instruments, Inc., Fullerton, CA). The

Figure 5. Calibration curve of authentic ZMP for HPLC ion-exchange chromatography.

Ten to 25 µl of 100 µM ZMP were injected on a Partisil SAX ion-exchange column (10 µm, 250 X 4.6 mm). The ZMP peak (retention time ≈ 8 min) monitored at 267 nm was cut out and weighed. The weight of each peak was plotted against the amount of ZMP injected. Chromatographic conditions: mobile phase, 5 mM $NH_4H_2PO_4$, pH 2.8, and 750 mM $NH_4H_2PO_4$, pH 3.9 (the Gradient is described in 'Materials & Methods'); flow rate, 2.0 ml/min.


Weight of peak area (mg)
0
10
20
30
40
50
60
70
80
90
0.0
0.5
1.0
1.5
2.0
2.5
3.0
ZMP (nmol)

mobile phase was 1.5% methanol in 10 mM $NH_4H_2PO_4$ (pH 3) at a flow rate of 1.0 ml/min. The peaks were monitored at 267 nm with a variable wavelength detector (LDC/Milton Roy, Riviera Beach, FL).

Determination of protein concentrations Protein concentrations were measured using a Bio-Rad microassay procedure (Richmond, CA) originally described by Bradford (1976). The mean soluble protein concentration was found to be 17.4 ± 2.3 $\mu g/10^6$ viable cells (mean ± SEM).

Preparation of enzyme sources

Preparation of cell extracts from cultured human lymphoblasts The procedure for preparing cell extracts for enzyme assays is summarized in figure 4. An aliquot of the cell suspension in PBS (before sonication) described in the previous section (page 25) was diluted using 25 mM Tris(hydroxymethyl)-aminomethane-HCl (Tris-HCl) buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA), and stored in aliquots at -70°C. Immediately before the enzyme assay, cells were disrupted by freezing and thawing three times, and then centrifuged (8,000 X g) for 10 min at 4°C. The supernatants were assayed for protein, and appropriate dilutions were made using 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA.

Separation of human peripheral blood mononuclear cells (PBMC) PBMC from patients with rheumatoid arthritis (RA) were separated using LeucoPREP (Becton Dickinson Labware, Lincoln Park, NJ). These RA patients had never been treated

with MTX. Ten ml of whole blood were transferred to a LeucoPREP tube and centrifuged (1,500 X g) for 20 min at room temperature. PBMC were collected and transferred to a 15 ml conical centrifuge tube. The cells were washed three times with 15 ml of PBS, suspended in PBS, and disrupted by freezing and thawing three times. Insoluble cell debris was removed by centrifugation (600 X g).

Purified SAH hydrolase from rabbit erythrocytes

Commercially available SAH hydrolase was diluted 100 times using 20% glycerol containing 25 mM Tris, pH 7.4, 1 mM dithioerythritol, and 1 mM EDTA. Final protein concentration of the diluted enzyme was 1.2 μg/ml.

Development of an assay for the determination of ZMP transformylase activity

Principle of the assay ZMP transformylase catalyzes the transfer of a formyl group from 10-HCO-H_4PteGlu to ZMP with the formation of 5-formamino-4-imidazolecarboxamide (fZMP) and H_4PteGlu as shown below.

$$\text{ZMP} + \text{10-HCO-}H_4\text{PteGlu} \rightarrow \text{fZMP} + H_4\text{PteGlu} \qquad (5)$$

The C-N^{10} bond of H_4PteGlu, but not of 10-HCO-H_4PteGlu, is cleaved by alkaline permanganate oxidation to form p-aminobenzoyl glutamate (pABG). The p-amino group of pABG is detected by the Bratton-Marshall assay (Bratton and Marshall 1939). In the Bratton-Marshall assay, pABG reacts with nitrite in acidic solution to form the diazonium salt. The excess nitrite is destroyed with ammonium sulfamate, and the diazonium salt is coupled with N-(1-naphthyl)ethylenediamine to give a stable purple dye.

Preparation of L-10-formyl-tetrahydrofolic acid L-10-formyltetrahydrofolic acid (L-10-HCO-H_4PteGlu) was prepared by the method of Rabinowitz (1963). Fifty milligrams of L-5-HCO-H_4PteGlu were dissolved in 3 ml of 1 M 2-mercaptoethanol, and the pH of the solution was adjusted to 1.5-1.6 with HCl. The solution was stored at 4°C overnight, and the precipitate which formed was washed twice with 250 µl of 5 mM HCl/10 mM 2-mercaptoethanol and dissolved in 5 ml of 5 mM HCl/10 mM 2-mercaptoethanol. An aliquot of the solution was diluted 10-fold, and the concentration of L-5,10-methenyltetrahydrofolic acid (L-5,10-CH^+-H_4PteGlu) was quantified using a molar extinction coefficient of 25.0 x 10^3 $M^{-1}cm^{-1}$ at 360 nm (Rabinowitz 1963) with a DMS 200 UV-visible Spectrophotometer (Varian Techtron Pty., Mulgrave, Australia). L-5,10-CH^+-H_4PteGlu was stored at -20°C. Immediately before the enzyme assay, it was converted into L-10-HCO-H_4PteGlu by adjusting the pH to 7.4 with 50 mM phosphate buffer.

Determination of ZMP transformylase activity in cultured human lymphoblasts ZMP transformylase activity was measured as described previously (Ha et al. 1990). Cell extracts were incubated at 37°C for 1.5-3 hr with 50 mM potassium phosphate buffer, pH 7.4, 10 mM 2-mercaptoethanol, 20 µM or 100 µM 10-HCO-H_4PteGlu, and 100 µM ZMP in a total volume of 500 µl. The enzyme reaction was terminated by adding 50 µl of 0.17 M $HgCl_2$ and 50 µl of 1 M ammonium bicarbonate, pH 9.2. Five min after the addition of 100 µl

of 2% (w/v) $KMnO_4$, 400 µl of 0.3% (v/v) H_2O_2 were added, and the tubes were centrifuged at 2,000 x g for 5 min. A portion (0.5 ml) of the supernatant was transferred to a test tube on ice, and 50 µl of 3 M NaCl, 300 µl of 5 M H_2SO_4, and 100 µl of 1% (w/v) $NaNO_2$ were added. After a 3-min incubation on ice, 100 µl of 5% (w/v) ammonium sulfamate was added. One min later, 100 µl of 1% (w/v) N-(1-naphthyl) ethylenediamine was added. The purple color was allowed to develop at room temperature overnight and the absorbance of the azo dye of pABG was measured at 560 nm.

Determination of SAH hydrolase activity

Principle of the assay SAH hydrolase activity is measured either in the hydrolytic or synthetic direction.

Adenosine + L-Homocysteine → SAH (6)

The fact that the equilibrium strongly favors synthesis over hydrolysis (De la Haba and Cantoni 1959) complicates the determination of SAH hydrolase in the hydrolytic direction. Measurements of this reaction require adenosine deaminase (ADA) in the assay system to remove the products since adenosine is an inhibitor of the hydrolytic reaction (Ueland 1982a). Therefore, most assays of SAH hydrolase have been performed in the synthetic reaction.

The activity in the synthetic direction was measured using the method described by Magnuson et al. (1984) with slight modifications. Enzyme is incubated with homocysteine and radioactive adenosine as substrates. An ADA inhibitor (deoxycoformycin) is included in the assay mixture to pre-

vent deamination of adenosine to inosine. The amount of radioactive SAH formed is measured after SAH is separated from adenosine by paper chromatography.

Kinetic studies of SAH hydrolase SAH hydrolase from rabbit erythrocytes (30 μl) or human PBMC (100 μl) was incubated at 37°C for 1-3 hr with 500 μM DL-homocysteine, 1 mM dithioerythritol, 25 mM potassium phosphate buffer, pH 7.4, 500 μM magnesium acetate, 0.5 μM deoxycoformycin, and various concentrations of [8-^{3}H] adenosine (0.1 - 10 μM) in a total volume of 200 or 300 μl, respectively. In order to determine if Z-riboside is an inhibitor of SAH hydrolase, Z-riboside was added to the assay mixture at final concentrations of 100, 200, and 500 μM. The reaction was terminated by the addition of 20 μl of 2 M formic acid containing 10 mM SAH. An aliquot of the mixture (25 μl) was spotted onto Whatman 3MM paper (Whatman International Ltd., Maid Store, England) and the chromatogram was developed with ethanol, acetic acid, and water (64:1:35). After being dried and visualized under UV light, the SAH spot (Rf ≈ 0.3) was cut out, and SAH was extracted using 2 ml of water. A portion of the extract (1 ml) was counted using 10 ml of scintillation liquid.

To investigate the time-dependent inactivation of SAH hydrolase by purine metabolites, enzyme from rabbit erythrocytes or human PBMC was preincubated with various concentrations of purine metabolites in the assay mixture without adenosine at 37°C. Total volume of the preincubation mixtures was 160 and 240 μl for purified enzyme and human PBMC

enzyme, respectively. After 15- to 60-min preincubation, 40 or 60 μl of [8-^{3}H] adenosine was added to a final concentration of 10 μM in order to measure the enzyme activity. The activity was expressed as percent of control (i.e., without purine metabolites during preincubation).

To determine whether the inactivation of SAH hydrolase by Z-metabolites is reversible or irreversible, enzyme from rabbit erythrocytes was preincubated with various concentrations of Z-riboside or ZMP in 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 1 mM dithioerythritol in a total volume of 500 μl. After 2- or 24-hr preincubation, 50 μl were removed from each tube and diluted 10-fold with the same buffer. SAH hydrolase activity was determined using the diluted enzyme mixture (50 μl) with a final concentration of 10 μM [8-^{3}H] adenosine in a total volume of 200 μl. In order to remove Z-metabolites, the rest of the preincubation mixture was dialyzed three times against 100 volumes of 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 1 mM dithioerythritol at 4°C for a total of 24 hr. After dialysis, SAH hydrolase activity and protein concentration of the dialysate were measured. The specific enzyme activity was expressed as percent of control (i.e., without Z-metabolites during preincubation).

Determination of SAH hydrolase activity in cultured human lymphoblasts SAH hydrolase activity from cell extracts was measured using the method described in the previous section except that only one concentration of [8-^{3}H] adenosine (1

μM) was used in a total volume of 100 μl. This concentration of adenosine was approximately equal to its Km. Thus, the assay is relatively sensitive to potential inhibitors. After the incubation for 5 min at 37°C, the reaction was terminated by the addition of 10 μl of 2 M formic acid containing 10 mM SAH. The enzyme activity was calculated as nmol of SAH formed per hr per mg protein and expressed as percent of control (i.e., without inhibitors).

Determination of kinetic parameters All enzyme activities were measured at one time point, and substrate concentrations were approximated as the average of the initial and final concentrations of substrates present during the incubation (Lee and Wilson 1971). Kinetic parameters and inhibitor constants were obtained using unweighted data and the Ez-fit program developed by Perrella (E.I. Du Pont de Nemour, Wilmington, DE). Kinetic parameters for the inactivation of SAH hydrolase were determined as described by Kitz and Wilson (1962).

Determination of ADA activity

Principle of the assay ADA activity is determined by UV, colorimetric, or radiochemical methods. In this study, a radiochemical method was used in which radioactive adenosine is converted to radioactive inosine (Mudawwar et al. 1976). After the enzyme reaction, the assay mixture is heated in acid to hydrolyze adenosine and inosine to their free bases, adenine and hypoxanthine. Hypoxanthine is separated by

paper chromatography, and the amount of radioactive hypoxanthine is measured.

Kinetic studies of ADA Extracts of human PBMC were incubated with various concentrations of [8-^{14}C] adenosine (80-600 μM) in 0.1 M Tris buffer, pH 7.4, in a total volume of 50 μl at 37°C for 2 hr. In order to determine the effect of Z-riboside on ADA activity, Z-riboside was added to the assay mixture at final concentrations of 2, 3, and 5 mM. The enzyme reaction was terminated by adding 50 μl of 30% trichloroacetic acid, 10 μl of 1.6 N HCl, and 50 μl of 10 mM inosine and adenosine. The mixture was heated for 20 min at 110°C in a screw-capped tube. After cooling, 25 μl of the mixture were spotted onto paper and developed with butanol (saturated with water) and concentrated NH_4OH (100:1). The hypoxanthine spot (Rf ≈ 0.12) was located using UV light and extracted. The amount of radioactivity was counted.

Determination of ADA activity in cultured human lymphoblasts ADA activity in cell extracts was measured using the same method described in the previous section. For this determination 200 μM of [8-^{14}C] adenosine were used. The concentration of adenosine used in this assay is above the Km (≈ 50 μM). The incubation was carried out for 10 min for MOLT-4 cells and 40 min for Ramos cells. Enzyme activity was calculated as μmol of inosine formed per hr per mg soluble protein and expressed as percent of control.

Statistical analysis

The data expressed as percent of control were transformed to square roots in order to normalize the distribution and were analyzed by analysis of variance (Sokal and Rohlf 1981). Scheffe's test was used to determine significant differences between two groups. Statistical tests were done by GB-STAT computer program (Dynamic Microsystems, Inc., Silver Spring, MD). The means of transformed data were squared again for reporting means, and standard errors of the mean (SEM) were estimated from the confidence limits of the transformed data. All the data were expressed as mean ± SEM.

RESULTS

Effect of 5-amino-4-imidazolecarboxamide riboside (Z-riboside) on the activities of S-adenosyl-L-homocysteine (SAH) hydrolase and adenosine deaminase (ADA)

5-Amino-4-imidazolecarboxamide riboside (Z-riboside) was tested for its ability to inhibit S-adenosyl-L-homocysteine (SAH) hydrolase and adenosine deaminase (ADA). Enzyme activities were measured at various concentrations of adenosine with or without Z-riboside. Z-riboside was a competitive inhibitor of SAH hydrolase with respect to adenosine. Double reciprocal plots of SAH hydrolase from human peripheral blood mononuclear cells (PBMC) and rabbit erythrocytes are shown in figure 6 and figure 7. K_i values were 0.19 and 0.07 mM for the enzyme from human PBMC and rabbit erythrocytes, respectively. Z-riboside was also a competitive inhibitor of ADA from human PBMC with respect to adenosine. K_i value was 0.54 mM. K_i and K_m values are given in table 4, and K_i values are approximately 10- to 200-fold higher than K_m values, suggesting that Z-riboside concentration would have to be relatively high in order to exert effective inhibition of these enzymes.

Effect of methotrexate (MTX) on the activities of SAH hydrolase and ADA

In order to test whether methotrexate (MTX) inhibits SAH hydrolase and ADA in a cell-free system, extracts of

Figure 6. Competitive inhibition by Z-riboside of SAH hydrolase from human PBMC.

Extracts of human PBMC (100 μl) were incubated at 37°C for 3 hr with various concentrations of [8-^{3}H] adenosine (0.1, 0.3, 1, 3, or 10 μM) and Z-riboside (0, 100, 200, or 500 μM) in the presence of 500 μM DL-homocysteine, 25 mM potassium phosphate buffer, pH 7.4, 500 μM magnesium acetate, 0.5 μM deoxycoformycin in a total volume of 300 μl. The reciprocal of the velocity is plotted against the reciprocal of the concentration of adenosine. Each line is a regression line from 5 points (some of the points are above the scale), and each point is an average of duplicate measurements.

