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To Mom and Dad,

With Love

ABSTRACT

A comprehensive study of the mechanism by which the inhibition of the *de novo* biosynthesis of purine nucleotides is toxic to mouse T lymphoma (S-49) cells is presented. Equitoxic concentrations of 6-methylmercaptopurine ribonucleoside (6MMPR), an inhibitor of early *de novo* purine nucleotide biosynthesis, and mycophenolic acid (MA), an inhibitor of *de novo* guanine nucleotide biosynthesis, had similar effects on nucleic acid synthesis and on cell cycle progression. However, an equitoxic concentration of L-alanosine, an inhibitor of *de novo* adenine nucleotide biosynthesis, had minimal effects on nucleic acid synthesis and a dramatically different effect on cell cycle progression than either 6MMPR or MA. Furthermore, only an exogenous source of purine nucleotides which returned guanine nucleotide levels to normal could reverse the DNA synthesis inhibition caused by 6MMPR. These results indicate that the biological consequences of purine starvation are associated with the depletion of guanine nucleotides rather than that of adenine nucleotides. The selective depletion of GTP to levels similar to that produced by MA resulted in all of the biological consequences produced by MA. However, the selective depletion of dGTP to levels similar to that produced by MA did not affect cell growth. Studies with cells synchronized by centrifugal elutriation show that the toxicity of MA

is specific to the S-phase of the cell cycle. Addition of actinomycin D at a concentration that inhibited RNA synthesis increased the availability of GTP and reestablished normal DNA synthesis in MA treated cells. These results suggest that the depletion of GTP rather than that of dGTP produces toxic effects in S-49 cells and that GTP is required for a process involved in DNA synthesis. The relatioship of GTP to DNA synthesis was studied on the molecular level. Three possible links between GTP and DNA synthesis 1) inhibition of RNA synthesis, 2) inhibition of protein synthesis, and 3) inhibition of primer RNA oligonucleotide synthesis, were disproven. However, a tentative relationship *via* the phosphorylation of histone H1 was observed. First, in whole cells MA caused a 70% inhibition of the phoshorylation of H1, while inhibition of DNA synthesis by methods not involving the depletion of GTP did not have that effect. Second, phosphate from [γ-^{32}P]GTP is much more effectively incorporated into H1 than phosphate from [γ-^{32}P]ATP. Therefore, purine starvation may act primarily *via* GTP depletion to interrupt the normal control of chromatin structure required for DNA replication and thereby cause cellular toxicity.

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The filling of this dissertation is the culmination of both my work and my life in San Francisco. The time here has been a pleasure because of the city and the people around me. I thank my collaborators, Dr. Buddy Ullman, Dr. Vincent Groppi, and Dr. Jonathan Maybaum, for their support and the pleasure of working with them. I have been deeply touched by Dr. David W. Martin Jr., who in addition to putting a great deal of effort into my scientific development, invited me to perform much of this work in his laboratory. I am grateful to my research advisor, Dr. Wolfgang Sadee, for his insight and for allowing me the freedom to develop as an independent researcher. I couldn't have gotten through this without the bar bunch, the baseball bunch, the beach bunch, or Roger and the Reno-Tahoe shuttle. Finally, the loving support of my parents, Shirley and Irving Cohen, will never be forgotten.

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CHAPTER I
INTRODUCTION

1. OVERALL OBJECTIVE AND RATIONALE

Purine antimetabolite chemotherapeutic agents have proven to be effective anticancer, antifungal, immunosuppressive, antiviral, and antipsoriatic agents (1). The mechanisms of action of these agents are complex. They may inhibit enzymes of the purine nucleotide biosynthetic pathway, incorporate into RNA and DNA, or inhibit DNA repair enzymes (1).

My project has investigated the mechanisms involved in toxicity resulting from the inhibition of enzymes of the purine biosynthetic pathway. I have chosen model compounds which only inhibit purine nucleotide biosynthesis. Their mechanisms of action do not seem to be complicated by other sites of action since the effects of each are totally reversible by the addition of an alternate supply of purine nucleotides and no incorporation into RNA or DNA has been observed (15,36,42).

Work on the mechanisms of action of these agents had generally been limited to enzyme inhibition studies and to examinations of the gross biochemical effects (*e.g.*, inhibition of nucleic acid synthesis). Little has been published regarding how the perturbation of purine nucleotide levels lead to these gross biochemical effects. It has been accepted, with little experimental proof, that an imbalance

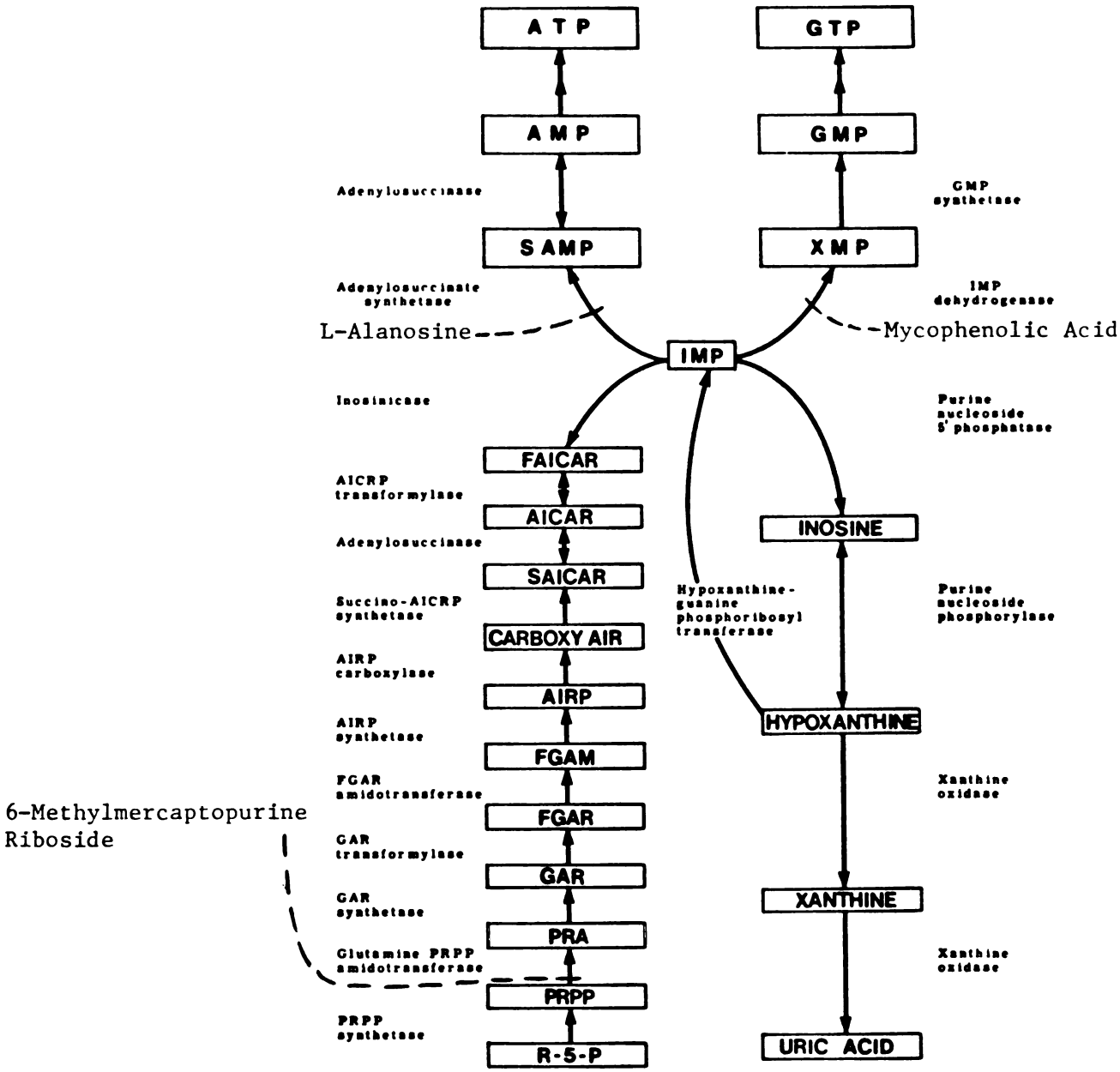

Fig. I-1. Points of Inhibition of the Purine Biosynthetic Pathway by Compounds Used in This Study.

ABREVIATIONS

AICAR: aminoimidazole carboxamide ribonucleotide

AICRP: AICribose ribose phosphate

AIRP : alribonucleotide phosphate

FAICAR: formyl AICAR

FGAM : formyl glycineamide

GAR : glycine amidoribonucleotide

FGAR : formyl GAR

PRA : phosphoribosylaine

PRPP : phosphoribosylpyrophosphate

R-5-P: ribose-5-phosphate

SAICAR: succinoAICAR

SAMP : succinoAMP

or depletion in the supply of deoxyribonuceoside triphosphates disrupts DNA synthesis and is responsible for toxicity (16,45,50,52). Thus, the goal of this project has been to determine systematically which perturbations of the nucleotide levels are responsible for cellular toxicity and to investigate, at the molecular level, how these perturbations lead to the gross biochemical effects. Understanding the specific molecular events involved should allow the development of agents which are more selective.

2. PURINE BIOSYNTHESIS AND MALIGNANCY

PP-ribose-P amidotransferase catalyzes the first uniquely committed step in the *de novo* synthesis of purine nucleotides, the formation of 5-phosphoribosylamine from PP-ribose-P and glutamine. A series of nine reactions then convert 5-phosphoribosylamine to the first recognizable purine nucleotide, inosinate. Inosinate is the branch point for the synthesis of either guanine or adenine nucleotides. Inosinate dehydrogenase and adenylosuccinate synthetase are pivotal enzymes in that they catalyze the rate limiting reactions which govern the proportion of *de novo* purine biosynthesis directed toward guanine or adenine nucleotides, respectively (Fig. I-1).

The activities of the purine biosynthetic enzymes have been studied in a series of rat hepatomas of different degrees of malignancy as measured by growth rate. The

activities of the three "key enzymes" mentioned above which are normally rate limiting for the the synthesis of purine nucleotides are altered during malignancy (2,3). In malignant cells the purine nucleotide biosynthetic pathway accelerates to provide the greater quantities of purine nucleotides required for accelerated cell growth (2,3). In the series of liver hepatomas the activity of PP-ribose-P amidotransferase increased 150% to 250% over that of normal liver. However, the observation is not significant since the activity in liver regenerating from a partial hepatectomy also increased to 180% over normal liver (4). The activity of adenylosuccinate synthetase in the hepatomas increased 160% to 370% over that of normal liver, while it was not affected by partial hepatectomy (5). Inosinate dehydrogenase has the lowest activity in normal liver of any enzyme in the purine nucleotide biosynthetic pathway. In slow growing hepatomas it increased to 200% over that of normal liver, while in fast growing hepatomas it increased to 1400% over that of normal liver. The activity in regenerating liver was 500% of that in normal liver (6).