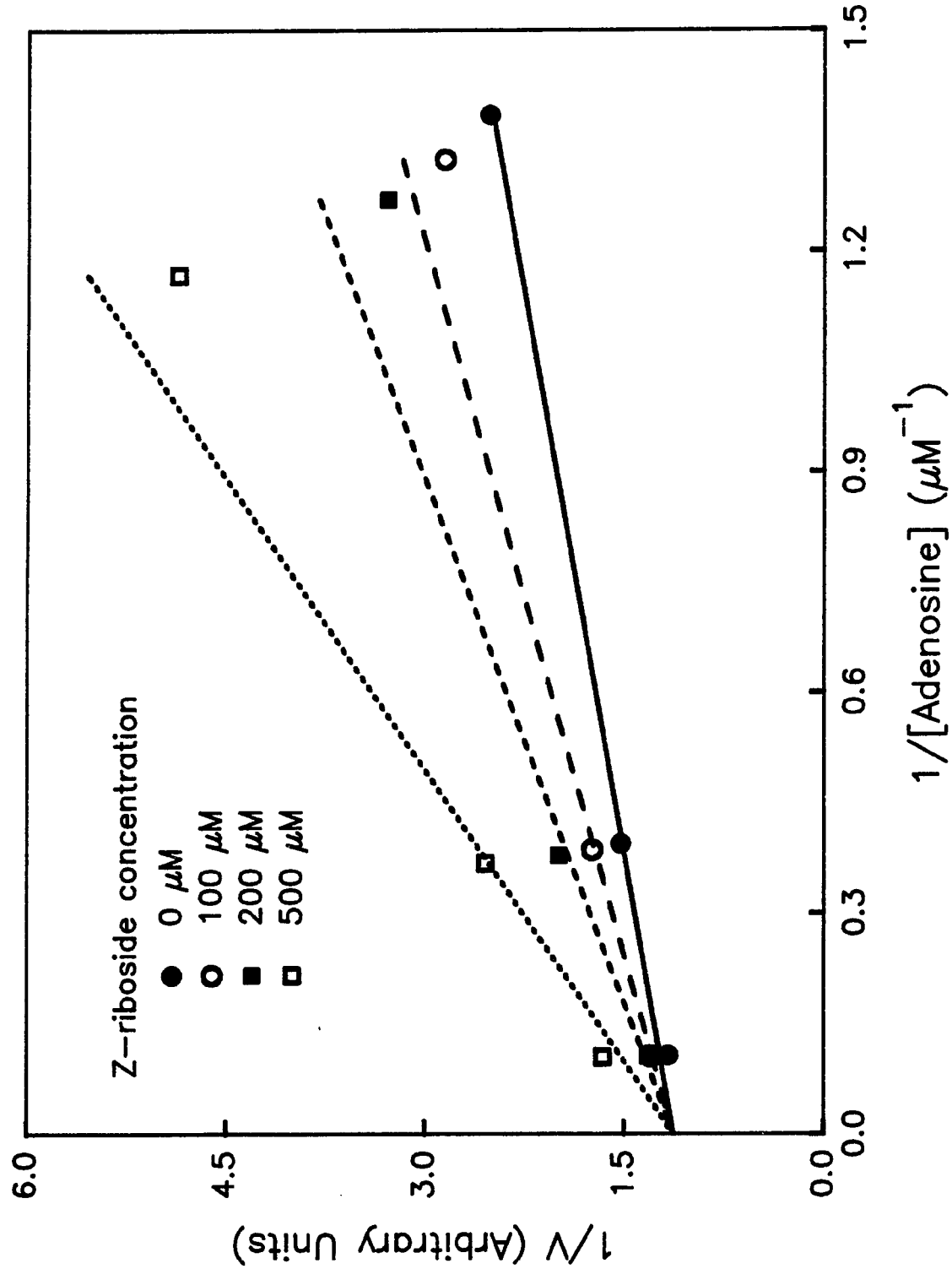

Z-riboside concentration
0 μM
100 μM
200 μM
500 μM
1/V (Arbitrary Units)
1/[Adenosine] (μM⁻¹)
6.0
4.5
3.0
1.5
0.0
0.0
0.3
0.6
0.9
1.2
1.5

Figure 7. Competitive inhibition by Z-riboside of SAH hydrolase purified from rabbit erythrocytes.

SAH hydrolase purified from rabbit erythrocytes (30 μl) was incubated at 37°C for 3 hr with various concentrations of [8-^{3}H] adenosine (0.1, 0.3, 1, 3, or 10 μM) and Z-riboside (0, 100, 200, or 500 μM) in the presence of 500 μM DL-homocysteine, 25 mM potassium phosphate buffer, pH 7.4, 500 μM magnesium acetate, 0.5 μM deoxycoformycin in a total volume of 200 μl. The reciprocal of the velocity is plotted against the reciprocal of the concentration of adenosine. Each line is a regression line from 5 points (some of the points are above the scale), and each point is a mean of duplicate measurements.

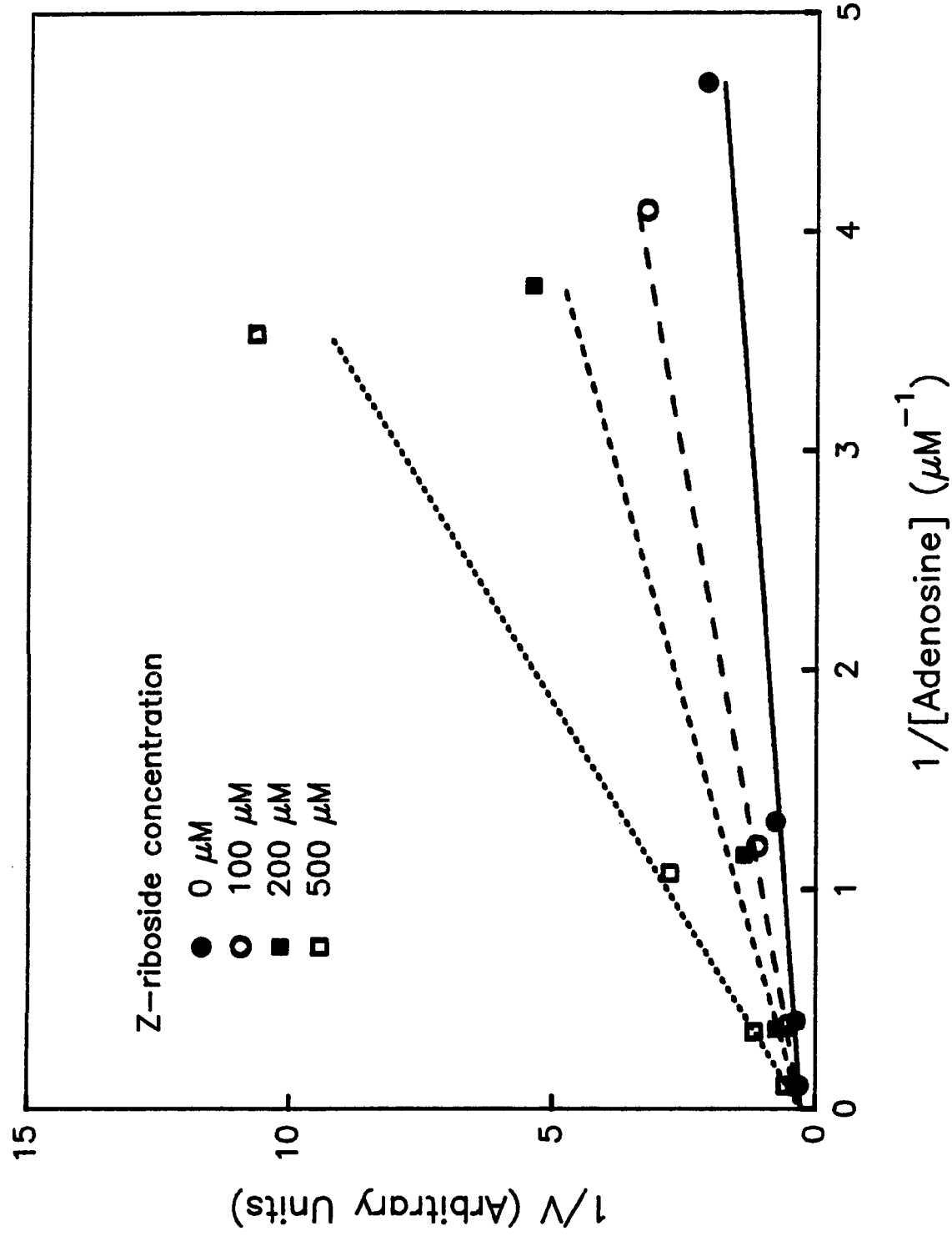

Z-riboside concentration
0 μM
100 μM
200 μM
500 μM
1/V (Arbitrary Units)
1/[Adenosine] (μM⁻¹)
0
5
10
15
0
1
2
3
4
5

Table 4. Competitive inhibition of SAH hydrolase and ADA by Z-riboside

Enzyme	Source	K_i (mM)	K_m for adenosine (μM)
SAH hydrolase	PBMC [a]	0.19 ± 0.03 [c]	0.9 ± 0.1
SAH hydrolase	RE [b]	0.07 ± 0.01	1.2 ± 0.1
ADA	PBMC	0.54 ± 0.14	54 ± 15

a : Human peripheral blood mononuclear cells
b : Rabbit erythrocytes
c : Standard error

MOLT-4 cells were used for enzyme sources. When SAH hydrolase was assayed with 5 μM adenosine and 100 μM MTX, less than 4% inhibition was observed when compared to the control activity (i.e., without MTX). ADA activity was inhibited less than 1% when 1 or 2 mM MTX were added to the assay mixture (100 μM adenosine). Kazmers et al. (1983) also reported that MTX did not inhibit ADA of MOLT-4 cells in a cell-free system.

Inactivation of SAH hydrolase by purine metabolites

In order to test if Z-riboside and 5-amino-4-imidazolecarboxamide ribotide (ZMP) inactivate SAH hydrolase, SAH hydrolase from rabbit erythrocytes or human PBMC was preincubated with Z-riboside or ZMP in the presence of 500 μM DL-homocysteine. Table 5 shows the remaining activity of the enzyme after 1 hr preincubation with Z-riboside, ZMP or other purine metabolites. The inactivating effect was similar in these two different sources. SAH hydrolase was inactivated by Z-riboside, ZMP, and adenosine monophosphate (AMP). This inactivation was dependent on the concentration of the metabolites. In contrast, there was little or no inactivation by inosine and IMP, which suggests that further metabolism of Z-riboside and ZMP relieves this potential metabolic block. 5-Amino-4-imidazolecarboxamide (Z-base) was also tested at a concentration of 1 mM, but less than 1% inactivation was observed.

The absence of homocysteine during preincubation did not change the effects of Z-riboside, ZMP, AMP, and IMP on

Table 5. Inactivation of SAH hydrolase from rabbit erythrocytes (RE) and human peripheral blood mononuclear cells (PBMC) after 1 hr preincubation with purine metabolites in the presence of 500 μM DL-homocysteine

Inhibitors	Conc.(mM)	Remaining activity of control [a] (%)	
		RE	PBMC
Z-riboside	0.01	73 ± 6 (6) [b]	74 ± 7 (2)
	0.1	27 ± 5 (4)	33 ± 3 (2)
	1.0	4 ± 2 (4)	19 ± 1 (2)
ZMP	0.01	95 ± 4 (5)	95 ± 1 (2)
	0.1	88 ± 7 (4)	81 ± 5 (2)
	1.0	3 ± 1 (3)	7 ± 1 (2)
AMP	0.01	75 ± 12 (3)	57
	0.1	11 ± 3 (4)	30
	1.0	3 ± 2 (4)	23
IMP	0.01	106 ± 15 (2)	93
	0.1	88 ± 5 (3)	116
	1.0	66 ± 1 (2)	102
Inosine	0.01	100 ± 1 (2)	115
	0.1	93 ± 3 (3)	96
	1.0	86 ± 6 (4)	79

a : Control experiments contained 500 μM DL-homocysteine and no inhibitor.
b : Mean ± SEM (Number of assays)

enzyme inactivation when these metabolites were tested at a concentration of 1 mM. However, after preincubation with 1 mM inosine, only 20% of enzyme activity remained in the absence of homocysteine, while 86% remained in the presence of 500 μM DL-homocysteine (table 5). This result indicates that homocysteine protects the enzyme from the inactivating effects of inosine.

In order to further investigate time-dependent inactivation by Z-riboside, SAH hydrolase from rabbit erythrocytes was preincubated in the presence of Z-riboside and homocysteine (500 μM) for different time periods. The logarithm of percent of remaining activity was plotted against preincubation time as shown in figure 8. The inactivation was a pseudo-first order process. First order rate constants (k) were obtained from the half-life ($t_{1/2}$) of the enzyme activity.

$$k = 0.693 \div t_{1/2} \qquad (7)$$

A double reciprocal plot for the first order rate constants against the concentrations of Z-riboside was linear (figure 9). This line has a slope of K_{Inact}/V_{Imax}, and an intercept of $1/V_{Imax}$ on the 1/k axis. (K_{Inact} is the binding constant for inactivation site and V_{Imax} is maximal rate of inactivation) (Kitz and Wilson 1962). From this relationship, V_{Imax} and K_{Inact} were estimated to be 0.07 min^{-1} and 0.11 mM, respectively. Deoxyadenosine, which was the most powerful irreversible inhibitor among the natural purine nucleosides tested by Chiang et al. (1981), was reported to have a V_{Imax}

Figure 8. Time-dependent inactivation of SAH hydrolase purified from rabbit erythrocytes by Z-riboside.

SAH hydrolase purified from rabbit erythrocytes (30 μl) was preincubated with various concentrations of Z-riboside (0, 0.01, 0.1, 1 mM) in the presence of 500 μM DL-homocysteine, 25 mM potassium phosphate buffer, pH 7.4, 500 μM magnesium acetate, 0.5 μM deoxycoformycin in a total volume of 160 μl. After 15, 30, 45, and 60 min, 40 μl of [8-^{3}H] adenosine was added to each tube to a final concentration of 10 μM, and the mixture was incubated at 37°C for 3 hr. The control had no Z-riboside during preincubation. Percent of the remaining activity compared to control was plotted against preincubation time on semilog graph paper. Each point is an average of duplicate measurements.

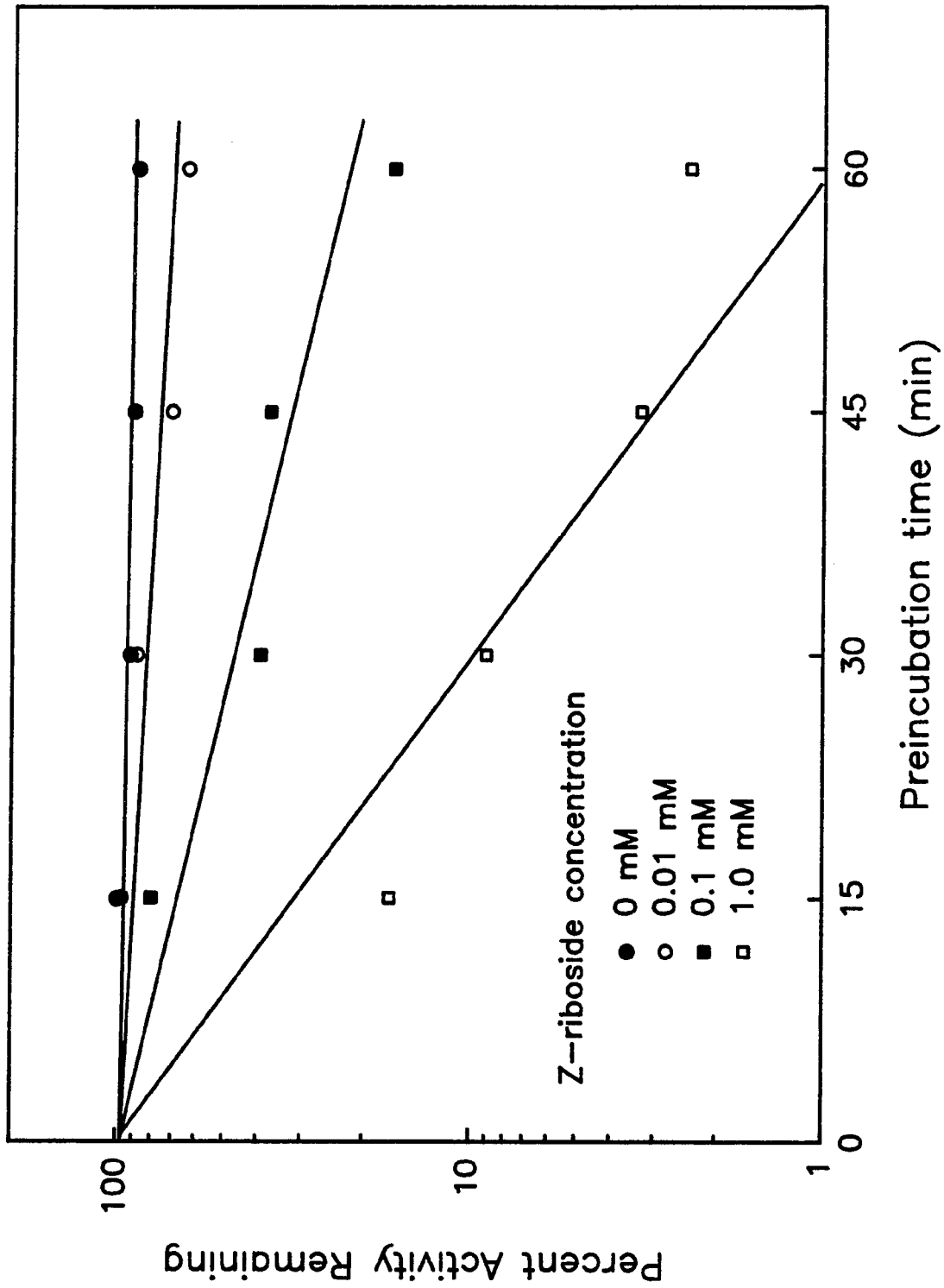
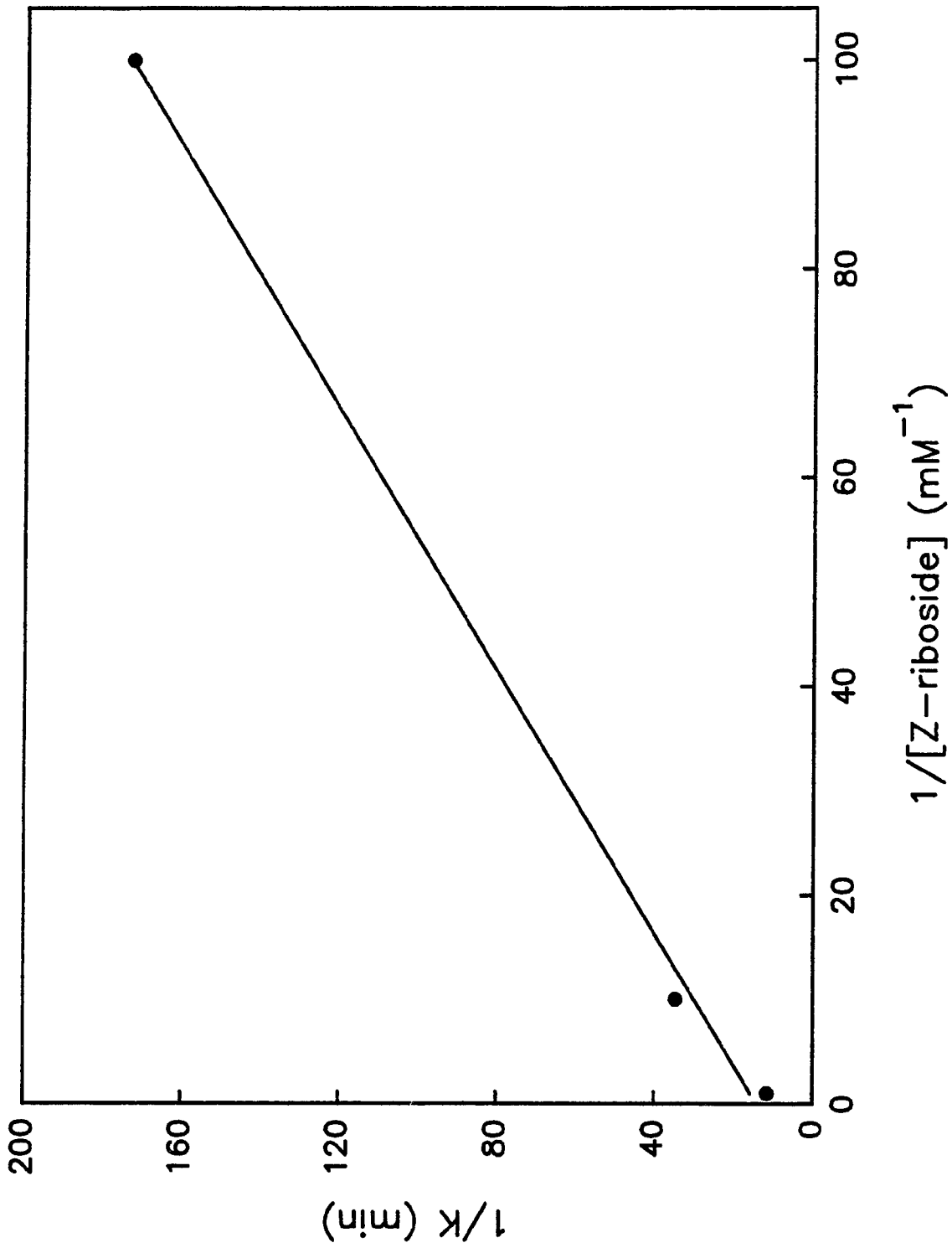

Percent Activity Remaining
100
10
1
0
15
30
45
60
Preincubation time (min)
Z-riboside concentration
0 mM
0.01 mM
0.1 mM
1.0 mM

Figure 9. Kinetics of inactivation of SAH hydrolase purified from rabbit erythrocytes by Z-riboside.

The reciprocal of the first order rate constant calculated from the data in figure 7 is plotted against the reciprocal of the concentration of Z-riboside. This line has a slope of K_{Inact}/V_{Imax} and an intercept of $1/V_{Imax}$ on the 1/k axis. K_{Inact} is the binding constant for inactivation site and V_{Imax} is maximal rate of inactivation.


1/K (min)
200
160
120
80
40
0
0
20
40
60
80
100
1/[Z-riboside] (mM^{-1})

of 0.12 min^{-1}, and a K_{Inact} of 66 μM (Hershfield 1979). Thus, Z-riboside is almost as effective as deoxyadenosine in its ability to inactivate SAH hydrolase.

SAH hydrolase was dialyzed in an attempt to restore the activity by removing Z-riboside or ZMP. Inactivation of the enzyme activity by Z-riboside or ZMP was only partially restored by dialysis (table 6). This result indicates that the inactivation is irreversible. The increase in SAH hydrolase activity following dialysis most likely represents the effect of removing reversible competitive inhibition.

Effect of MTX and Z-metabolites on the growth of cultured human lymphoblasts

In order to determine the effect of Z-metabolites on the growth of MOLT-4 and Ramos cells and to determine whether Z-metabolites potentiate the toxic effect of methotrexate (MTX), Z-riboside was added to cell culture media with or without MTX. Z-riboside was used because it is readily taken up by the cell and it can be phosphorylated to ZMP in the cells (Sabina et al. 1985). Preliminary studies were carried out to evaluate the effect of various concentrations of Z-riboside and MTX on the growth of MOLT-4 cells (tables 7 and 8). At concentrations of 0.1 and 0.3 μM MTX (with or without Z-riboside), cell proliferation was suppressed and cell viability was virtually zero after a 48- or 72-hr culture (table 7). When Z-riboside was added after 10- or 24-hr exposure to MTX (0.01, 0.02, or 0.03 μM), cell growth was apparently inhibited at concentrations of 100 and

Table 6. Effect of dialysis (24 hr) on inactivation of SAH hydrolase purified from rabbit erythrocytes by Z-metabolites

Preincubation condition			Remaining activity (% of control) [a]	
Inhibitor	Conc. (mM)	Preincubation time (hr)	Before dialysis	After diaysis
Z-riboside	0.01	24	64 ± 1 [b]	61 ± 12
	0.1	24	3 ± 1	29 ± 1
	1.0	2	5 ± 1	27 ± 4
ZMP	0.1	24	85	95 ± 3
	1.0	24	59 ± 5	50 ± 4

a : Control experiments contained no inhibitor. After dialysis, 64% of enzyme activity remained.
b : Mean ± SEM

Table 7. Effect of MTX and Z-riboside on the growth of MOLT-4 cells [a] (preliminary study I)

Incubation time	Z-riboside [b] (μM)	MTX (μM)				
		0	0.01	0.03	0.1	0.3
24 hr	0	100 [c]	58	46	29	18
48 hr	0	100	51	1	0	0
	1	106	39	1	0	0
	3	98	49	1	0	0
	10	91	39	1	0	0
	30	66	43	0	0	0
	100	105	34	0	0	0
72 hr	0	100	53	1	0	0
	1	86	46	3	0	0
	3	109	60	1	0	0
	10	106	53	1	0	0
	30	101	49	0	0	0
	100	69	30	0	0	0

a : The number of viable cells is expressed as percent of control.
b : Z-riboside was added 24 hr after the cells were exposed to MTX.
c : Values are means of duplicate determinations.

Table 8. Effect of MTX and Z-riboside on the growth of MOLT-4 cells [a] (preliminary study II)

Incubation time	Z-riboside [b] (μM)	MTX (μM)		
		0	0.01	0.02
10 hr	0	100 [c]	63	67
34 hr	0	100	31	34
	30	28	24	3
	100	45	34	10
	200	10	45	14
58 hr	0	100	27	3
	30	140	29	1
	100	102	22	1
	200	87	21	0

a : The number of viable cells is expressed as percent of control.
b : Z-riboside was added 10 hr after the cells were exposed to MTX.
c : Values are means of duplicate determinations.

200 μM Z-riboside (tables 7 and 8). Based on these preliminary studies, it was decided to add 0, 100, or 200 μM concentrations of Z-riboside to the media 8 hr after the addition of 0, 0.01, or 0.02 μM MTX. The number of cells were counted 24 and 48 hr after the addition of MTX. It was assumed that this 8-hr preincubation with MTX was sufficient for MTX to be taken up by the cell and polyglutamylated.

Tables 9 and 10 show the effect of MTX and Z-riboside on the number of viable cells. Growth of MOLT-4 cells was significantly inhibited by exposure to 0.01 and 0.02 μM MTX for both 24 and 48 hr (table 9). The addition of Z-riboside alone also inhibited the growth of MOLT-4 cells. The decreased growth of MOLT-4 cells caused by MTX was potentiated by Z-riboside. For example, at 48 hr, 200 μM Z-riboside potentiated the effect of 0.02 μM MTX by decreasing cell growth from 19 to 5% of control.

In contrast to MOLT-4 cells, Ramos cells were resistant to the effect of MTX (table 10). Even at 48 hr, exposure to 0.02 μM MTX alone did not inhibit the growth of Ramos cells. However, the addition of Z-riboside alone inhibited cell growth, and the addition of 200 μM Z-riboside to 0.02 μM MTX-treated cells significantly inhibited cell growth from 109 to 67%. The effect of Z-riboside is, however, less striking in Ramos cells than in MOLT-4 cells. These results indicate that MOLT-4 cells are more sensitive to the effects of MTX and Z-riboside on cell growth than are Ramos cells.

Table 9. Effect of MTX and Z-riboside on the growth of MOLT-4 cells [a]

Incubation time	Z-riboside [b] (μM)	MTX (μM)		
		0	0.01	0.02
24 hr	0	100 ± 4 [c]	63 ± 2 ++	57 ± 4 ++
	100	89 ± 4	60 ± 2 ++	47 ± 3 ++
	200	73 ± 4 **	58 ± 3	47 ± 3 ++
48 hr	0	100 ± 4	57 ± 6 +	19 ± 5 ++
	100	93 ± 4	57 ± 6	6 ± 3 ++
	200	68 ± 5	41 ± 3	5 ± 2 ++,*

a : The number of viable cells is expressed as percent of control.
b : Z-riboside was added 8 hr after the cells were exposed to MTX.
c : Mean ± SEM. Values were obtained from four independent experiments. Each experiment was done in duplicate or triplicate.
+ : $p < 0.05$ vs. MTX control (i.e., 0 μM MTX in this row)
++ : $p < 0.01$ vs. MTX control
* : $p < 0.05$ vs. Z-riboside control (i.e., 0 μM Z-riboside in this column)
** : $p < 0.01$ vs. Z-riboside control

Table 10. Effect of MTX and Z-riboside on the growth of Ramos cells [a]

Incubation time	Z-riboside [b] (μM)	MTX (μM) 0	0.01	0.02	
24 hr	0	100 ± 4 [c]	86 ± 6	104 ± 6	
	100	91 ± 4	91 ± 5	103 ± 4	
	200	85 ± 4	81 ± 4	92 ± 5	
48 hr	0	100 ± 2	95 ± 4	109 ± 9	
	100	88 ± 4	85 ± 5	97 ± 8	
	200	71 ± 4	67 ± 3	67 ± 4	**

a : The number of viable cells is expressed as percent of control.
b : Z-riboside was added 8 hr after the cells were exposed to MTX.
c : mean ± SEM. Values were obtained from four independent experiments. Each experiment was done in duplicate.
** : $p < 0.01$ vs. Z-riboside control (i.e., 0 μM Z-riboside in this column)

Tables 11 and 12 show these experimental data expressed as percent of total cells which are viable. The results were qualitatively similar to those of cell growth, indicating that MOLT-4 cells are more sensitive to MTX toxicity and its potentiation by Z-riboside than Ramos cells.