3. SPECIFIC AIMS

Chapters II and III present methodologies developed for the purpose of studying the nucleotide metabolic pathways and used extensively for the work presented in this dissertation. These methodologies include a high performance

liquid chromatographic (HPLC) assay for the simultaneous measurement of the major purine ribo- and deoxyribonucleoside mono-, di-, and triphosphates and techniques which avoid the artifacts inherent in previous methods for determining the rates of nucleic acid synthesis by the incorporation of radiolabeled precursors.

Chapters IV and V present studies on which nucleotide perturbations are primarily responsible for cellular toxicity. Chapter IV presents research on the depletion of guanine nucleotides by mycophenolic acid, a specific inhibitor of inosinate dehydrogenase (Fig. I-1,Fig. I-2). Experiments are described which selectively depleted either guanine ribonucleotides or guanine deoxyribonucleotides, thereby allowing us to determine unambiguously that the depletion of the ribonucleotide, GTP, rather than that of the deoxyribonucleotide, dGTP, causes cellular toxicity. The chapter also contains evidence showing that it is the disruption of DNA synthesis by GTP depletion which leads to toxicity and discusses theories of the possible links between guanine nucleotides and this essential cellular process. Chapter V presents research which shows that the inhibition of early *de novo* purine biosynthesis by 6-methylmercaptopurine riboside (6MMPR) , a specific inhibitor of PP-ribose-P amidotransferase, causes cellular toxicity primarily *via* the depletion of guanine nucleotides (Fig. I-1,Fig. I-3). The biochemical consequences of total purine starvation are com-

pared to those of guanine nucleotide depletion caused by mycophenolic acid and of adenine nucleotide depletion caused by inhibition of adenylossucinate synthetase by L-alanosine (Fig. I-1,Fig. I-4).

Chapter VI contains two studies of the molecular mechanisms by which nucleotide depletion results in cellular toxicity. They investigate some processes involved in DNA synthesis for which GTP may be required.

Pertinent background literature information is presented within each chapter.

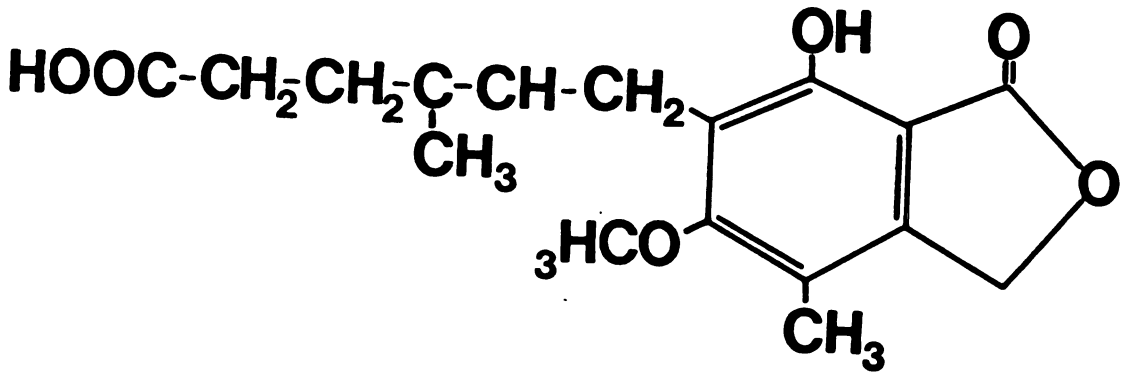

Fig. I-2. MYCOPHENOLIC ACID

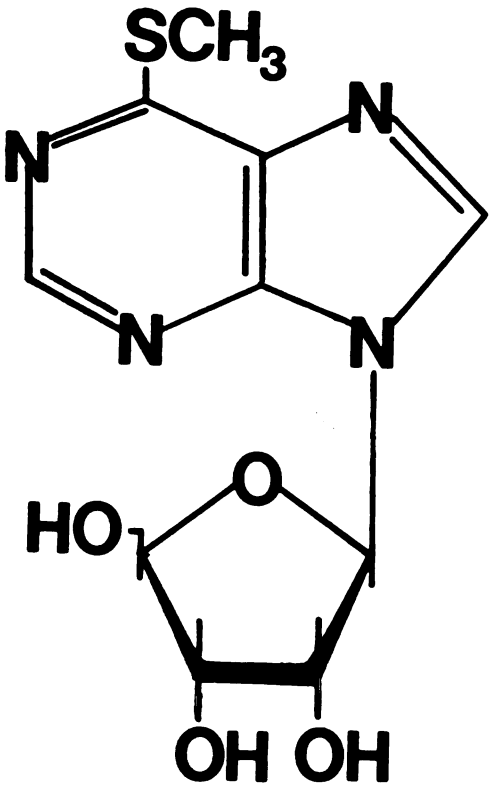

Fig. I-3. 6-METHYLMERCAPTOPURINE RIBOSIDE

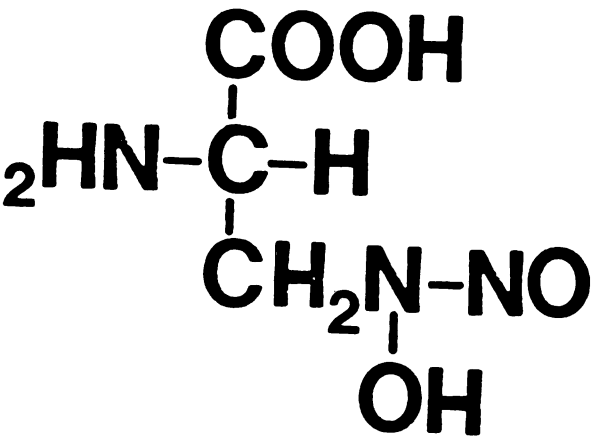

Fig. I-4. L-ALANOSINE

CHAPTER II
ANALYSIS OF PURINE RIBONUCLEOTIDES AND DEOXYRIBONUCLEOTIDES IN CELL EXTRACTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

1. INTRODUCTION

The measurement of the acid-soluble purine and pyrimidine mononucleotide pools is important in the study of the regulation of cellular function and its alteration by enzyme abnormalities or antimetabolite chemotherapeutic treatment. Current assay procedures for the measurement of the purine and pyrimidine mononucleotides are not capable of detecting all of the major ribonucleotides and 2'-deoxyribonucleotides at physiological concentrations (7-10). High-performance liquid chromatographic (HPLC) assays are usually limited to the analysis of ribonucleotides by the incomplete separation of 2'-deoxyribonucleotides from the more abundant ribonucleotides (7,8). HPLC analysis of ribonucleoside monophosphates is often hindered by their low intracellular concentrations and interfering endogenous compounds such as dinucleotide cofactors. The selective chemical degradation of ribonucleotides by periodate oxidation has been used to measure 2'-deoxyribonucleotide triphosphates, but not the corresponding mono- or diphosphates (14). The *in vitro* DNA template assay is also limited to quantitation of 2'-deoxyribonucleoside triphosphates (10).

We now report an HPLC-UV assay procedure for the determination of all the major purine mononucleotides. The assay

consists of an initial separation of the nucleotides by anion-exchange HPLC followed by hydrolysis of the collected nucleotides to nucleosides with alkaline phosphatase and quantitation by reversed-phase HPLC with UV detection. Our laboratory has previously reported an assay procedure for the measurement of pyrimidine mononucleotides (11), based on the same analytical principles, which can be combined with the purine assay presented here to allow a comprehensive analysis of cellular mononucleotide pools.

2. MATERIALS AND METHODS

2.1- Reagents- Nucleosides and nucleotides of analytical grade and alkaline phosphatase (orthophosphoric monoester phosphohydrolase; E.C. 3.1.3.2) type III, from *Escherichia coli*, at an activity of 22 I.U./mg protein, were obtained from Sigma, St. Louis,MO. [2-^{3}H]adenosine 5'-monophosphate (^{3}H-AMP), specific activity 15.8 Ci/mmol. was purchased as the ammonium salt from Amersham, Arlington, IL. Mycophenolic acid was provided by Eli Lilly and Co., Indianapolis, IN. 1,1,2 trichlorotrifluoroethane (freon) and tri-n-octylamine (amine) were purchased from Aldrich Chem. Co., Milwaukee, WI.

2.2- Apparatus- HPLC analysis was performed on a Model ALC/GPC 204 liquid chromatograph with an additional Model 6000A solvent delivery system and a Model 660 solvent programmer (Waters Assoc., Milford, MA.).

2.3- Cell Culture- The growth and lymphocytic properties of wild type mouse T lymphoma (S-49) cells have been described in detail previously (12). Cells were grown to a density of 10^6 cells/ml in Dulbecco's modified Eagle's medium with 10% horse serum at 37°C. A 100 ml volume of the cell suspension was then incubated in the presence or absence of 1.2 uM mycophenolic acid for 3 hr. An acid-soluble extract was obtained from the cells by centrifugation, PBS wash of the cell pellet, perchloric acid extraction of the cell pellet, and neutralization of the extract by ion pair extraction. The neutralization method consists of adding freon/amine solution (78:22, v/v) 1.5 times the volume of 0.4 N $HClO_4$ used. The mixture is vortexed for 30 sec then centrifuged for 5 min at ca. 1000g. The upper layer is the neutralized cell culture extract (13).

2.4- HPLC Separation of Nucleotides- Nucleotides were separated by anion-exchange HPLC on an Aminex A-29 column (30 cm x 4.0 mm I.D.; resin from Bio-Rad Labs., Richmond, CA) using the chromatographic conditions previously reported by this laboratory for the analysis of pyrimidine nucleotides (11). Briefly, a 30 min isocratic elution using 0.025 M sodium citrate, pH 8.2, was followed by a 2-hr. linear gradient to a final eluent concentration of 0.500 M sodium citrate, pH 8.2. The flow rate was maintained at 0.3 ml/min throughout the procedure and the column temperature was 50°C. Nine effluent fractions were collected (Fig. II-1)

and concentrated by lyophilization.

2.5- Enzymaic Conversion of Nucleotides to Nucleosides- Lyophilized nucleotide fractions 1-7 were reconstituted with 1 ml water. Fractions 8 and 9 were reconstituted with 1.5 ml of warm water (ca. 60°C) to dissolve the larger amounts of citrate present. One unit of alkaline phosphatase was added and the fractions were incubated at 37°C for 30 min. This enzyme preparation replaces the potato acid phosphatase previously used for this procedure (11). E. coli alkaline phosphatase has the following advantages over potato acid phosphatase: 1) the commercially available enzyme has a sufficiently high activity that protein denaturation and centrifugation are no longer required before HPLC analysis; rather the incubation mixture can be directed subjected to HPLC analysis, with a resultant increase in analytical recovery; 2) no pH adjustment is necessary since the pH of the anion-exchange HPLC eluent is close to the pH optimum of the enzyme; 3) a component of potato acid phosphatase degrades adenosine and 2'-deoxyadenosine to the base adenine which precludes the separate analysis of adenine ribonucleotides and 2'-deoxyribonucleotides with this enzyme.

2.6- Analysis of Resultant Nucleosides- A 10 ul volume of 99.7% glacial acetic acid was added to samples containing inosine, guanosine, or 2'-deoxyguanosine after phosphate hydrolysis in order to obtain reproducible retention times during reversed-phase chromatography. Fraction 4, containing

xanthosine, required the addition of 100 ul of 99.7% glacial acetic acid to obtain reproducible retention times. An aliquot of each sample up to the entire volume was injected onto a LiChrosorb RP-18 reversed-phase column (25 cm x 10 mm I.D.; Altex Scientific, Berkely, CA, .) Samples were eluted with acetonitrile-water (3.5:96.5) at a flow rate of 7.0 ml/min. Absorbances at 254 nm and 280 nm were monitored. Peaks were quantiated by peak height measurement and comparison with an external standard solution.

2.7- High Sensitivity Analysis of Nucleosides- Five to ten- fold higher sensitvity was attained, when necessary, by collecting the appropriate effluent fraction from the 10 mm I.D. reversed-phase column, concentrating it to approximately 250 ul under a stream of nitrogen at 70°C, and reinjecting it onto a uBondapak C_{18} reversed-phase column (30 cm x 3.9 mm I.D.; Waters Assoc.) using an optimized eluent at a flow rate of 2.5 ml/min. Guanosine, 2'-deoxyguanosine, inosine and xanthosine were eluted wth acetonitrile-water (2.5:97.5) buffered at pH 4.7 with 0.01 M sodium acetate. Adenosine and 2'-deoxyadenosine were eluted with acetonitrile-water (2.0:98.0) buffered at pH 3.0 with 0.01 m potassium phosphate.

3. RESULTS AND DISCUSSION

3.1 HPLC Analysis of Nucleotides

3.1.1- Separation- Fig. II-1 shows (A) the separation of a solution of purine nucleotide standards and (B) the separation of nucleotides from an extract of S-49 cells on Aminex A-29. Weak anion-exchange with citrate buffer at pH 8.2 was chosen over strong anion-exchange with phosphate buffer at acidic pH (2) because phosphate buffer inhibits alkaline phosphatase, an integral part of this assay. The fully porous anion-exchange resin Aminex A-29 was chosen over pellicular resins because of its higher sample capacity. However, this prous resin requires lower flow rates for proper performance and therefore a longer total analysis time of 4 hr. Fig. II-2 shows the nucleosides derived from nucleotides in an extract of S-49 cells on the 10 mm I.D. reversed-phase column. This high-capacity column was used because of the large amount of citrate in samples after anion-exchange, which can overload a smaller column. Fig. II-3 shows the increased sensitivity attainable when the nucleosides eluted from the 10 mm I.D. column are collected, concentrated, and reinjected onto an analytical reversed-phase column.

3.1.2- Minimun Detection Limit- The detection limit for nucleosides isolated from cell extracts on the 10 mm I.D. revesed-phase column ranges from 40 pmol/sample for guanosine to 100 pmol/sample for 2'-deoxyadenosine. A sensitivity

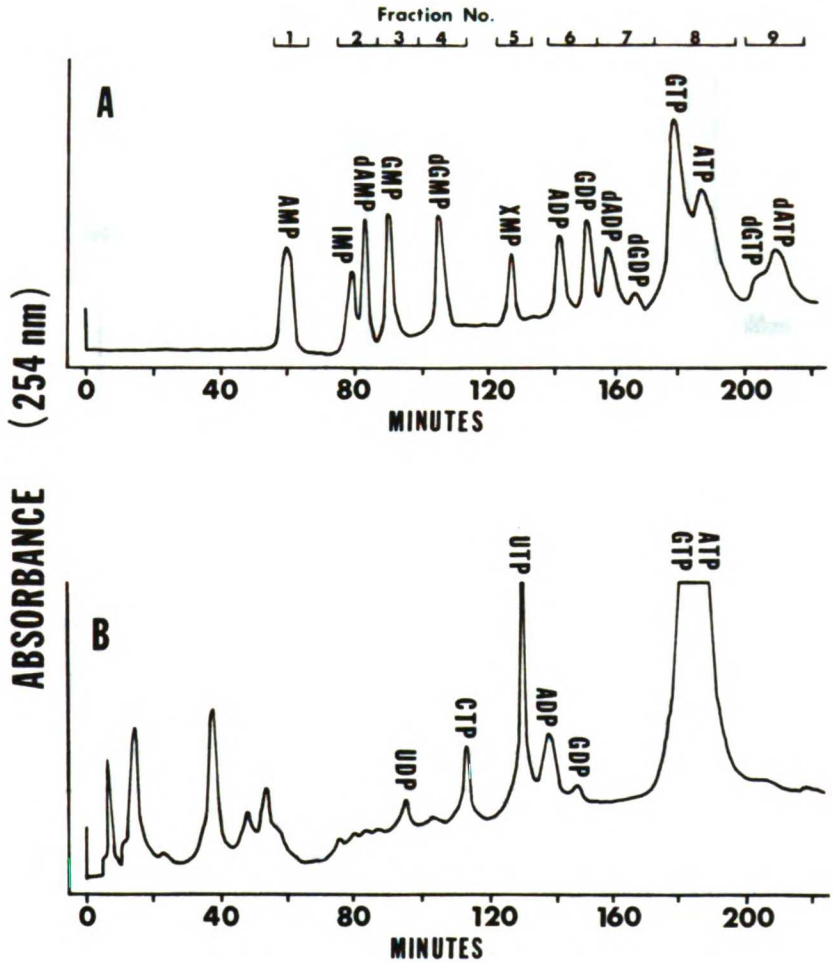

Fig. II-1 Separation of purine nucleotides by Aminex A-29 anion-exchange HPLC. (A) Chromatogram of purine nucleotide standards and fraction collection intervals. (B) Chromatogram of an acid-soluble extract from mouse T lymphoma (S-49) cells. The detector sensitivity is 0.5 absorbance units full scale.

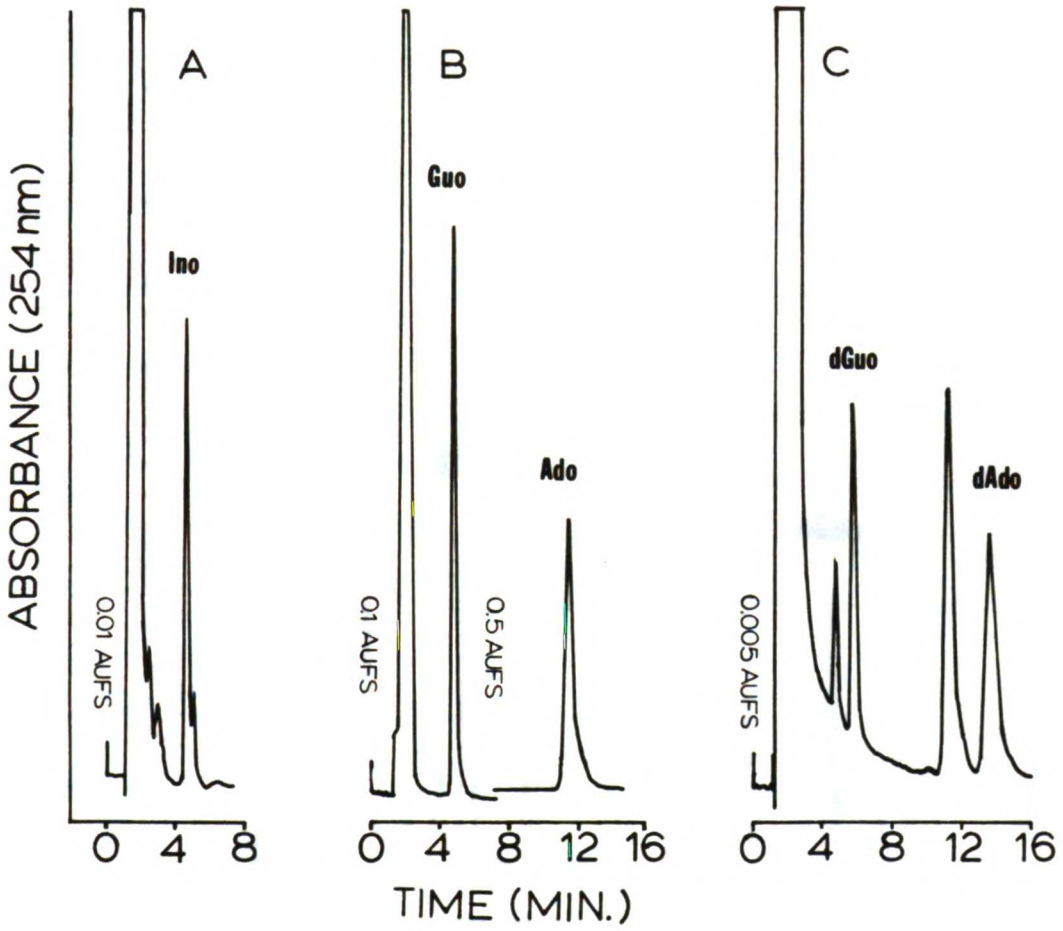

Fig. II-2. Chromatography of nucleosides derived from the nucleotides in a biological sample consisting of the extract of S-49 cells on LiChrosorb RP-18 reversed-phase column. (A) 1.48 nmol of inosine derived from IMP. (B) 13.3 nmol of guanosine derived from GTP and 74.7 nmol of adenosine derived from ATP. (C) 0.59 nmol of deoxuguanosine derived from dGTP and 1.08 nmol of deoxyadenosine derived from dATP. The peaks immediately preceding each deoxyribonucleoside are small amounts of the respective ribonucleoside derived from GTP and ATP.