Accumulation of Z-metabolites during MTX or Z-riboside treatment in cultured human lymphoblasts

In order to test if Z-metabolites accumulate during MTX treatment, MOLT-4 and Ramos cells were incubated with various concentrations of MTX for 24 hr, and intracellular levels of ZMP and Z-riboside were measured. Table 13 shows intracellular ZMP levels in MOLT-4 and Ramos cells. In MOLT-4 cells, ZMP accumulated after exposure to 0.01 and 0.02 μM MTX, but not after exposure to 0.1 μM MTX. The representative chromatograms of a 50 μl sample of ultrafiltered MOLT-4 cell extracts after exposure to 0.02 and 0.1 μM MTX are shown in figure 10 and figure 11, respectively. These results suggest that 0.1 μM MTX completely inhibited GAR transformylase in MOLT-4 cells. Thus, ZMP synthesis was suppressed and no accumulation of ZMP occurred. These results are similar to the results reported by Bokkerink et al. (1986). In contrast to MOLT-4 cells, ZMP was detected in Ramos cells only after exposure to 0.1 μM MTX. This result may explain the finding that Ramos cells were more resistant to MTX than MOLT-4 cells in the cell growth studies. Bokkerink et al. (1988) reported less accumulation of ZMP in human B-lymphoblasts (RAJI) than in T-lymphoblasts

Table 11. Effect of MTX and Z-riboside on the viability of MOLT-4 cells [a]

Incubation time	Z-riboside [b] (μM)	MTX (μM)		
		0	0.01	0.02
24 hr	0	83 ± 1 [c]	75 ± 2	69 ± 2 +
	100	82 ± 1	74 ± 2	65 ± 3 ++
	200	77 ± 2	72 ± 2	61 ± 3 ++
48 hr	0	85 ± 1	72 ± 2 +	31 ± 5 ++
	100	83 ± 2	73 ± 2	12 ± 4 ++,*
	200	74 ± 3	59 ± 3	10 ± 3 ++,*

a : Viability is expressed as percent of total cells which are viable.
b : Z-riboside was added 8 hr after the cells were exposed to MTX.
c : mean ± SEM. Values were obtained from four independent experiments. Each experiment was done in duplicate or triplicate.
+ : $p < 0.05$ vs. MTX control (i.e., 0 μM MTX in this row)
++ : $p < 0.01$ vs. MTX control
* : $p < 0.05$ vs. Z-riboside control (i.e., 0 μM Z-riboside in this column)
** : $p < 0.01$ vs. Z-riboside control

Table 12. Effect of MTX and Z-riboside on the viability of Ramos cells [a]

Incubation time	Z-riboside [b] (μM)	MTX (μM)		
		0	0.01	0.02
24 hr	0	92 ± 2 [c]	91 ± 2	91 ± 2
	100	91 ± 2	91 ± 2	92 ± 2
	200	90 ± 2	89 ± 2	90 ± 2
48 hr	0	92 ± 1	89 ± 2	88 ± 2
	100	88 ± 2	89 ± 2	89 ± 2
	200	81 ± 4	82 ± 4	77 ± 4

a : Viability is expressed as percent of total cells which are viable.
b : Z-riboside was added 8 hr after the cells were exposed to MTX.
c : mean ± SEM. Values were obtained from four independent experiments. Each experiment was done in duplicate.

Table 13. ZMP levels in human lymphoblasts cultured with various concentrations of MTX for 24 hr [a]

MTX (μM)	MOLT-4	Ramos
0	*	*
0.01	1.3 ± 0.6 ; 22.8 ± 10.8 [b] (0.2 - 2.5)[c]	*
0.02	1.5 ± 0.4 ; 25.5 ± 5.5 (0.9 - 2.0)	*
0.1	*	1.7 ± 0.4 ; 29.9 ± 3.3 (1.4 - 2.1)

a : Expressed as nmol/mg soluble protein; nmol/10^9 cells
b : Mean ± SEM. Values are from two independent cultures, and each sample (50 μl of cell extracts) was analyzed in duplicate by HPLC.
c : Range of the values (nmol/mg soluble protein)
* : Below the detection limit (i.e., less than 0.1 nmol/mg soluble protein)

Figure 10. A typical HPLC ion-exchange chromatogram of the extracts of 0.02 μM MTX-treated MOLT-4 cells.

A 50 μl sample obtained as described in 'Materials & Methods' was injected on a Partisil SAX ion-exchange column (10 μm, 250 X 4.6 mm) to separate ZMP. This chromatogram is monitored at 267 nm. Other conditions are the same as described in figure 5.

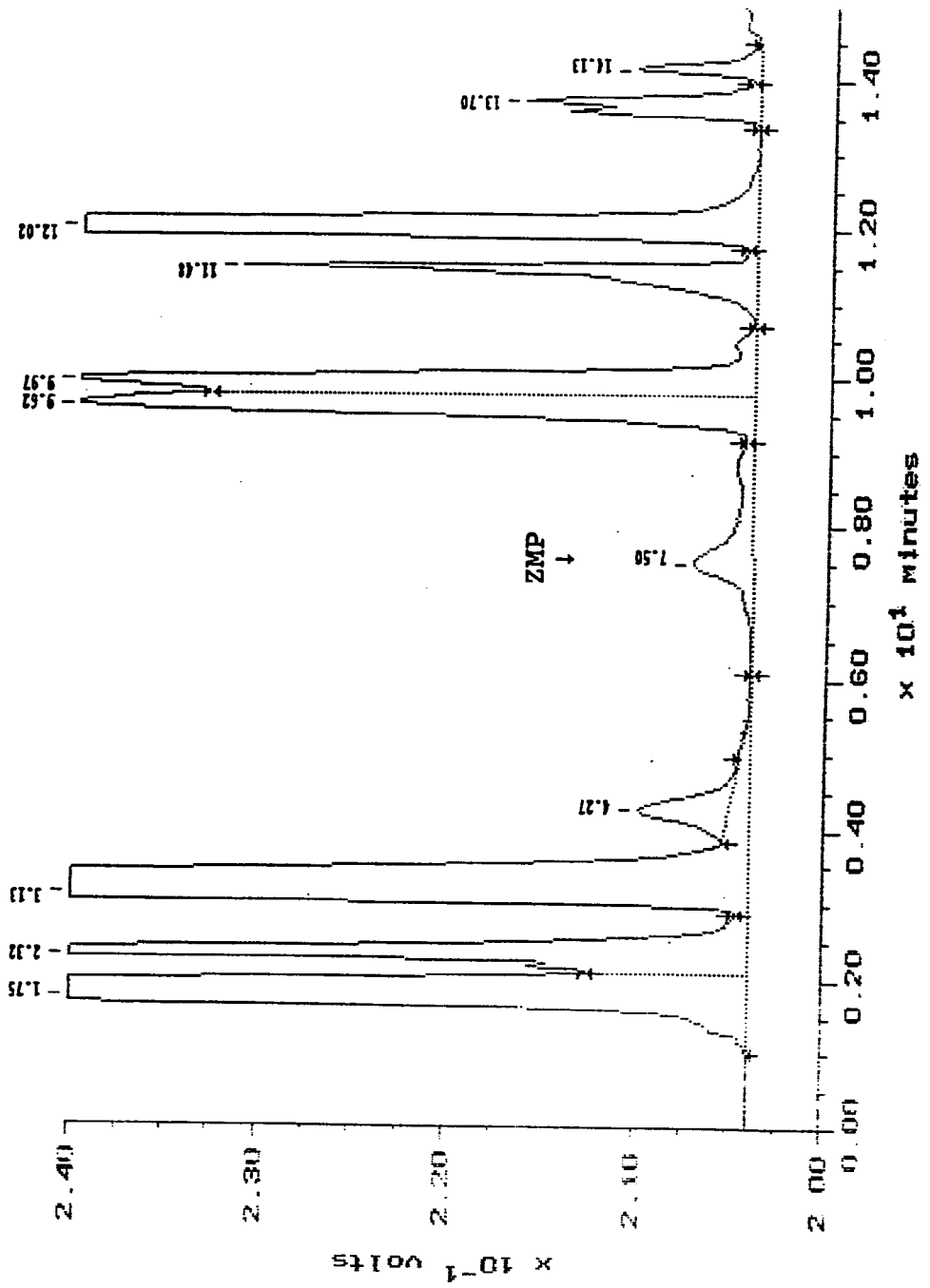
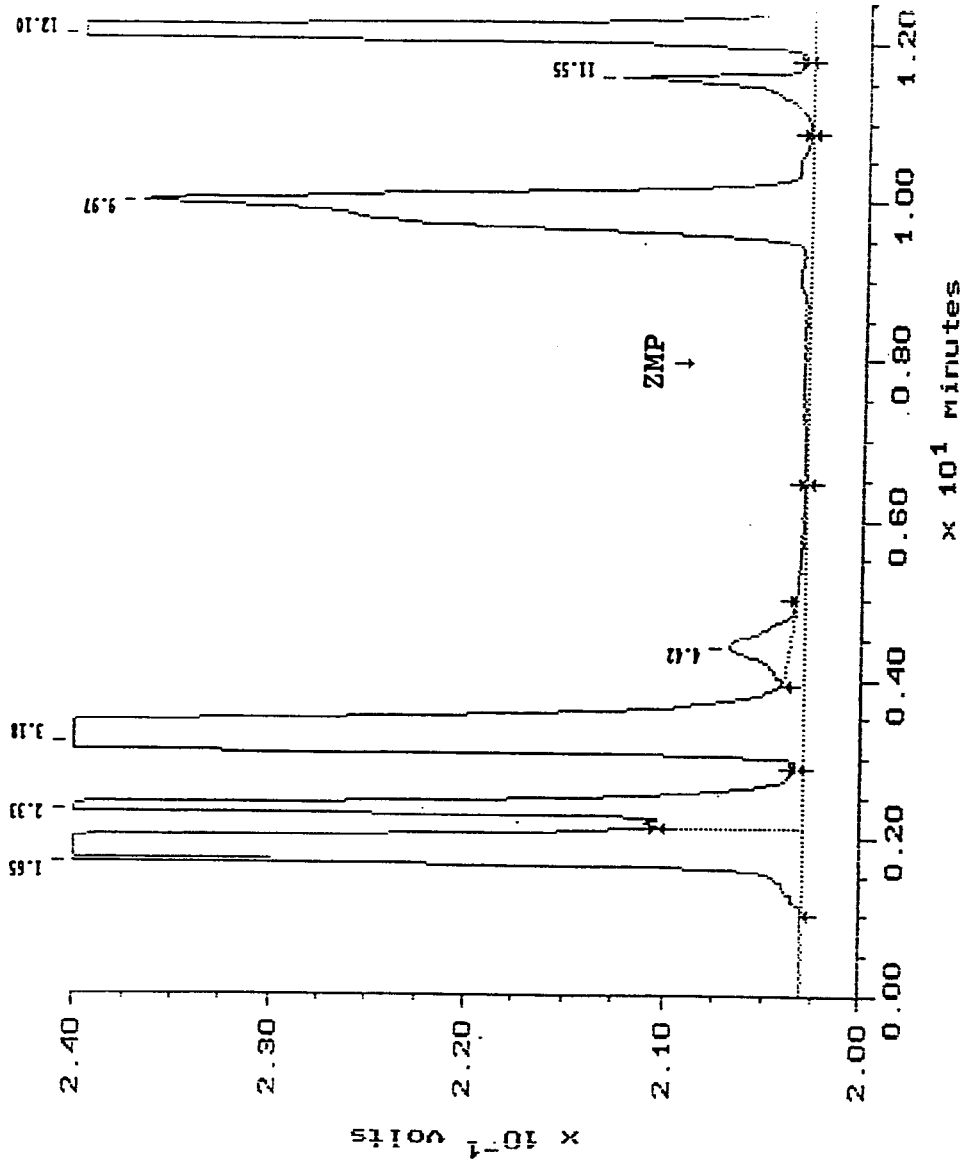

1.75
2.32
3.13
4.27
ZMP
7.50
9.62
9.97
11.48
12.02
13.70
14.13
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
x 10¹ minutes
2.00
2.10
2.20
2.30
2.40
x 10⁻¹ volts

Figure 11. A typical HPLC ion-exchange chromatogram of the extracts of 0.1 μM MTX-treated MOLT-4 cells.

A 50 μl sample obtained as described in 'Materials & Methods' was injected on a Partisil SAX ion-exchange column (10 μm, 250 X 4.6 mm) to separate ZMP. Other conditions are the same as described in figure 5.


x 10⁻¹ volts
2.40
2.30
2.20
2.10
2.00
0.00
0.20
0.40
0.60
0.80
1.00
1.20
x 10¹ minutes
1.65
2.33
3.18
4.42
ZMP
9.97
11.55
12.10

(MOLT-4) when the cells were incubated with 0.02 μM MTX for 24 hr, and no accumulation of ZMP in both T- and B-lymphoblasts after incubation with 0.2 μM MTX.

Z-riboside accumulation could not be detected at any of the MTX concentrations we tested. These results were confirmed with two different HPLC methods. Since Z-riboside is able to cross cell membranes, it is possible that Z-riboside leaked out from the cells while they were in the culture media and being washed.

After the cells were incubated with 30, 100, or 200 μM Z-riboside in the absence of MTX, accumulation of Z-riboside and ZMP in the cells was determined. However, the intracellular concentrations of both Z-riboside and ZMP were less than the detection limits. As can be seen by comparing figure 12 and figure 13, a new peak appeared with a retention time of 10.10 min in the reverse-phase column. Fractions containing the peak were collected. The U.V. spectrum of the compound had a peak at 266 nm (pH 6.35) similar to that of a Z-metabolite (figure 14). In addition, the compound gave a purple color in the Bratton-Marshall reaction, and the colored product had a peak at 550 nm and a shoulder at approximately 600 nm. This spectral property is characteristic of the Bratton-Marshall azo dye of a Z-metabolite (figure 15) (Lukens and Buchanan 1959a; 1959b). ZMP, 5-amino-4-imidazolecarboxamide riboside triphosphate (ZTP), and 5-amino-4-imidazole-N-succinocarboxamide ribotide (sZMP), which is the immediate precursor of ZMP, have been

Figure 12. A typical reverse-phase HPLC chromatogram of the extracts of untreated Ramos cells (i.e., without Z-riboside and MTX treatment during culture).

A 25 μl sample obtained as described in 'Materials & Methods' was injected on a C_{18} column (5 μm, 250 X 4.6 mm) to separate Z-riboside. This chromatogram is monitored at 267 nm. Chromatographic conditions: mobile phase, 50 mM potassium phosphate buffer, pH 6.35; flow rate, 1.0 ml/min.

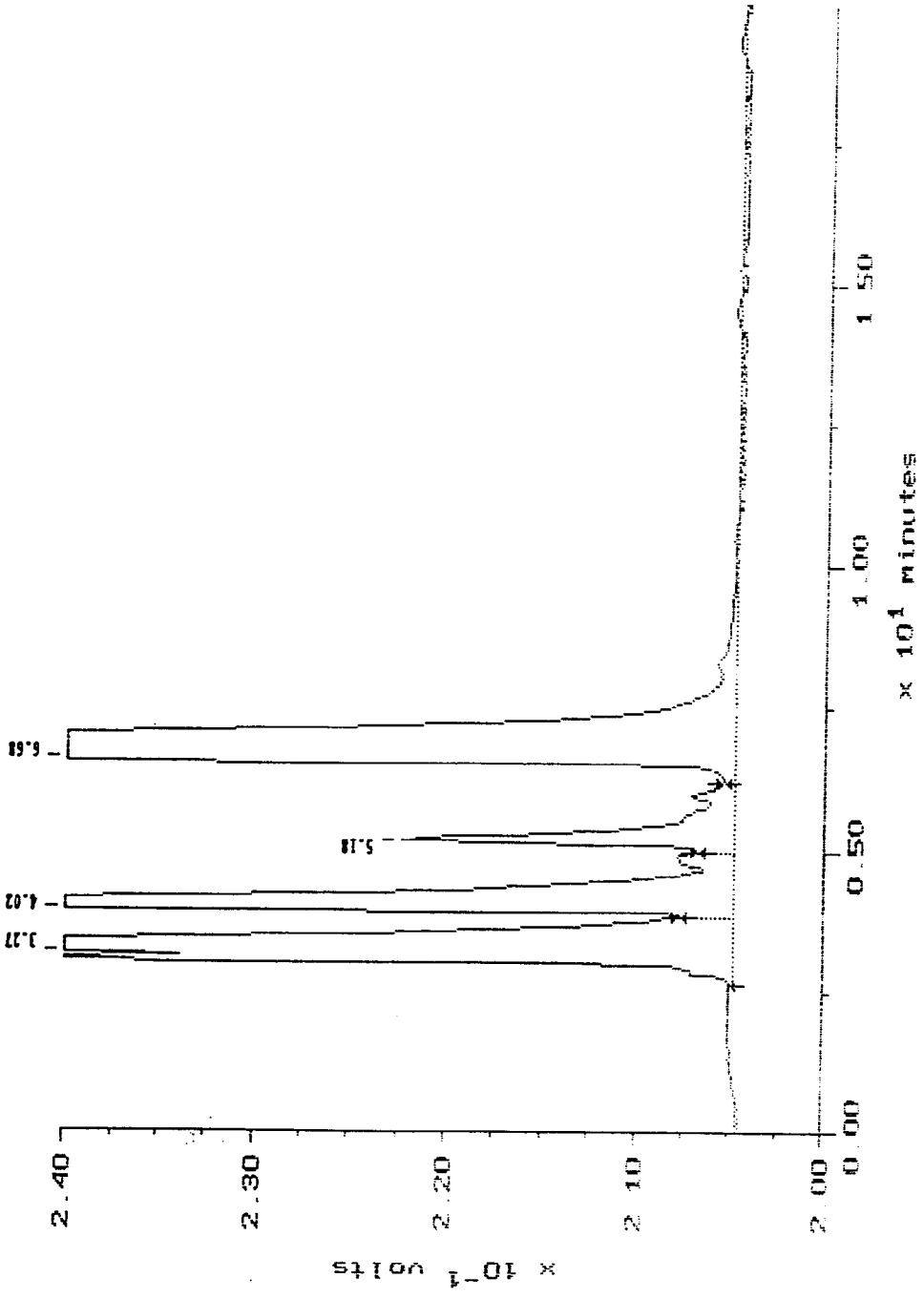

2.40
2.30
2.20
2.10
2.00
x 10^-1 volts
0.00
0.50
1.00
1.50
x 10^1 minutes
6.68
5.18
4.02
3.37

Figure 13. A typical reverse-phase HPLC chromatogram of the extracts of 100 μM Z-riboside-treated Ramos cells.

A 25 μl sample obtained as described in 'Materials & Methods' was injected on a C_{18} column (5 μm, 250 X 4.6 mm) to separate Z-riboside. Other conditions are the same as described in figure 12.

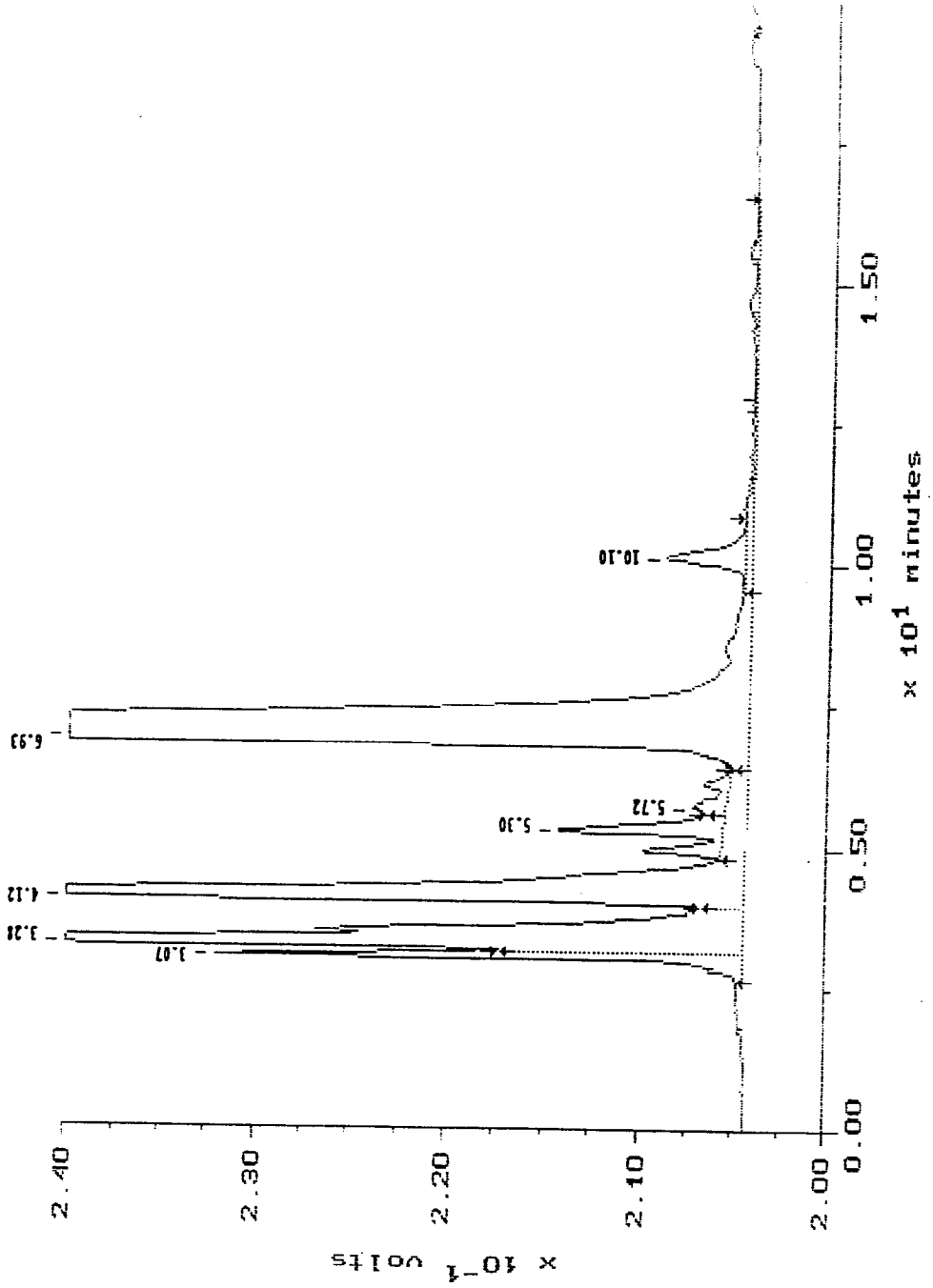

x 10^-1 volts
2.40
2.30
2.20
2.10
2.00
0.00
0.50
1.00
1.50
x 10^1 minutes
10.10
6.93
5.72
5.30
4.12
3.28
3.07

Figure 14. U.V. spectrum of the unidentified compound eluting at 10.10 min from the reverse-phase HPLC column chromatogram of the extracts of MOLT-4 cells exposed to Z-riboside (100 μM) for 24 hr (see figure 13).

Fractions containing the peak were collected, and the U.V. spectrum of the compound was scanned from 220 to 300 nm *versus* the elution buffer (50 mM potassium phosphate buffer, pH 6.35) as blank.

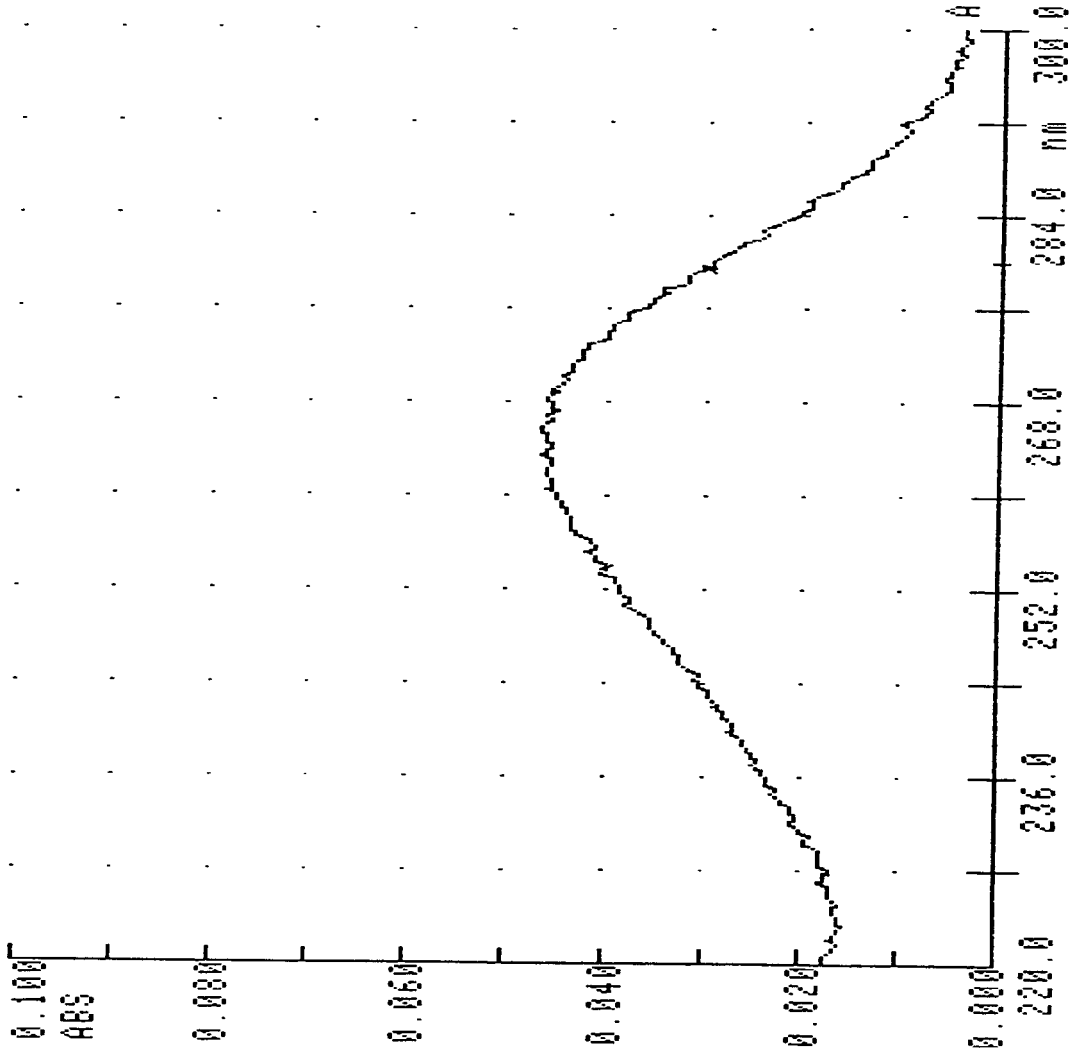
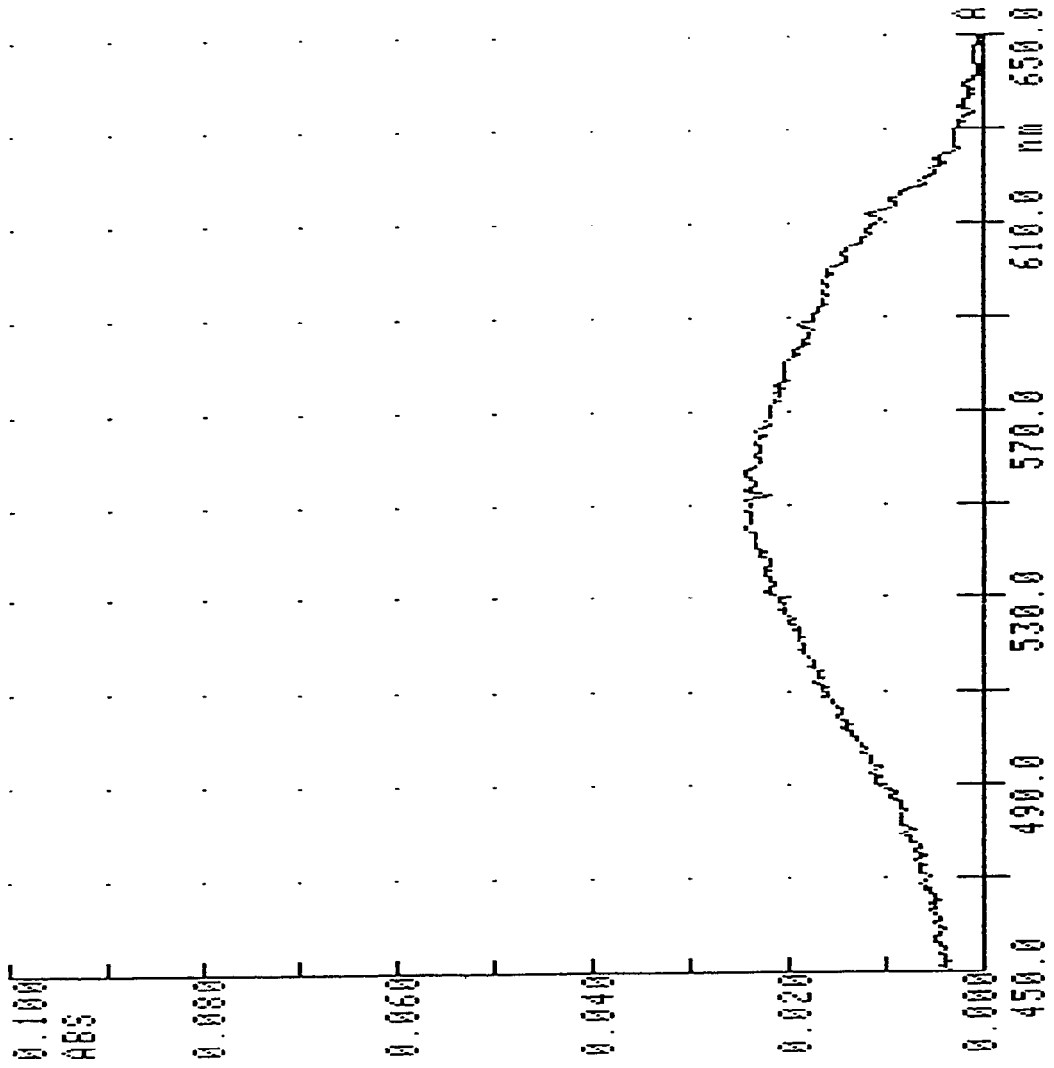

0.100
ABS
0.080
0.060
0.040
0.020
0.000
220.0
236.0
252.0
268.0
284.0
nm
300.0
A

Figure 15. U.V. spectrum of the Bratton-Marshall azo dye of the unidentified compound in figure 13.