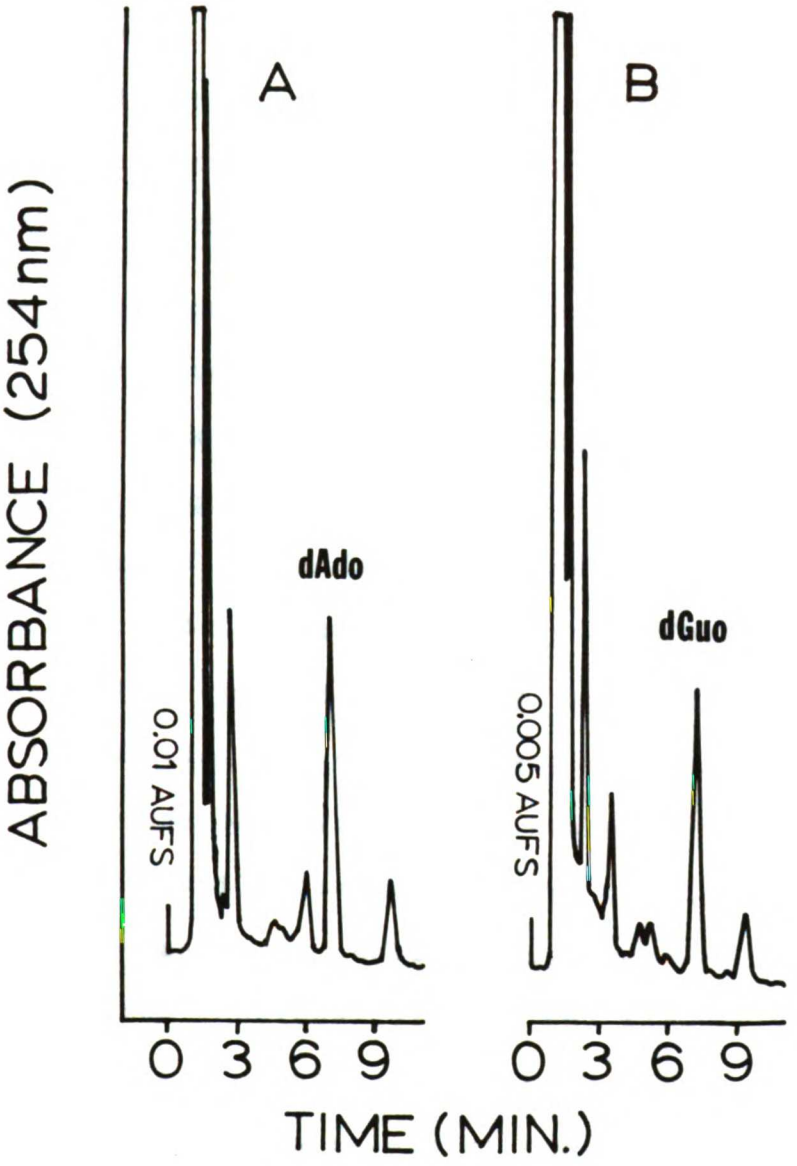

Fig. II-3. Chromatography of nucleosides derived from the nucleotides in an extract of S-49 cells which were collected from the 10 mm ID column and reanalyzed on an analytical reversed-phase column (3.9 mm ID). A) 0.103 nmol of deoxyguanosine derived from dGDP. B) 0.218 nmol of doxyadenosine derived from dADP.

of 10 pmol/sample is attainable for all nucleosides by reanalysis on the analytical reversed-phase column. In a sample of 10^8 cells only dGMP and dAMP are present at levels that require the higher sesitivity of the final analytical step. XMP was not present in detectable quantities (<10 pmol/10^8 cells) in the S-49 cell line.

3.1.3- Analytical Recovery- The analytical recovery of the method was determined by the addition of trace amounts of ^{3}H-AMP to samples at the beginning of the procedure. ^{3}H-AMP recovery after quantitation on the 10 mm I.D. column was 85+3%. The effect of the different citrate concentrations of the mono-,di-,and triphosphate fractions on quantitation by reversed-phase HPLC was studied by analyzing nucleoside standards in solutions of citrate ranging from 0.1 M to 2.5 M. No difference was observed. There was also not a significant difference (<5%) observed between quantitation on the 10 mm I.D. column and upon collection, concentration, and reanalysis of the nucleosides on the analytical column. Alkaline phosphatase quantitatively hydrolyzes nucleotides present in amounts far in excess of those found in biological samples in 30 min. Leaving samples in solution with the active enzyme longer than this does not result in any degradation of the nucleosides.

3.1.4- Precision- The precision of the assay was studied by the measurement of the nucleotide pools of three separate aliquots of the same S-49 cell extract. The stan-

dard deviations of these measurements for each nucleotide, given in Table II-1, are below 10% except for the dNMPs, which are present in levels approaching the sensitivity limit of the assay.

3.1.5- Specificity- The specificity of the method for the desired nucleosides has been confirmed by retention times on both anion-exchange and reversed-phase HPLC, peak symmetry, and the ratio of their absorbances at 254 nm to those at 280 nm. dADP cannot be quantitated under the conditions used for the high capacity reversed-phase HPLC step because an interfering compound with a UV absorbance ratio different from that of authentic 2'-deoxyadenosine; however, the interference is separated from 2'-deoxyadenosine under the conditions used for high sensitivity analysis, pH 3.0. Dinucleotide cofactors are resistant to treatment by the phosphatase enzyme, i.e. , NAD is not converted to adenosine + P_i + NMN by alkaline phosphatase, and therefore do not interfere with the assay (14).

3.1.6- Comparison With Other Methods- Table II-2 shows the nucleotide levels in S-49 cells determine by this method and those determined by other methods. There is consistency between the different methods.

Table II-1

Precision of Nucleotide Analysis

The nucleotide content of three aliquots from the same extract of S-49 cells were separately determined.

	nmol/10^8 cells	% SD		nmol/10^8 cells	% SD
AMP	3.66	6	dAMP	0.023	12
ADP	12.4	6	dADP	0.12	3
ATP	141	6	dATP	1.25	10
GMP	0.18	a	dGMP	0.009	19
GDP	2.27	1	dGDP	0.062	5
GTP	24.8	5	dGTP	0.80	9
IMP	0.42	a			
XMP	b				

a - single determination

b - not present in detectable amounts

Table II-2

Nucleotide Content of S-49 Cells as Determined by This and Alternative Methods

	Nucleotide Content (nmol/10^8 cells)				
	IMP	ATP	GTP	dATP	dGTP
This Method[a]	0.59(0.07)	130(10)	23(3)	2.0(0.1)	0.78(0.74)
Alternative Methods		185[b]	24[b]	1.4[c]	0.98[c]

a - values are the means obtained from six separate cultures parentheses contain SD

b - determined by anion exchange with UV absorbance detection (18)

c - determined by DNA polymerase assay (19)

3.2- Nucleotide Pool Size Changes in S-49 Cells Following Treatment With Mycophenolic Acid

Mycophenolic acid is a potent inhibitor of the enzyme inosinate dehydrogenase, an essential enzyme in the biosynthesis of guanine ribo- and deoxyribonucleotides from IMP (14). We are studying the toxic effects resulting from the depletion of either guanine ribonucleotides or guanine deoxyribonucleotides in S-49 cells. As a part of this study, we incubated wild type S-49 cells with 1.2 uM mycophenolic acid for 3 hr and observed the changes in purine nucleotide pool sizes. Mycophenolic acid reduced the concentrations of GTP and dGTP to 30 and 55% of control values, respectively, while the IMP level increased to approximately 170% of control. These changes can be attributed to inosinate dehydrogenase inhibition. In addition, the level of ATP was relatively unaffected while the level of dATP increased slightly to 130% of control values (Table II-3). Similar purine nucleotide pool size changes caused by mycophenolic acid have been observed in the murine lymphoma L5178Y cell line (16,17).

Table II-3

Effects of Mycophenolic Acid on the Levels of Selected Nucleotides in S-49 Cells

Exponentially growing S-49 cells were incubated for three hours with 1.2 uM mycophenolic acid. Control values are in Table II-2.

Nucleotide Content (% of Control)				
IMP	GTP	ATP	dGTP	dATP
170	30	83	55	130

4. CONCLUSION

The presented assay for purine mononucleotides yields reproducible results which are consistent with previous methods for the analysis of selected mononucleotides. Moreover, the analytical approach combining high resolution anion-exchange and reversed-phase HPLC with one enzymatic step allows the flexibility to determine any or all of the endogenous purine or pyrimidine mononucleotides as well as nucleotide derivatives of administered antimetabolite drugs. The analytical features of the method make it a more generally applicable tool than previous procedures in the study of the mononucleotide metabolism of cells.

CHAPTER III
IN VIVO RATES OF ENZYMATIC PROCESSES: THE ACCURATE DETERMINATION OF DNA SYNTHESIS RATES

1. INTRODUCTION

The rate of an enzymatic process in intact cells cannot be measured by the appearance of product as for an *in vitro* reaction because: 1)The newly formed product cannot be differentiated from that already present and 2)the product is usually immediately available as the precursor for another enzymatic process in the metabolic pathway. Radiolabeled compounds have been used in intact cells to circumvent these problems since the product formed can be distinguished from that already present and labeled molecules further along the metabolic pathway can still be identified as products of the original enzymatic process.

For the study of nucleotide metabolism the method consists of administering a radioactive necleotide precursor (*i.e.* ring constituent, base, or nucleoside), to control or treated cells and then monitoring the disposition of the label into nucleotides and nucleic acids (20,21).

The most common use of the method has been the measurement of DNA synthesis rate by the incorporation of [^{3}H]dThd into DNA. However, many reports have shown that the correlation of incorporation to synthesis rate is not universally true (22,23), especially in systems treated with nucleotide antimetabolites (24).

The inconsistency is partially due to the difficulty of determining the specific radioactivity of the dTTP pool used for DNA synthesis which, because of compartmentation, is not necessarily the same as the specific radioactivity of the total cellular dTTP pool. For example, in HeLa cells dTTP derived from the salvage pathway (dThd → dTMP) is used for DNA synthesis in preference to de novo dTTP (dUMP → dTMP) (24).

We have conducted studies which provide further evidence of errors in rates determined by the use of radiolabeled precursors. Distortions can result from: 1)the effect of drug treatment on the kinetics of label incorporation into nucleotide pools and 2)the effect of cell cycle distribution on the specific activity of nucleotide pools, as well as 3)the above mentioned compartmentation effects.

We have developed an approach to the measurement of enzymatic rates in intact cells which minimizes the distortions causd by these factors. Also, by determining the specific activities of precursor pools we are able to calculate absolute enzymatic rates, rather than the relative rates between drug-treated and control cells presented in previous approaches.