All reactions were carried out at 0°C. To the fractions containing the peak (400 µl), 50 µl of 10 N H_2SO_4 and 150 µl of 1% (w/v) $NaNO_2$ were added. After 3-min incubation, 150 µl of 5% (w/v) ammonium sulfamate were added, followed by the addition of 150 µl of 1% (w/v) N-(1-naphthyl)ethylenediamine after 1-min interval. The test tube was removed from the ice bath, and after 30 min, the spectrum was scanned from 450 to 650 nm *versus* a blank which contained 400 µl of the elution buffer (50 mM potassium phosphate buffer, pH 6.35) and the Bratton-Marshall reagents.


0.100
ABS
0.080
0.060
0.040
0.020
0.000
450.0
490.0
530.0
570.0
610.0
650.0
nm

shown to accumulate in Chinese hamster ovary fibroblasts which were exposed to 700 μM Z-riboside (Sabina et al. 1985). The new compound is likely to be sZMP or 5-amino-4-imidazole-N-succinocarboxamide riboside (sZ-riboside), because ZTP would elute before ZMP (retention time of 3.6 min in this column). The intracellular concentrations of the new compound were estimated to be 13-24 nmol/10^9 cells in MOLT-4 cells and 14-17 nmol/10^9 cells in Ramos cells assuming a molar extinction coefficient of sZMP at 267 nm equal to that of ZMP (Flaks et al. 1957).

Development of ZMP transformylase assay

The colorimetric assay for ZMP transformylase developed by Flaks and Lukens (1963) has the following limitations: (1) since the assay measures the disappearance of the azo dye of ZMP, the measurement of a small difference in large absorbances is susceptible to error; (2) the color of the azo dye of ZMP resulting from the Bratton-Marshall assay begins to fade within 2 hr; (3) no antioxidant is included in the assay to protect labile folates, i.e., 10-formyl-tetrahydrofolic acid (10-HCO-H_4PteGlu) and tetrahydrofolic acid (H_4PteGlu); (4) the assay is not suitable for detecting low activity in crude extracts of some tissues.

The new assay was developed by Ha et al. (1990). This method is based upon the observation that H_4PteGlu, a product of ZMP transformylase, was oxidatively cleaved to p-aminobenzoyl glutamate (pABG) by alkaline permanganate treatment, whereas 10-HCO-H_4PteGlu, a substrate of ZMP

transformylase, was oxidized primarily to 10-formylfolic acid (10-HCO-PteGlu) (Maruyama et al. 1978). The color of the Bratton-Marshall azo dye of pABG which was formed from H_4PteGlu by permanganate oxidation was measured.

In order to validate the new assay, several experiments were performed. The standard curve of pABG with alkaline permanganate oxidation did not show great difference from the standard curve of the Bratton-Marshall azo dye of pABG without oxidation (figure 16), indicating that the pABG ring is not destroyed by permanganate oxidation. Both pABG and ZMP are Bratton-Marshall positive compounds, but ZMP was not stable to permanganate oxidation. Therefore, after permanganate oxidation, the presence of a relatively high concentration of ZMP (i.e., 500 μM) does not produce color in the Bratton-Marshall reaction (table 14). Thus, there was essentially no difference in color development when various concentrations of ZMP were added to the Bratton-Marshall assay of pABG with permanganate oxidation. The color of the azo dye obtained in the Bratton-Marshall assay of pABG was stable for 24 hr. In this assay, mercaptoethanol is also included to protect labile folates, 10-HCO-H_4PteGlu and H_4PteGlu, from oxidation. The oxidation products of these compounds (i.e., 10-HCO-H_2PteGlu, 10-HCO-PteGlu, H_2PteGlu, or PteGlu) are either substrates with different activities or inhibitors of the transformylase (Baggott et al. 1986).

Figure 16. Effect of permanganate oxidation on the standard curve of pABG Bratton-Marshall reaction product.

In the absence of enzyme, various concentrations of pABG were mixed with 500 μM ZMP, 50 mM phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol in a total volume of 500 μl. To the mixture, 50 μl of 0.17 M $HgCl_2$ and 50 μl of 1 M ammonium bicarbonate, pH 9.2, were added. In one set of tubes, 100 μl of 2% (w/v) $KMnO_4$ were added, and after 5 min, 400 μl of 0.3% (v/v) H_2O_2 were added. In the other set of tubes, water was substituted for $KMnO_4$ and H_2O_2. The color was developed as described in 'Materials & Methods'.

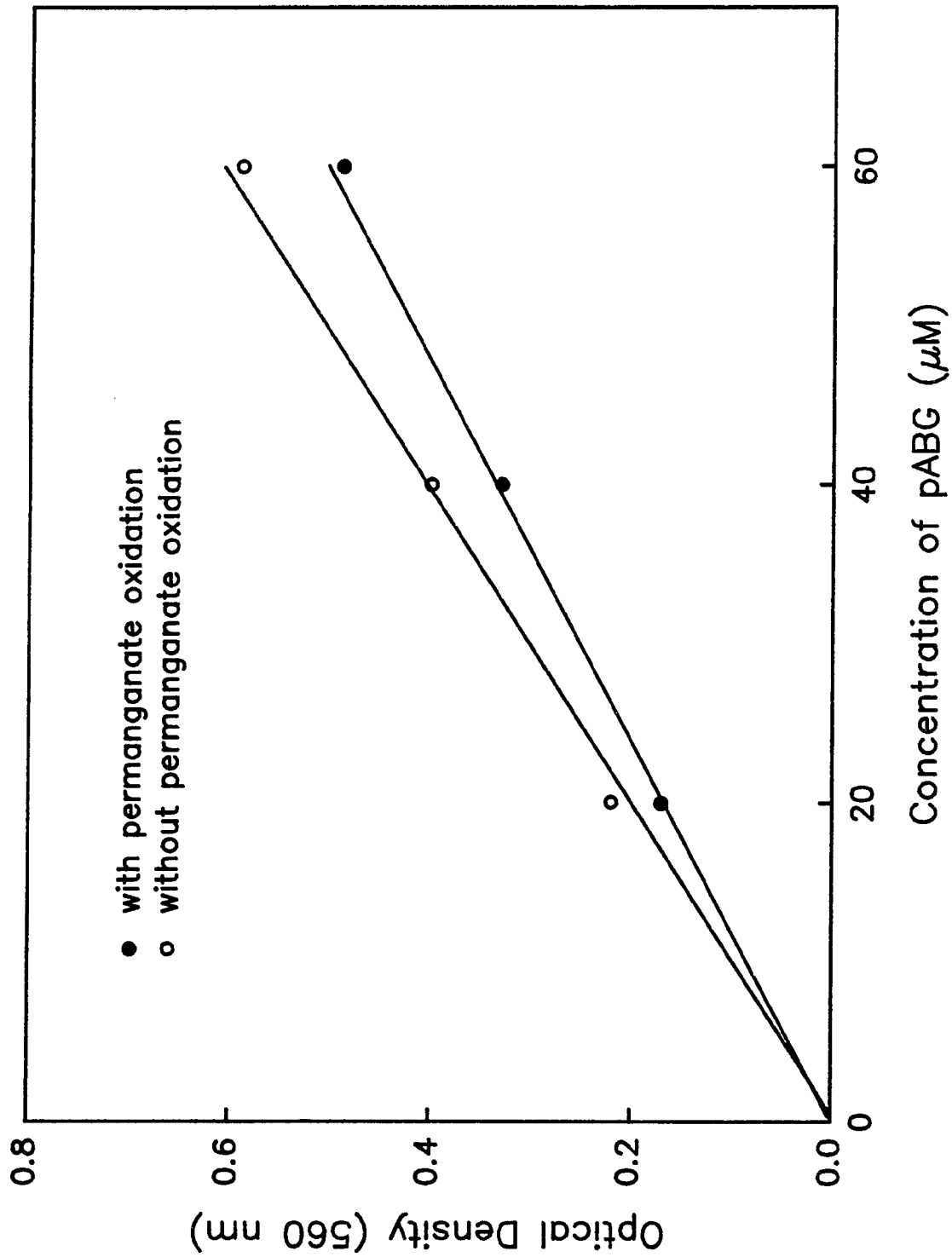

Optical Density (560 nm)
0.0
0.2
0.4
0.6
0.8
0
20
40
60
Concentration of pABG (μM)
● with permanganate oxidation
o without permanganate oxidation

Table 14. Effect of various concentrations of ZMP on the standard curve of pABG in the oxidative cleavage and Bratton-Marshall reaction

pABG (μM) [a]	ZMP (μM) [a]		
	10	200	500
10	0.081 [b]	0.081	0.086
20	0.171	0.168	0.180
40	0.330	0.319	0.329
100	0.684	0.738	0.781

a : Concentrations of pABG and ZMP in the original solutions before oxidative cleavage and Bratton-Marshall reactions.

b : Optical density of the Bratton-Marshall assay product of pABG at 560 nm.

Determination of ZMP transformylase activity in cultured human lymphoblasts

The activity of ZMP transformylase in MOLT-4 and Ramos cells cultured with various concentrations of MTX for 24 hr was measured using the newly developed assay. The enzyme activity was linear with time up to 3 hr and with protein concentration up to 12.5 μg. Approximately 10 μg of protein were used for the assay. The ZMP transformylase activity of cells cultured without MTX was 160 ± 3 and 131 ± 20 μM/hr/mg soluble protein in MOLT-4 and Ramos cells, respectively.

When the enzyme was assayed with 100 μM 10-HCO-H_4PteGlu, inhibition of enzyme activity by MTX was not evident in either cell-line. Less than 10% inhibition was observed in the extracts of cells exposed to 0.01, 0.02, and 0.1 μM MTX. In order to decrease the ratio of added 10-HCO-H_4PteGlu to $MTXGlu_n$ (presumably present in the cell extracts), the concentration of 10-HCO-H_4PteGlu in the assay was decreased from 100 μM to 20 μM. The assay was also performed at two time points (1.5 and 3 hr). Inhibition of the transformylase activity was then detected in MTX-treated MOLT-4 cells. ZMP transformylase activity of MOLT-4 cells was inhibited more than 50% by 24 hr exposure to MTX at concentrations of 0.01, 0.02, and 0.1 μM (figure 17). The inhibition of ZMP transformylase was not dose-dependent and approximately the same at 0.01 μM as 0.1 μM MTX concentration. In contrast to MOLT-4 cells, the inhibition of ZMP transformylase of Ramos cells could not be demonstrated (figure 18). It is important to recognize that

Figure 17. ZMP transformylase activity of MOLT-4 cells cultured with various concentrations of MTX for 24 hr.

Extracts of MOLT-4 cells containing 20 μg of protein were incubated at 37°C for 1.5 and 3 hr with 20 μM L-10-HCO-H_4PteGlu, 100 μM ZMP, 50 mM potassium phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol in a total volume of 500 μl. The assay was performed as described in 'Materials & Methods'.

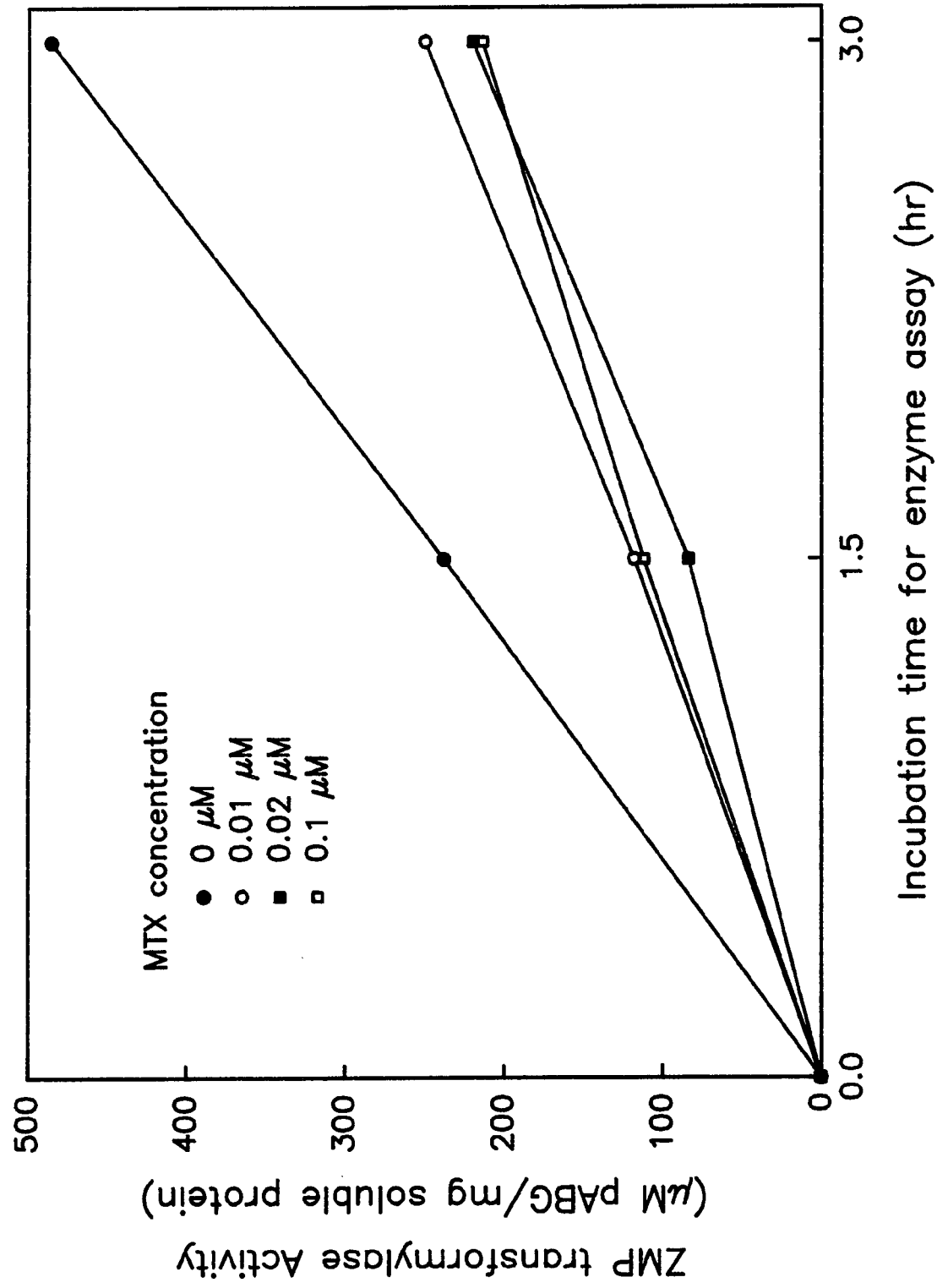
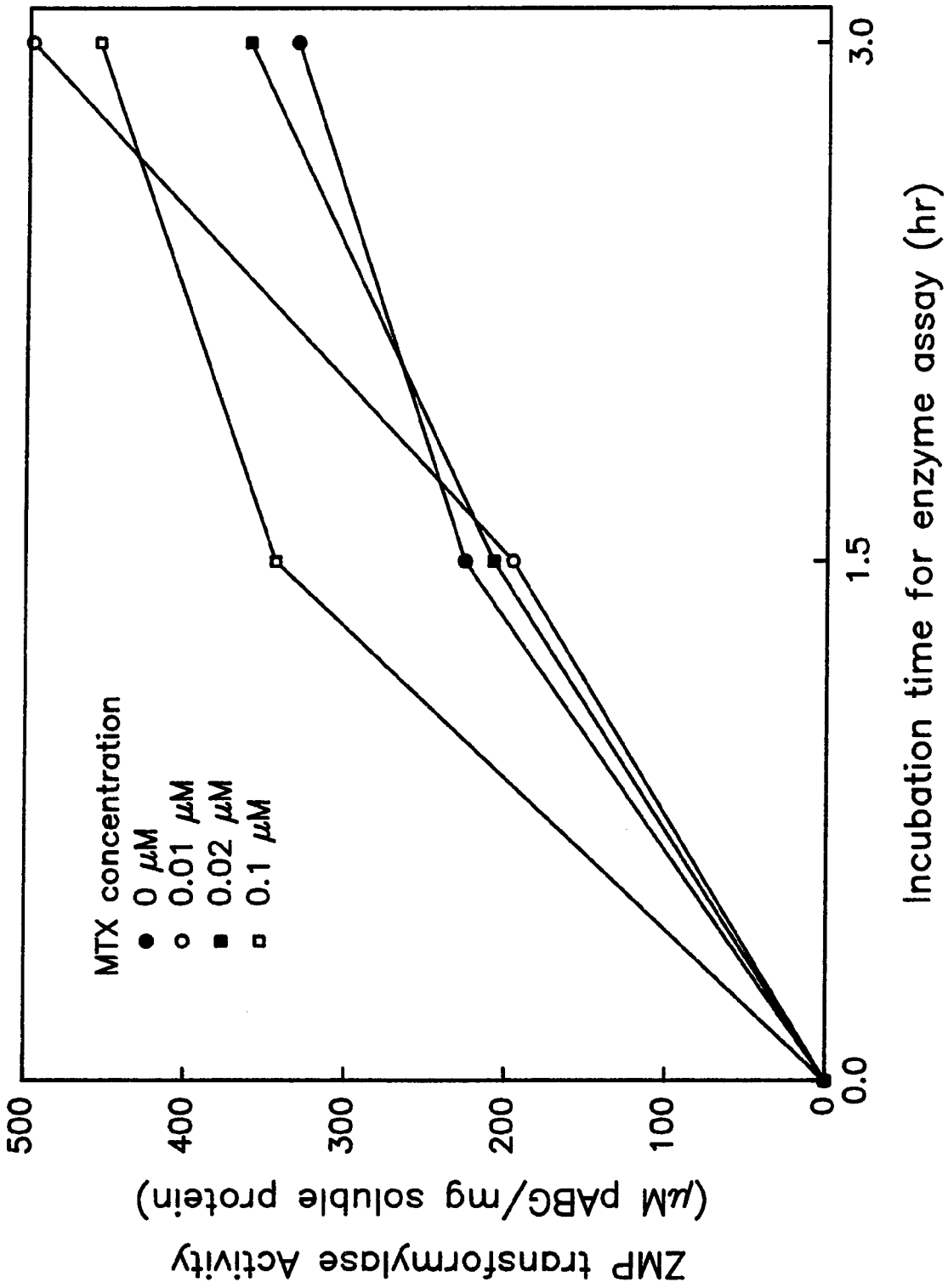

ZMP transformylase Activity
(μM pABG/mg soluble protein)
500
400
300
200
100
0
0.0
1.5
3.0
Incubation time for enzyme assay (hr)
MTX concentration
0 μM
0.01 μM
0.02 μM
0.1 μM

Figure 18. ZMP transformylase activity of Ramos cells cultured with various concentrations of MTX for 24 hr.

Extracts of Ramos cells containing 20 μg of protein were incubated at 37°C for 1.5 and 3 hr with 20 μM L-10-HCO-H_4PteGlu, 100 μM ZMP, 50 mM phosphate buffer, pH 7.4, and 10 mM 2-mercaptoethanol in a total volume of 500 μl. The assay was performed as described in 'Materials & Methods'.


ZMP transformylase Activity
(μM pABG/mg soluble protein)
500
400
300
200
100
0
0.0
1.5
3.0
Incubation time for enzyme assay (hr)
MTX concentration
0 μM
0.01 μM
0.02 μM
0.1 μM

intracellular concentrations of MTX and $MTXGlu_n$ are decreased when cell extracts are made and when the extracts are added to the assay system, therefore, it is likely that the observed inhibition of ZMP transformylase is an underestimate of the inhibition that occurs inside the cell.

Determination of SAH hydrolase activity in cultured human lymphoblasts

The activity of SAH hydrolase in MOLT-4 and Ramos cells cultured with various concentrations of Z-riboside or MTX for 24 hr was measured. The enzyme activity was linear with time up to 10 min and with protein concentrations between 0.5 μg and 1 μg per assay mixture. The enzyme activity without bovine serum albumin (BSA) (0.5 mg/ml) was 78-80% in MOLT-4, and 84-90% in Ramos when compared with the activity with BSA. Therefore, BSA was included in the assay.

The SAH hydrolase activity of cells cultured without MTX and Z-riboside was 264 ± 9 and 285 ± 9 nmol SAH formed/hr/mg soluble protein in MOLT-4 cells and Ramos cells, respectively. The enzyme from both sources was stable at -70°C for at least a month. However, only 5% and 1% of enzyme activity of MOLT-4 cells remained after storing the extracts for 2 and 4 months at -70°C, respectively. The enzyme of Ramos cells was more stable than that of MOLT-4 cells with 12% and 7% of enzyme activity remaining after 2 and 4 months, respectively.

Table 15 shows the activity of SAH hydrolase when the cells were incubated with various concentrations of Z-riboside for 24 hr. There was significant inhibition of SAH

Table 15. SAH hydrolase activity of human lymphoblasts cultured with various concentrations of Z-riboside for 24 hr [a]

Z-riboside (μM)	MOLT-4	Ramos
0	100 ± 1 [b] (10) [c]	100 ± 2 (10)
30	36 ± 1 ** (2)	68 ± 1 (2)
100	30 ± 3 ** (6)	52 ± 10 ** (6)
200	23 ± 2 ** (7)	40 ± 3 ** (7)

a : Expressed as percent of control activity (i.e., 0 μM Z-riboside)
b : Mean ± SEM
c : Number of assays from two independent cultures
** : $p < 0.01$ vs. Z-riboside control (i.e., 0 μM Z-riboside)

Table 16. SAH hydrolase activity of human lymphoblasts cultured with various concentrations of MTX for 24 hr [a]

MTX (μM)	MOLT-4	Ramos
0	100 ± 1 [b] (14) [c]	100 ± 1 (14)
0.01	91 ± 4 (10)	100 ± 6 (10)
0.02	84 ± 3 * (14)	84 ± 2 * (13)
0.1	105 ± 6 (11)	85 ± 6 (11)

a : Expressed as percent of control activity (i.e., 0 μM Z-riboside)
b : Mean ± SEM
c : Number of assays from two independent cultures
* : $p < 0.05$ vs. Z-riboside control (i.e., 0 μM Z-riboside)

hydrolase activity at all concentrations of Z-riboside tested in MOLT-4 cells and at 100 and 200 μM Z-riboside in Ramos cells. The inhibitory effect of Z-riboside on SAH hydrolase was greater in MOLT-4 than in Ramos cells. This result is consistent with the observation that MOLT-4 cells are more sensitive to the effect of MTX plus Z-riboside on cell growth than are Ramos cells (tables 9 and 10).

Table 16 shows the activity of SAH hydrolase when the cells were incubated with various concentrations of MTX for 24 hr. In MOLT-4 cells, SAH hydrolase activity was inhibited by a low concentration (i.e., 0.02 μM), but not by a high concentration (i.e., 0.1 μM) of MTX present in the media. This result is consistent with the finding that ZMP accumulated only at low concentrations of MTX and that exposure of Z-riboside inhibited SAH hydrolase in MOLT-4 cells. In Ramos cells, SAH hydrolase activity was also inhibited at 0.1 μM MTX, which is consistent with the finding that ZMP was detected at high concentration (i.e., 0.1 μM) of MTX in Ramos cells. It is important to recognize that irreversible inhibition of SAH hydrolase by Z-riboside or ZMP would not be underestimated by dilution of the intracellular volume which occurs when cell extracts are made and the components of the assay are added. However, as with ZMP transformylase, the reversible competitive inhibition of SAH hydrolase would be underestimated.

Determination of ADA activity in cultured human lymphoblasts

The activity of ADA in MOLT-4 and Ramos cells cultured with various concentrations of Z-riboside or MTX for 24 hr was measured. ADA activity was linear with time up to 10 min in MOLT-4 cells and up to 40 min in Ramos cells, and with protein concentrations up to 1.5 μg per reaction mixture. The enzyme activity without BSA (0.05 mg/ml) was about 36% in MOLT-4 cells when compared with the activity with BSA. Therefore, BSA was included in the assay.

The enzyme activity of cells cultured without Z-riboside and MTX was 123 ± 7 μmol inosine/hr/mg soluble protein in MOLT-4 cells and 9.1 ± 0.9 μmol/hr/mg soluble protein in Ramos cells. Thus, ADA activity in MOLT-4 cells was approximately 14-fold higher than that in Ramos cells. This result is consistent with the observation that ADA activity is greater in T- than B-cells (Sullivan et al. 1977; Huang et al. 1976) and underscores the metabolic differences in adenosine homeostasis that exist between lymphocytes and cell lines from these precursors. The stability of the deaminase during storage at -70°C was similar in these two different types of cells. Therefore, even after 4 months, MOLT-4 cells had an approximately 14-fold higher activity than Ramos cells. The enzyme was stable at -70°C for at least a month. Approximately 60% and 50% of enzyme activity remained after 2 and 4 month storage, respectively.

ADA activity of human lymphoblasts cultured with Z-riboside for 24 hr is shown in table 17. There was no

Table 17. ADA activity of human lymphoblasts cultured with various concentrations of Z-riboside for 24 hr [a]

Z-riboside (μM)	MOLT-4		Ramos	
0	100 ± 2 [b]	(8) [c]	100 ± 2	(10)
30	105 ± 18	(4)	96 ± 7	(4)
100	101 ± 11	(6)	92 ± 7	(6)
200	104 ± 11	(7)	92 ± 5	(7)

a : Expressed as percent of control activity (i.e., 0 μM Z-riboside)
b : Mean ± SEM
c : Number of assays from two independent cultures

Table 18. ADA activity of human lymphoblasts cultured with various concentrations of MTX for 24 hr [a]

MTX (μM)	MOLT-4		Ramos	
0	100 ± 2 [b]	(11) [c]	100 ± 3	(13)
0.01	93 ± 4	(8)	87 ± 4 *	(8)
0.02	87 ± 4	(11)	89 ± 2	(10)
0.1	88 ± 7	(7)	103 ± 4	(12)

a : Expressed as % of control activity (i.e., 0 μM MTX)
b : Mean ± SEM
c : Number of assays from three independent cultures
* : $p < 0.05$ vs. MTX control (i.e., 0 μM MTX)

detectable difference in ADA activity when cells were incubated with Z-riboside. The values were highly variable between cultures. Since the ADA activity of MOLT-4 cells was much higher than Ramos cells, MOLT-4 cell extracts had to be diluted 2.5-8 times more than Ramos cell extracts. Therefore, it may be more difficult to detect the inhibition in MOLT-4 cells because the putative inhibitor (Z-riboside) was diluted prior to the assay.

When the cells were incubated with MTX for 24 hr, the inhibition of ADA was detected only in Ramos cells after exposure to 0.01 μM MTX (table 18). However, it is difficult to interpret this finding because we could not detect any inhibition of ADA in Ramos cells after exposure to Z-riboside.

DISCUSSION

5-Amino-4-imidazolecarboxamide riboside (Z-riboside) and 5-amino-4-imidazolecarboxamide ribotide (ZMP) inhibited S-adenosyl-L-homocysteine (SAH) hydrolase in a cell-free system and cultures of human T- and B-cell lines. The addition of certain concentrations of methotrexate (MTX) to cell culture media caused accumulation of ZMP and inhibition of SAH hydrolase. However, MTX itself did not inhibit this enzyme in a cell-free system. The addition of Z-riboside inhibited cell growth and potentiated growth inhibition of MTX in a T-cell line. These findings support our hypothesis that MTX indirectly inhibited SAH hydrolase via the accumulation of ZMP. In contrast to SAH hydrolase, adenosine deaminase (ADA) inhibition by Z-riboside was detected only in a cell-free system. A new colorimetric assay for ZMP transformylase was developed. Using the assay, ZMP transformylase was found to be inhibited in cultured T-cell line (but not in a B-cell line) when the cells were exposed to MTX. Our findings will be discussed below in the order described in the preceding sections. In addition, the mechanisms of the inability of Z-riboside and MTX to inhibit ADA in a cell-culture system will be discussed.

Effect of Z-riboside and ZMP on the activities of SAH hydrolase and ADA

Z-riboside is structurally similar to adenosine, therefore, it is likely that enzymes which utilize adenosine, such as adenosine kinase, SAH hydrolase, and ADA, bind Z-riboside as a substrate or as an inhibitor. For example, adenosine kinase is known to use Z-riboside as a substrate (Sabina et al. 1985). We evaluated the effect of Z-riboside as an inhibitor of two adenosine metabolizing enzymes, SAH hydrolase and ADA. Z-riboside was found to inhibit these enzymes by a competitive mechanism.

K_m values for adenosine of SAH hydrolase in human peripheral blood mononuclear cells (PBMC) and rabbit erythrocytes were determined to be 0.9 μM and 1.2 μM, respectively. Other researchers have reported Km values of adenosine in the range of 0.2 μM to 1.8 μM when assayed in the synthetic direction: 0.2 μM in mouse liver (Doskeland and Ueland 1982), 0.4 μM in guinea pig heart (Schrader et al. 1981), 0.5 μM in horse liver (Magnuson et al. 1984), 0.6 μM in rat liver (Kajander et al. 1976), 1 μM in human lymphoblasts (Hershfield and Kredich 1978), and 1.8 μM in beef liver (Guranowski et al. 1981). Differences in species or assay condition may explain the differences among the values. Our data obtained from human PBMC are comparable to the data from human lymphoblasts (Hershfield and Kredich 1978).

K_m value for adenosine of ADA in human PBMC was found to be 54 μM, and similar values have been obtained in other

human tissues: 48 μM in leukemic granulocytes (Wiginton et al. 1981), 52 μM in erythrocytes (Daddona and Kelley 1977), and 66 μM in lung (Akedo et al. 1972).