Only a short summary of data and results is presented here. A complete presentation has been published (25,26).

2. MATERIALS AND METHODS

2.1- Reagents and Apparatus- All nucleotide and nucleoside standards and HPLC solvents were of the highest quality available. [U-^{14}C]cytidine (485 mCi/mmol) was obtained from Amersham Inc., Arlington Heights, IL and [methyl-^{3}H]thymidine (49.8 Ci/mmol) from New England Nuclear, Boston, MA. Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) type III, from *E. coli* , and DNase I (deoxyribonucleate 5'-oligonucleotidohydrolase, E.C. 3.1.4.5) type I, from bovine pancreas, and phosphodiesterase I (oligonucleate 5'-nucleotidohydrolase, E.C. 3.1.4.1) type VI, from *Crotalus adamanteus*, were purchased from Sigma Co., St. Louis, MO. Radioactive analyses were carried out on a Beckman LS-9000 liquid scintillation counter, cell counting on a Coulter Counter equipped with a channel analyzer, and chromatography on a Waters Model ALC/GPC 204 liquid chromatograph.

2.2- Cell Culture- Mouse T lymphoma (S-49) cells were grown as described in chapter II. 3 uCi of [^{14}C]Cyd or 10 uCi of ^{3}H]dThd were added to 100 ml cell suspensions 30 min prior to harvest. Cells were harvested and nucleotides extracted as described in chapter II.

2.3- Nucleotide Content and Specific Activity Measurements- Pyrimidine nucleotides were analyzed as previously described (25). Peaks containing radioactive nucleosides derived from radioactive nucleotide metabolites of

administered precursors were collected, concentrated to 1 ml and after addition of 15 ml Aquasol, analyzed for ^{14}C and ^{3}H which permitted the calculation of the specific activities of these pools.

2.4- Conversion of RNA and DNA to Free Nucleosides- The acid-insoluble cell pellet was washed with water and then solubilized in 500 ul 0.3 N KOH overnight. Acidification with 200 ul of ice-cold $HClO_4$ gave a supernatant containing free ribonucleotides from hydrolyzed RNA and a pellet containing DNA. The supernatant was brought to alkaline pH with 400 ul of 0.5 M Tris buffer, pH 8.5, and then treated with 2 units of alkaline phosphatase for 2 hr to yield free ribonucleosides. The DNA pellet was suspended in 1 ml of 0.1 M Tris buffer (pH 8.5), 50 ul 0.3 M MgCl, and 200 units of DNase I, and incubated for 4 hr at 37°C, after which 0.05 units of phosphodiesterase I and 2 units of alkaline phosphatse were added and the incubation continued for 4 hr more. Free nucleosides obtained from RNA and DNA were chromatographed by HPLC and analyzed for radioactivity in Cyd and dCyd (25).

2.5- Nucleoside Content of Culture Medium- 200 ul 4.0 N $HClO_4$ was added to 4.5 ml of incubation medium collected during cell harvest. After 10 min in ice, the resulting supernatant was collected, neutralized by ion-pair extraction as in chapter II, and lyophilized. Nucleosides were chromatographed by HPLC and analyzed for radioactivity (25).

3. CALCULATION OF IN VIVO RATES

3.1- Introduction- The method uses equation III-1 to calculate the rate of an enzymatic process.

$$\text{RATE} = \frac{(\Sigma \text{ nCi of radioactivity found in all products})/\text{unit time}}{\text{average specific activity of precursor pool}} \quad \text{(EQ. III-1)}$$

The products and precursor pools for each process are listed in Table III-1. The denominator of equation III-1, time average specific activity, must be determined for the precursor pool of each enzymatic process to be measured [^{14}C]CTP for RNA synthesis and [^{14}C]dCTP for DNA synthesis) as they each exhibit a unique variation with time. For this purpose we constructed a simple model of the pyrimidine nucleotide metabolic pathway and used this model to derive equations III-2 and III-3 which give the time average specific activity of CTP and dCTP.

3.2- Model-

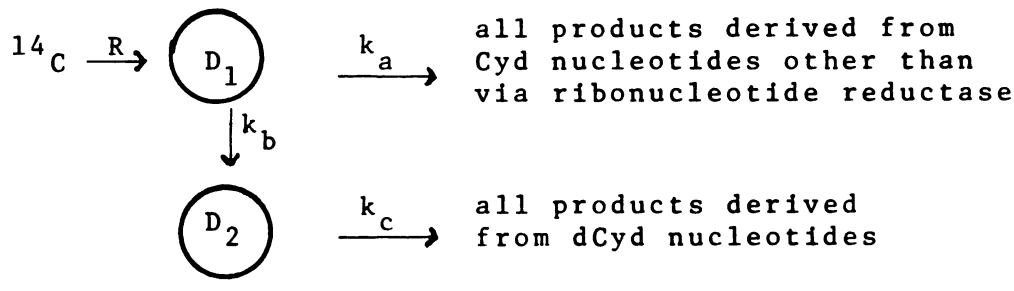

Table III-1

Precursor and Product Pools for *in vivo* Rate Calculations.

Enzymatic Process	Precursor Pool	Product Pool
RNA Synthesis	CXP	Cyd in RNA
DNA Synthesis	dCXP	dCyd in DNA
DNA Synthesis*	dTXP	dThd in DNA

*when using labeled dThd

3.3- Definition of Terms in This Section-

R= zero order input rate of [^{14}C]Cyd into CTP

D_1= DPM as CTP (varies with time)

D_2= DPM as dCTP (varies with time)

k_a, k_b, k_c= 1st order rate constants for disposition of ^{14}C

k_{ab}= $k_a + k_b$

P_1= pool size of CTP (nmole)

P_2= pool size of dCTP (nmole)

$S.A._{1,Avg}$= time average specific activity of CTP (DPM/nmole)

$S.A._{2,Avg}$= time average specific activity of dCTP (DPM/nmole)

$S.A._{1,SS}$= steadt state specific activity of CTP

3.4- Calculation of Time Average Specific Activity-

$$S.A._{1,Avg} = \frac{R}{P_1 k_{ab} t}\left[t + \frac{e^{-k_{ab}t} - 1}{k_{ab}}\right] \quad \text{(EQ. III-2)}$$

$$S.A._{2,Avg} = \frac{R}{k_{ab} P_1} + \frac{[k_b R k_{ab}^2 (e^{-k_c t} - 1) + k_c^2 (e^{-k_{ab} t})]}{P_2 t k_c^2 k_{ab}^2 (k_{ab} - k_c)} \quad \text{(EQ. III-3)}$$

The following parameters must be determined: P_1, P_2, R, k_{ab}, k_b, k_c.

P_1 and P_2 are pools sizes which can be measured directly.

R may be obtained by one of two methods:

R= DPM in nucleotides, nucleic acids, and medium other than [^{14}C]Cyd

$$R = S.A._{1,SS}\ k_{ab}\ P_1$$

$k_{ab} = \frac{.693}{t_{1/2}^{CTP}}$ where $t_{1/2}^{CTP}$ is the time for the specific activity of CTP to reach one-half of steady-state.

$$k_b = \frac{\text{DPM in dCTP, dUMP, dTTP, dUrd in medium, and DNA}}{\text{DPM in all pools derived from CTP}} (k_{ab})$$

$$k_c = \frac{k_b P_1}{P_2}$$

4. RESULTS AND DISCUSSION

In control cells 7.2 nmol/10^8cells/30 min of dCTP was incorporated into DNA using [^{14}C]Cyd as a precursor, while 31.9 nmol10^8cells/30 min of dTTP was incorporated using [^{3}H]dThd as a precursor. An indenpendent measure of the rates of incorporation of dCTP and dTTP into DNA was performed. The actual amount of dC and dT in the DNA of S-49 cells was measured as 4.79 and 6.10 nmol/10^8cells, respectively, by the chromatographic analysis of hydrolyzed DNA. Also, an exponentially growing cell population with a doubling time of 20 hr (observed) produces 1.71 X 10^6new cells/10^8cells/30 min. Therefore, the expected rates of dCTP and dTTP incorporation into DNA are 8.14 and 10.43 nmol/10^8cells/30 min, respectively. The experimental value for the rate of dCTP incorporation into DNA using [^{14}C]Cyd as a precursor agrees well with the expected value, while the experimental value for the rate of dTTP incorporation into DNA using [^{3}H]dThd as a precursor is substantially higher than the expected value. This may result from preferential use of the salvage pathway for nucleotides for DNA synthesis (24). Furthermore, when the growth of cells was inhibited by 50% with 5-fluorouracil (FUra) the DNA synthesis rate determined by the incorporation of [^{14}C]dCTP decreased propotionally to 4.9 nmol/30 min/10^8cells, while the rate according to [^{3}H]dTTP incorporation actually

increased to 64.3 nmol/30 min/10^8cells. This paradoxical increase may result from the inhibition of de novo thymidine nucleotide biosynthesis by FUra, making DNA synthesis more dependent on the labeled nucleotide from the salvage pathway. Finally, there is great variability of the specific activity of [^{3}H]dTTP derived from [^{3}H]Thd in the different phases of the cell cycle, while that for [^{14}C]dCTP derived from [^{14}C]Cyd is relatively constant (Table III-2). Therefore, agents which affect the cell cycle distribution might lead to erroneous results from the incorporation of [^{3}H]dTTP, while having minimal effects on results from the incorporation of [^{14}C]dCTP.

In conclusion, the DNA synthesis rates determined by the incorporation of [^{14}C]dCTP from [^{14}c]Cyd into DNA is more reliable than those determined by the incorporation of [^{3}H]dTTP from [^{3}H]dThd. By introducing the label through CDP-reductase, the only source of dCyd nucleotides in the absence of exogenous dCyd, the factors which distort the measurement of nucleic acid synthesis rates can be minimized.

Table III-2

Pool Sizes and Specific Activities of Selected Nucleotide Pools in Synchronized S-49 Cells

Nucleotide	Cell Fraction		
	I(G_1)	II(S)	III(late S + G_2 + M)
	Nucleotide Content (nmol/10^8cells)		
dCTP	0.87	1.71	1.52
CTP	20.80	20.70	29.70
dTTP	0.99	0.72	2.02
	Specific Activity (DPM/nmol)		
dCTP[1]	12356	19128	15591
CTP[1]	16028	15266	13443
dTTP[2]	106768	26491	49886

[1]using [^{14}C]Cyd as a precursor

[2]using [^{3}H]dThd as a precursor

CHAPTER IV
GUANINE NUCLEOTIDE DEPLETION AND TOXICITY IN MOUSE T LYMPHOMA (S-49) CELLS

1. INTRODUCTION

MA, virazole, and 6-chloropurine, three drugs which have shown antineoplastic or antiviral activity, each inhibit the *de novo* synthesis of guanine nucleotides. None of these compounds has been found to incorporate into either RNA or DNA (15,27,28). The toxicity of these agents appears to be associated with the depletion of guanine nucleotides.

MA, an inhibitor of two enzymes which are essential for the *de novo* synthesis of guanine nucleotides (inosinate dehydrogenase and guanylate synthetase), causes a depletion of both GTP and dGTP, a partial reduction of RNA synthesis, and a drastic inhibition of DNA synthesis. However, these effects are preventable by an exogenous supply of guanine nucleotides *via* the salvage pathways (15,29). It has been suggested that toxicity is caused by an inhibition of DNA synthesis resulting from the depletion of dGTP (16). However, there are cells which grow normally despite low dGTP levels (32).

The purpose of this study was to determine the mechanism by which guanine nucleotide depletion produces toxic effects and to resolve whether GTP depletion or dGTP depletion is responsible for the toxicity. We present evidence that it is the depletion of GTP rather than that of dGTP

which is toxic to cells, that GTP depletion is asociated with the inhibition of DNA synthesis, and that cells which are replicating DNA suffer the majority of toxity.

2. MATERIALS AND METHODS

2.1- Reagents and Apparatus- Nucleotides and nucleosides of analytical grade, alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) type III, from *E. coli* , DNase I (deoxyribonucleate 5'-oligonucleotidohydrolase, E.C. 3.1.4.5) type I, from bovine pancreas, and phosphodiesterase I (oligonucleate 5'-nucleotidohydrolase, E.C. 3.1.4.1) type VI from *Crotalus adamanteus* were purchased from Sigma Co., St. Louis, MO. [U-^{14}C]cytidine (485 mCi/mmol), was purchased from Amersham Inc.,Arlington Heights, IL. HPLC analyses were performed on a model ALC/GPC 204 liquid chromatograph (Waters Assoc., Milford, MA), cell density measurements on a model Z_f Coulter counter (Coulter Electronics, Hialea, FL), radiactive analyses on a Beckman 9000LS liquid scintillation counter, and centrifugal elutrations with a Beckman JE-6 elutriator (Beckman Inst., Palo Alto, CA).

2.2- Cell Culture- Mouse T lymphoma (S-49) cells were grown as described in chapter II.

Growth experiments were performed in Costar (24 wells) tisuse culture plates. Two ml of cell suspension (2 X 10^5 cells/ml) were added to the combination of agents to be

tested. After 24 hr, cell density was measured by Coulter counter. Percent growth is defined as

$$\frac{\text{(Final treated density)-(initial density)}}{\text{(Final control density)-(initial density)}}$$

Treatment was considered lethal when the final cell density was less than the initial density after a 24 hr incubation. Pulse exposure of asynchronous or synchronized S-49 cells to MA was performed on 10 ml of cell suspension (2.5×10^5 cells/ml). The cells were collected and resuspended twice in fresh medium to remove all traces of MA before being pipetted into tissue culture plates. Control cells were handled in the same way.

Nucleotide content and nucleic acid synthesis rates were measured in cells grown to density of 10^6 cells/ml in 100 ml of medium, then incubated in the combination of agents to be tested for 3 hr. Cells were harvested and nucleotides extracted as described in chapter II. 0.3 M KOH was added to the acid-insoluble pellet.

2.3- Analysis of Acid-soluble Extract- Concentrations of purine and pyrimidine ribonucleotides and deoxyribonucleotides in the acid-soluble extract were determined by a modification of the assay procedure reported in chapter II. Briefly, the nucleotides are initially separated by anion-exchange HPLC on a Aminex A-29 column (4.0 x 300 mm) using a 16 min isocratic elution with 0.175 M sodium citrate, pH 8.2, followed by isocratic elution with 0.500 M sodium

citrate, pH 8.2. The flow rate is 0.5 ml/min and the column temperature is 50°C. The nucleotides are then hydrolyzed to their respective nucleosides by alkaline phosphatase and quantified by reverse phase HPLC with UV absorbance detection. Pyrimidine nucleosides were chromatographed as previously described (11).

2.4- Analysis of RNA and DNA Synthesis Rates- The rates of RNA and DNA synthesis were measured by the incorporation of [U-^{14}C]cytidine into acid-insoluble material corrected for specific activity of the appropriate precursor, i.e. CTP or dCTP. The RNA and DNA in the acid-insoluble material was cleaved to ribonucleosides and deoxyribonucleosides as described in chapter III and the radioactivity in Cyd and dCyd separated by HPLC and measured by liquid scintillation counting.

2.5- Cell Sorting by Centrifugal Elutriation- Three hundred ml of exponentially growing cells at a density of 10^6 cells/ml were collected, resuspended in 10 ml of medium, and immediately introduced into the elutriator. Three fractions (f_1, f_2, and f_3) which consist mainly of cells in the G_1, S, and G_2/M phases of the cell cycle, respectively, were collected as previously described (33).

3. RESULTS

3.1- Toxicity of Mycophenolic Acid- The growth inhibitory and cytotoxic effects of MA on wild type S-49 cells and on HGPRTase-deficient (6MPR-3-3) S-49 cells were examined. After a 24 hr incubation with 1.2 uM MA, there was no net growth of either wild type or HGPRTase-deficient cells. No significant difference in sensitivity to MA was observed for wild type and HGPRTase-deficient mutant cells (Fig. IV-1).

3.2- Toxicity of Deoxyadenosine- After a 24 hr incubation of wild type S-49 cells with 10 uM dAdo and 10 uM EHNA to prevent dAdo deamination, there was 27% of control growth. However, addition of 10 uM dCyd to the medium allowed the normal growth of cells in concentrations of dAdo up to 10 uM.

3.3- Prevention of Mycophenolic Acid Toxicity- The ability of Guo or dGuo to prevent MA toxicity in wild type S-49 cells and the growth inhibition caused by the nucleosides themselves were investigated. Forty uM Guo caused slight growth inhibition over a 24 hr incubation (Fig. IV-2) while dGuo (>20 uM) resulted in drastic growth inhibition (Fig. IV-3). Guo and dGuo at concentrations to 20 uM were equally effective at preventing MA toxicity in wild type cells, but at higher concentrations, Guo provided additional protection (Fig. IV-2), while dGuo exhibited its own inhibitory effect (Fig. IV-3).

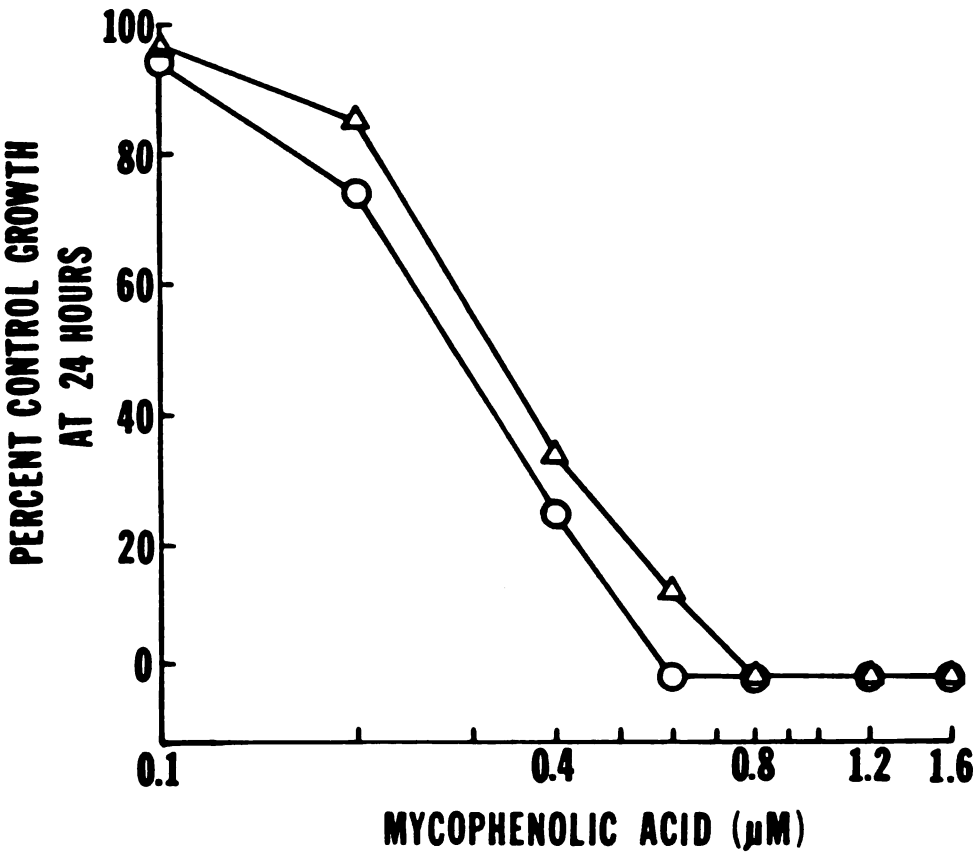

Fig. IV-1. Growth Inhibition of S-49 Cells by Mycophenolic Acid. Wild type (○) and HGPRTase deficient (△) S-49 cells were grown in the presence of increasing concentrations of mycophenolic acid for 24 hr. Results are those of a single typical experiment.