K_i values for Z-riboside of ADA and SAH hydrolase in human PBMC were 10- and 200-fold higher than K_m values for adenosine, respectively (table 4). The intracellular concentration of adenosine is normally so low that its detection is difficult. Some investigators have reported adenosine concentration in the mammalian brain as 0.5-2 nmol/g wet weight tissues, which is equivalent to approximately 1 μM (Newman and McIlwain 1977; Nordstrom et al. 1977). The plasma concentration of adenosine in normal individuals has been found between 0.05 to 0.4 μM (Kredich and Hershfield 1989). Since nucleosides readily enter and exit cells, the intracellular concentrations of adenosine in lymphocytes may be similar to the extracellular concentrations. Thus, the intracellular concentration of adenosine is most likely two or more orders of magnitude lower than its K_m of ADA and slightly lower than its K_m of SAH hydrolase.

The intracellular concentration of ZMP after 0.02 μM MTX exposure was 1.5 nmol/mg soluble protein or 26 nmol/10^9 cells (table 13). This represents approximately 25-30 μM and is slightly lower than the levels observed by Bokkerink et al. (1986) which were approximately 50 to 100 μM. Contrary to ZMP, the intracellular concentration of Z-riboside after MTX treatment has not been reported. We were unable to detect intracellular Z-riboside using the HPLC

method. Thus it is not known whether Z-riboside accumulates in the cells during MTX exposure and reaches levels which effectively inhibit ADA or SAH hydrolase. Z-riboside readily crosses cell membranes and could have leaked into the culture medium or during cell washing procedure. Therefore, the failure to detect Z-riboside may not prove that it was not present inside the cells.

Z-riboside has been previously shown to be a competitive inhibitor of ADA of purine-requiring mutants of E.Coli B (Kuramitsu et al. 1964) and of bovine intestinal mucosa (Baggott et al. 1986). In addition, Barankiewicz et al. (1991) reported by citing their unpublished results that Z-riboside inhibited both ADA and SAH hydrolase in human B-lymphoblasts.

We have demonstrated that Z-riboside and ZMP irreversibly inactivate SAH hydrolase (tables 5 and 6) and that this effect of Z-riboside is only slightly less potent than that of deoxyadenosine (figures 8 and 9). Z-riboside and ZMP have not been reported as inactivators of SAH hydrolase even though many naturally occurring purines have been shown to inactivate this enzyme. Adenine, adenosine monophosphate (AMP), and adenosine diphosphate (ADP) have been shown to induce a time-dependent inactivation of mouse liver enzyme, however, inorganic phosphate at the physiological concentration abolishes the inactivating effect of adenine and adenine nucleotides (Ueland and Saebo 1979; Ueland 1982b). Thus, it was thought that inactivation of SAH hydrolase by

adenine and adenine nucleotides occurs only under in vitro conditions. In contrast, our experiments, in which phosphate was included in the preincubation mixture, showed an ability to inactivate SAH hydrolase by Z-riboside, ZMP and AMP (table 5 and figure 8).

We have tested whether inosine monophosphate (IMP) or inosine inactivates SAH hydrolase. Our data, as shown in table 5, are similar to the findings of Knudsen and Yall (1972), Guranowski et al. (1981), and Zimmerman et al. (1980) who demonstrated that inosine and IMP have little or no capacity to inhibit or inactivate SAH hydrolase. In contrast, Hershfield (1981) demonstrated that inosine irreversibly inactivated SAH hydrolase of human placenta, erythrocytes, and cultured lymphoblastoid cells when the enzyme was preincubated in the absence of homocysteine. We found that the inactivating effect of inosine was greatly reduced when homocysteine was added to the preincubation mixture. Since 1 mM inosine did not inhibit the growth of mouse lymphoblasts (PNP-deficient) and human lymphoblasts (Ullman et al. 1979; Hershfield 1981), it seems unlikely that inosine induces sufficient inactivation of SAH hydrolase to cause substantial accumulation of SAH. Thus, the inactivating effect of Z-metabolites may be substantially reduced once the metabolites are converted to IMP or inosine.

Effect of MTX and Z-metabolites on the growth of cultured human lymphoblasts

Extrapolation of the data obtained from cell culture experiments to in vivo conditions is difficult. However, MTX concentrations which were tested in cell growth studies (0.01 and 0.02 μM) were similar to the levels in human serum measured after "low-dose" MTX therapy (Edelman et al. 1984; Leeb et al. 1989; Sinnett et al. 1989).

MTX was more toxic to MOLT-4 cells (T-lymphoblasts) than Ramos cells (B-lymphoblasts). Kazmers et al. (1983) also showed that the cytotoxic concentration of MTX, which was defined as the concentration resulting in a 50% decrease in the initial density of viable cells over 72 hr, was 8-fold higher in B- (MGL-8) than T-lymphoblasts (MOLT-4). Possible mechanisms of MTX resistance in B cells are inefficient transport of MTX, inefficient conversion of MTX to $MTXGlu_n$, or less inhibition of dihydrofolate reductase in B-cells.

Z-riboside inhibited cell growth in both MOLT-4 and Ramos cells. Since our results showed that Z-riboside inhibits SAH hydrolase and ADA (table 4), it is possible that SAH, adenosine, and deoxyadenosine accumulated in the cells. Accumulation of these metabolites has been shown to nhibit the growth of lymphoid cells (Kredich and Hershfield 1979; Henderson and Scott 1980; Henderson et al. 1980; Palella et al. 1982). Therefore, inhibition of cell growth by Z-riboside may occur through inhibition of SAH hydrolase

and ADA and a subsequent accumulation of SAH, adenosine and deoxyadenosine.

Effect of Z-riboside exposure on Z-metabolite accumulation and enzyme activities

ZMP, formed as an intermediate in purine de novo biosynthesis, is readily formylated by ZMP transformylase. Therefore, under normal conditions the intracellular levels of ZMP are below the level of detection. However, ZMP accumulation can be induced following Z-riboside exposure under both in vivo and in vitro conditions (Sabina et al. 1982; Sabina et al. 1985; Dixon et al. 1989; Gruber et al. 1991). Adenosine kinase is responsible for the phosphorylation of Z-riboside to ZMP, and ZMP is further metabolized to IMP, sZMP or ZTP depending on its intracellular concentrations (Sabina et al. 1985).

MOLT-4 and Ramos cells were exposed to Z-riboside for 24 hr, and the accumulation of Z-metabolites and their effects on the activities of SAH hydrolase and ADA were examined (tables 15 and 17). SAH hydrolase was inhibited in a dose-dependent manner by Z-riboside exposure (table 15). This result may be due to an irreversible inactivation by Z-metabolites (tables 5 and 6). However, the effect of Z-metabolites on synthesis or degradation of SAH hydrolase has not been determined. The intracellular levels of Z-riboside and ZMP were below the detection limit when MOLT-4 and Ramos cells were exposed to 30, 100, and 200 μM Z-riboside. However, Gruber et al. (1991) reported that when 200 μM radioactive Z-riboside were incubated with human peripheral

blood lymphocytes for 4 hr, 43% of radioactivity was detected in Z-nucleotides (i.e., ZMP, ZDP, ZTP), mainly in ZMP.

An unidentified compound accumulated after Z-riboside exposure and was tentatively identified as either 5-amino-4-imidazole-N-succinocarboxamide riboside (sZ-riboside) or 5-amino-4-imidazole-N-succinocarboxamide ribotide (sZMP). sZMP, the immediate precursor of ZMP in purine de novo biosynthesis, and its catabolites (sZ-riboside and 5-amino-4-imidazole-N-succinocarboxamide) have been shown to accumulate in concert with an increase of ZMP (Bochner and Ames 1982; Sabina et al. 1985). sZMP and its catabolites may accumulate either as a result of reversal of the bifunctional enzyme, adenylosuccinate AMP lyase (EC 4.3.2.2), or inhibition of this enzyme by high concentrations of ZMP. sZMP and sZ-riboside possess a charged dicarboxylic acid moiety at neutral pH and thus have a greater charge density than ZMP or Z-riboside. Therefore, sZMP and sZ-riboside cannot readily cross cell membranes and may be trapped inside the cell.

In contrast to the inhibition of SAH hydrolase activity by Z-riboside exposure, the inhibition of ADA activity was not detected (table 17). However, it may be difficult to detect any inhibition after Z-riboside (a reversible competitive inhibitor) was diluted and a high concentration of adenosine (200 μM) was added in the process of determining the enzyme activity.

Effect of MTX exposure on Z-metabolite accumulation and enzyme activities

To confirm the results of Bokkerink et al. (1986) indicating that "low-dose" MTX (0.02 μM) causes ZMP accumulation while "high-dose" MTX (0.2 μM) does not, MOLT-4 and Ramos cells were exposed to 0.01, 0.02 and 0.1 μM MTX. In MOLT-4 cells, ZMP accumulated after exposure to "low-dose" MTX (0.01 or 0.02 μM), but not "high-dose" MTX (0.1 μM). These results suggest that "high-dose" MTX exposure does not cause ZMP accumulation due to a complete inhibition of GAR transformylase. However, in Ramos cells, ZMP accumulated only after exposure to 0.1 μM MTX (table 13). These results are consistent with the findings that Ramos cells are not sensitive to MTX at "low-dose" in cell growth studies (tables 9 and 10).

To determine if the difference in ZMP accumulation between MOLT-4 and Ramos cells is due to difference in inhibition of ZMP transformylase by MTX, ZMP transformylase activity was measured using the newly developed colorimetric enzyme assay (Ha et al. 1990). ZMP transformylase activity of MOLT-4 cells was inhibited more than 50% by exposure to MTX, while the inhibition of ZMP transformylase activity of Ramos cells was not observed (figures 17 and 18). These results suggest that the transformylase in MOLT-4 cells is more sensitive to MTX inhibition or that MOLT-4 cells accumulate higher levels of $MTXGlu_n$ than Ramos cells.

The data showed that neither SAH hydrolase nor ADA was inhibited by MTX in a cell-free system (page 40). However,

when enzyme activities were measured after MTX exposure in a cell culture system, SAH hydrolase in MOLT-4 cells was inhibited at the concentrations of MTX where ZMP accumulates (0.01 and 0.02 μM), and not inhibited at the concentrations of MTX where ZMP did not accumulate (0.1 μM) (table 16). These results support our hypothesis that MTX treatment causes ZMP accumulation, which results in a secondary inhibition of SAH hydrolase. However, in Ramos cells, SAH hydrolase activity was inhibited at 0.02, and 0.1 μM MTX. This finding appears to be independent of ZMP accumulation.

The results of ADA activity are more difficult to interpret because inhibition was not detected in the cells even after exposure to an inhibitor (Z-riboside) (table 18). Therefore, to confirm that ADA activity is inhibited when cells were exposed to MTX, it may be necessary to determine if the concentration of adenosine increases inside the cell or in the medium.

Anti-inflammatory and immunosuppressive mechanisms

Recently, Cronstein et al. (1991) suggested that the anti-inflammatory action of MTX is mediated by increased release of adenosine at sites of inflammation. They also demonstrated that both Z-riboside and MTX have the same effect on adenosine release, and suggested that the effect of MTX on adenosine release is due to accumulation of Z-metabolites. Our results show that in a cell-free system MTX itself does not inhibit ADA, but Z-riboside does. Therefore, we speculate that Z-metabolites, which accumulate

due to MTX exposure, secondarily inhibit ADA and cause adenosine accumulation and release.

Nesher et al. (1991) suggested that the mechanism of action of MTX is related to the inhibition of SAM-dependent transmethylation reactions by the accumulation of SAH. They observed that MTX inhibited in vitro monocyte chemotaxis in culture media containing low concentrations of methionine, and that the inhibition was reversed by addition of methionine as well as SAM. These findings also support our hypothesis that MTX indirectly inhibits SAH hydrolase.

ZMP has been shown to accumulate under certain conditions, such as folate or vitamin B_{12} deficiency (Herbert et al. 1964; Middleton et al. 1964). Deficiencies of these vitamins cause ZMP accumulation since the further metabolism of ZMP by ZMP transformylase requires 10-formyltetrahydrofolic acid (10-HCO-H_4PteGlu), which is depleted in these deficiencies. ZMP accumulation was also shown in erythrocytes of patients with Lesch-Nyhan syndrome, who lack the purine salvage enzyme, hypoxanthine guanine phosphoribosyltransferase (HGPRT) (Sidi and Mitchell 1985). ZMP accumulates in these patients probably due to an increase of de novo purine biosynthesis. As described above (tables 1 and 2), administration of MTX also causes ZMP accumulation by inhibiting ZMP transformylase. It is worth noting that nutritional folate deficiency, defects of vitamin B_{12} metabolism (i.e., pernicious anemia, transcobalamin II deficiency), Lesch-Nyhan syndrome, and MTX treatment are all

associated with suppression of immune function (Allison et al. 1975; Gross and Newberne 1980; Beisel 1982; Cooper and Rosenblatt 1987; Segal et al. 1990). Thus, there seems to be a relationship between ZMP accumulation and impaired immune function. Based on these findings, it is reasonable to conclude that ZMP accumulation is at least one mechanism to explain the immunosuppressive effect of "low-dose" MTX.

SUMMARY

Using human peripheral blood mononuclear cells (PBMC), 5-amino-4-imidazolecarboxamide riboside (Z-riboside) was found to be a reversible competitive inhibitor of S-adenosyl-L-homocysteine (SAH) hydrolase and adenosine deaminase (ADA). In addition, Z-riboside and ZMP, but not inosine and IMP, were found to inactivate SAH hydrolase irreversibly in a time-dependent manner. Neither SAH hydrolase nor ADA was directly inhibited by methotrexate (MTX).

The growth inhibitory effect of 0.02 μM MTX was potentiated by the addition of Z-riboside to the culture media of MOLT-4 cells. MOLT-4 and Ramos cells are lymphoblasts derived from human T- and B-cells, respectively. In contrast, Ramos cells were resistant to the cytotoxic effects of 0.02 μM MTX.

ZMP accumulated in MOLT-4 cells treated with 0.01 and 0.02 μM MTX, but not with 0.1 μM MTX. On the other hand, ZMP accumulation was detected at 0.1 μM, but not at 0.01 or 0.02 μM MTX in Ramos cells.

A new colorimetric assay was developed to detect low levels of ZMP transformylase in crude extracts of tissues. Using this newly developed method, inhibition of ZMP transformylase was detected in extracts of MOLT-4 cells but not

of Ramos cells treated with 0.01 or 0.02 μM MTX. These results are consistent with the finding of ZMP accumulation.

Z-riboside inhibited SAH hydrolase in both MOLT-4 and Ramos cells in culture, and SAH hydrolase in MOLT-4 cells is more sensitive to Z-riboside than in Ramos cells. Although MTX itself is not an inhibitor of SAH hydrolase, inhibition of SAH hydrolase was detected in extracts of MOLT-4 cells but not of Ramos cells when these cells were treated with 0.01 μM MTX. These findings suggest that inhibition of SAH hydrolase by 0.01 μM MTX was secondary to the accumulation of ZMP. In contrast to SAH hydrolase, inhibition of ADA was not detected in a cell-culture system.

The data presented above are consistent with the hypothesis that "low-dose" MTX treatment inhibits ZMP transformylase, causes ZMP accumulation, and a subsequent inhibition of SAH hydrolase and possibly ADA. Our data might provide an immunosuppressive mechanism of action of MTX, which preferentially targets T-cells.

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