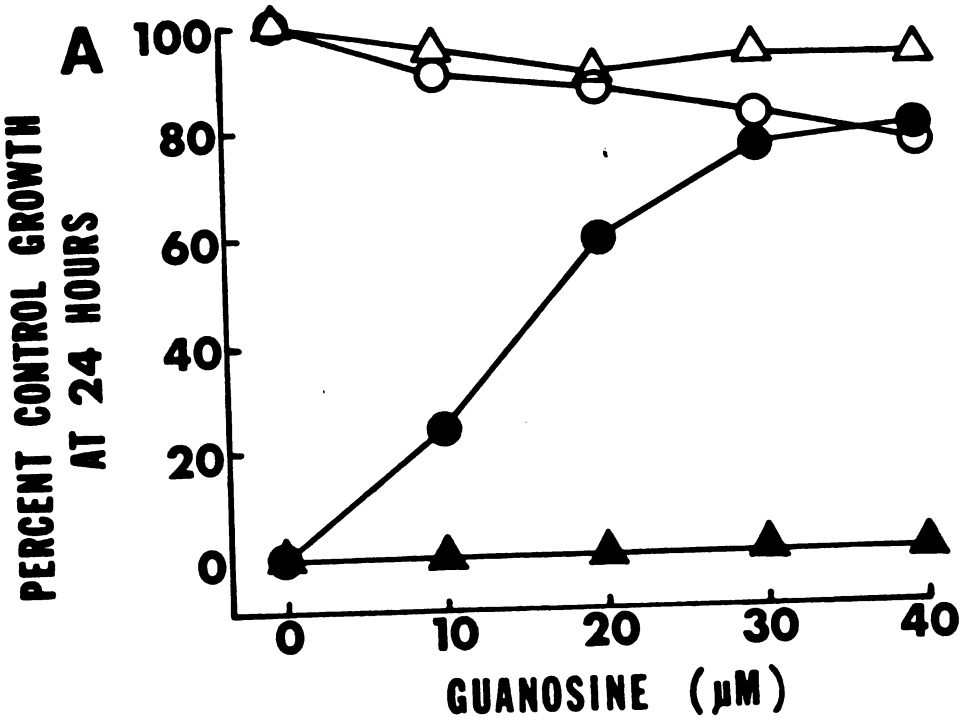

Fig. IV-2. Prevention of Mycophenolic Acid Toxicity by Guanosine in S-49 Cells. Wild type (●) and HGPRTase deficient (▲) S-49 cells were incubated with a toxic concentration of mycophenolic acid and increasing concentrations of guanosine for 24 hr. Wild type (△) and HGPRTase deficient cells (○) were also incubated with increasing concentrations of guanosine in the absence of mycophenolic acid. Results are those of a single typical experiment.

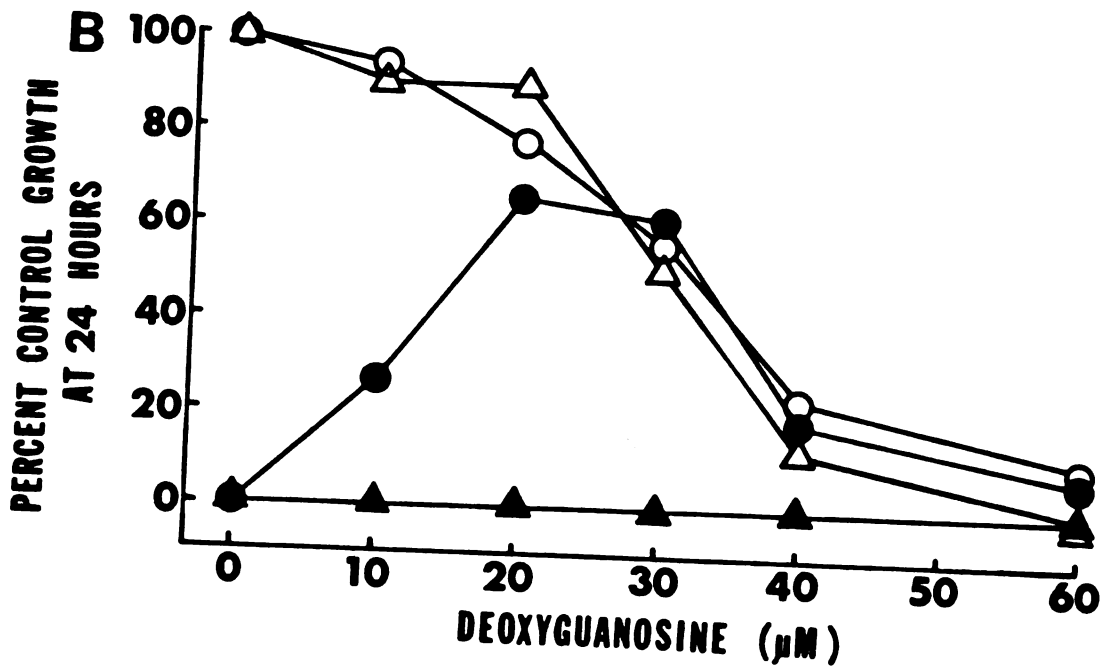

Fig. IV-3. Prevention of Mycophenolic Acid Toxicity by Deoxyguanosine in S-49 Cells. Wild type (●) and HGPRTase deficient (▲) S-49 cells were incubated with a toxic concentration of mycophenolic acid and increasing concentrations of deoxyguanosine for 24 hr. Wild type (○) and HGPRTase deficient cells (△) were also incubated with increasing concentrations of deoxyguanosine in the absence of mycophenolic acid. Results are those of a single typical experiment.

Neither Guo nor dGuo were able to prevent MA toxicity in HGPRTase-deficient S-49 cells. The growth inhibitory effect of dGuo in this mutant cell line was the same as in wild type cells, while Guo did not affect the growth of these cells (Fig. IV-2 and IV-3)(35).

3.4- Nucleotide Level Changes- The feedback mechanisms of the nucleotide biosynthetic pathways are complex and can cause wide ranging secondary changes in nucleotide levels (17). We therefore measured not only GTP and dGTP pools, but also a series of other relevant intracellular purine and pyrimidine ribo- and deoxyribonucleotide levels (Table IV-1) to determine whether these secondary effects are a factor in MA toxicity. Incubation of wild type S-49 cells with 1.2 uM MA for 3 hr reduced the concentration of GTP and dGTP to 30% and 55% of control values, respectively, while the level of IMP increased to 170% of control. The concentrations of the pyrimidine ribonucleotides and of the three other deoxyribonucleotides all increased, while the concentration of ATP was relatively unaffected (Table IV-1). These results are similar to those observed after inhibition of inosinate dehydrogenase in L1210 and L5178Y cells except that a decrease in dCTP levels was observed in L5178Y cells (16,30). Incubation of HGPRTase-deficient cells with 1.2 uM MA for 3 hr resulted in nucleotide level changes similar to those observed in wild type S-49 cells (Table IV-1).

Table IV-1

Nucleotide Levels in S-49 Cells After Various Treatments

The cultures were incubated and the nucleotide levels determined as described in methods.

	Nucleotide Content (nmol/10^8 cells)							
	GTP	dGTP	ATP	dATP	CTP	dCTP	UTP	dTTP
Wild Type								
control[a]	23(3)	0.78(0.04)	130(10)	2.0(0.1)	19(1)	2.0(0.3)	92(10)	2.0(0.4)
1.2 uM MA[b]	6.8(1.2)	0.43(0.04)	110(7)	2.6(0.1)	27(2)	3.1(0.6)	130(4)	6.0(1.2)
1.2 uM MA + 20 uM dGuo[c]	50(2)	1.6 (0.02)	110	2.0	28	2.3	98	2.3
1.2 uM MA + 0.05 uM act. D[c]	22(3)	0.93(0.18)	130	2.2	28	3.7	150	4.2
6 uM dAdo + 10 uM EHNA+ 10 uM dCyd[c]	23(3)	0.51(0.01)	120	2.5	23	2.0	72	3.9
HGPRTase Deficient								
control[c]	20(2)	1.2 (0.08)		2.5	29	2.4	96	3.1
1.2 uM MA[c]	4.3(1.0)	0.47(0.04)		3.6	46	3.4	290	5.0
1.2 uM MA + 20 uM dGuo[c]	4.4(0.4)	1.6 (0.03)		2.3	33	3.0	250	4.7

a - values are the means obtained from six separate cultures. Parentheses contain SD.

b - values are the means obtained from triplicate cultures. Parentheses contain SD.

c - values are the means obtained from duplicated cultures. Parentheses contain 1/2 of the range

Nucleotide levels in both wild type and HGPRTase-deficient S-49 cells were measured after a 3 hr incubation with 1.2 uM MA and 20 uM dGuo. In wild type S-49 cells the depletion of both GTP and dGTP by MA was prevented by dGuo. However, in HGPRTase-deficient S-49 cells the depletion of dGTP was prevented while the depetion of GTP was not. With the addition of dGuo to MA-exposed cells, the levels of many of the other nucleotides returned toward normal values in both cell lines, and no other nucleotides were depleted (Table IV-1).

Nucleotide levels in wild tyoe S-49 cells were also measured after a 3 hr incubation with 6 uM dAdo, 10 uM EHNA, and 10 uM dCyd, a combination that did not reduce growth of the cells and should selectively deplete dGTP. The concentration of dGTP was reduced to 65% of control while that of GTP remained normal. No other nucleotide levels were significanty perturbed by this treatment (Table IV-1).

Incubation of wild type S-49 cells for 3 hr with 1.2 uM MA and 0.05 uM actinomycin D resulted in GTP and dGTP levels which were close to normal. The levels of other nucleotide triphosphates were elevated by varying degrees (Table IV-1).

When wild type cells were incubated for 3 hr with 100 uM Guo or dGuo, the GTP level was elevated to the same extent, approximately 350%. However, incubation with dGuo was far more effective than Guo at elevating the dGTP level, 1500% <u>versus</u> 250%.

3.5- DNA and RNA Synthesis Rates- The relationship of DNA and RNA synthesis rates to the observed guanine nucleotide depletion was studied. Between 2.5 and 3 hr after the addition of MA, the rate of DNA synthesis was less than 5% of control in both wild type and HGPRTase-deficeint S-49 cells (Table IV-2). Wild type cells incubated with both MA and dGuo showed no inhibition of DNA synthesis during the same time period. However, HGPRTase-deficient mutants incubated with both MA and dGuo were only capable of DNA synthesis at 19% of control (Table IV-2). Similar results were seen for RNA synthesis rates. RNA synthesis in both wild type and HGPRTase-deficient cells dropped to approximately 60% of control during the labeling period. Addition of dGuo resulted in complete recovery of RNA synthesis in wild type cells, while there was only a partial recovery to 74% of control RNA synthesis in the HGPRTase-deficient cells (Table IV-2).

0.05 uM actinomycin D inhibited RNA synthesis without affecting DNA synthesis (Table IV-2), thereby reducing the demand for GTP. Inhibition of RNA synthesis in MA-treated cells by actinomycin D allowed DNA synthesis to proceed normally. (Table IV-2).

3.6- Cell Cycle Specificity of Mycophenolic Acid- Exposure of asynchronous cells to 10 uM MA for less than 3 hr did not affect cell growth. Exposure for longer periods of time resulted in increasing toxicity. Cells resumed normal

Table IV-2

Changes in Nucleic Acid Synthesis Rates in S-49 Cells

The cultures were incubated and the synthesis rates determined as described in methods. Values are the averages obtained from duplicate cultures.

	Nucleic Acid Synthesis (% of Control)	
Wild Type	RNA	DNA
1.2 uM MA	54	2
1.2 uM MA + 20 uM dGuo	112	96
0.05 uM act D	48	100
1.2 uM MA + 0.05 uM act D	26	97
HGPRTase Deficient		
1.2 uM MA	63	2
1.2 uM MA + 20 uM dGuo	74	19

growth within 24 hr after removal of the drug (Fig. IV-4).

Exposure of synchronized cells collected by centrifugal elutriation to 10 uM MA for 3 hr had a pronounced efect on cells in S-phase, the period of DNA synthesis, but minimal effect on cells in G_1-phase or G_2-phase. Untreated cells in G_2, S, or G_1 underwent mitosis at approximately 3, 7 and 14 hr, respectively, after elutriation. Treated cells in G_2 and G_1 divided along with their control counterparts, but cells in S were significantly inhibited (Fig. IV-5).

4. DISCUSSION

4.1- The Salvage of Guanosine and Deoxguanosine by S-49 Cells- An understanding of the salvage patterns

for Guo and dGuo is helpful for interpreting the data in this report. Guo is salvage to GTP *via* guanine by the sequential actions of purine nucleoside phosphorylase, HGPRTase, and kinases, and further converted to dGTP by ribonucleotide reductase (35) (Fig. IV-6). dGuo is salvaged to GTP and dGTP by the same enzymes as is Guo. However, dGuo is also directly phosphorylated to dGMP by deoxycytidine kinase followed by rapid conversion to dGTP by other nucleotide kinases (35) (Fig. IV-6). Guo is not directly phosporylated to GMP by any kinase in mammalian cells. Therefore, in cells deficient in the enzyme HGPRTase one would not expect the salvage of either Guo of dGuo to GTP to be possible, while the direct phosphorylation of dGuo

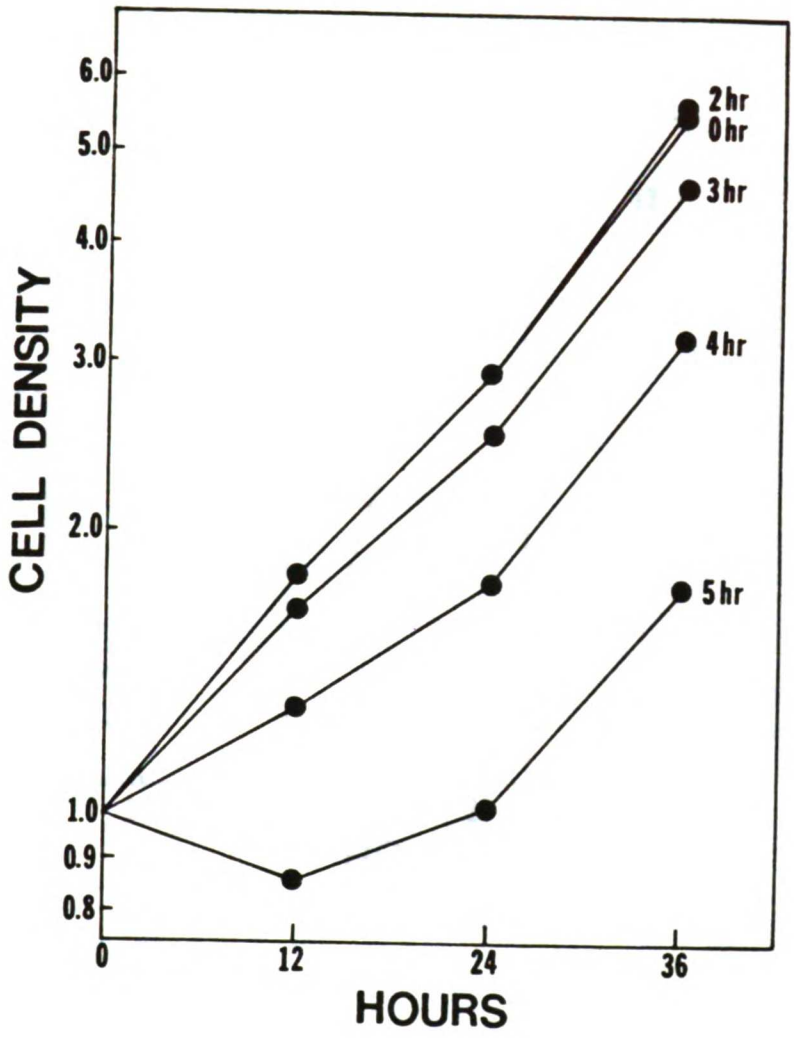

Fig. IV-4. Effects of a Pulse Exposure to 10 uM Mycophenolic Acid on the Growth Kinetics of Asynchronous S-49 Cells. Cells were exposed to mycophenolic acid for the time periods at the right and then incubated as described in methods. Each point represents the average cell density of duplicate wells which differed by <5%.

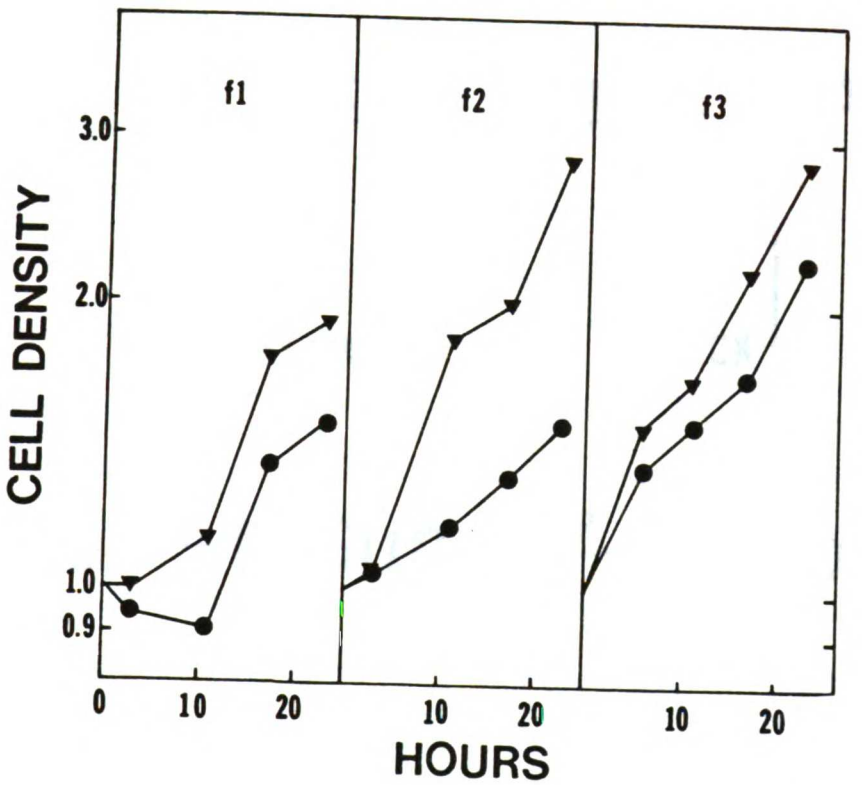

Fig. IV-5. Effects of a 3hr Exposure to 10 uM Mycophenolic Acid on the Growth Kinetics of Synchronized S-49 Cells. Elutriated Cells were collected into three fractions (f1,f2, and f3). Control cells (▼) and cells exposed to mycophenolic acid (●) were incubated as described in methods. Zero time is the beginning of the exposure.

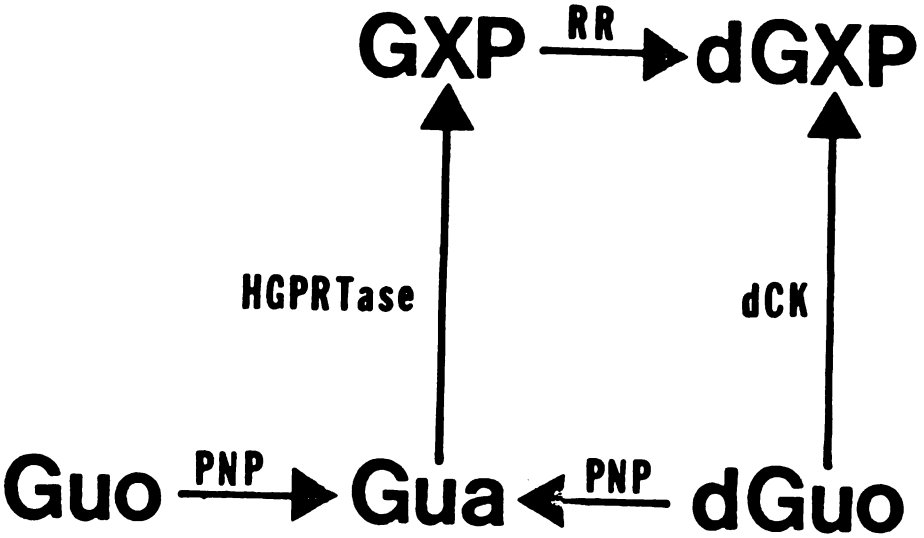

Fig. IV-6. Salvage Pathways of Guanosine and Deoxyguanosine in S-49 Cells. The enzymes involved are PNP, purine nucleoside phosphorylase; HGPRTase, hypoxanthine-guanine phosphoribosyltransferase; dCK, deoxycytidine kinase; R.R., ribonucleotide reductase. XP is defined as mono,di, or triphosphate.

to dGTP could still proceed.

4.2- Effect of Guanosine and Deoxyguanosine on Mycophenolic Acid Toxicity- The growth-inhibitory effects of MA are preventable by addition of an guanine to the culture medium as an alternate source of guanine nucleotides *via* the salvage pathway (15). The inability of Guo to prevent MA toxicity in the HGPRTase-deficient cells confirms that phosphoribosylation of the guanine base is required for the protective effect (Fig. IV-2).

If dGTP depletion were responsible for MA toxicity, then dGuo should be a more effective rescue agent than Guo since dGuo is more effective at elevating dGTP levels. The equal efficacy of Guo and dGuo at preventing MA toxicity in wild type cells suggests that salvage into GTP rather than into dGTP is responsible for alleviating the toxicity.

4.3- Selective Depletion of GTP and dGTP- The selective depletion of either GTP or dGTP directly addresses the question of whether GTP depletion or dGTP depletion is responsible for the toxicity resulting from the inhibition of *de novo* guanine nucleotide biosynthesis. We were able to deplete selectively dGTP to a level similar to that caused by MA by incubation of wild type S-49 cells with the nucleosides dAdo and dCyd along with the adenosine deaminase inhibitor EHNA to prevent rapid degradation of dAdo. Growth inhibition by dAdo results from depletion of dCTP, dTTP, and dGTP caused by an inhibition of ribonucleotide reductase by

elevated levels of dATP (19). Addition of dCyd to the culture medium provides an alternate source of both dCTP and dTTP, but not of dGTP, and is effective at preventing toxicity.

Selective depletion of GTP was achieved by incubation of HGPRTase-deficient S-49 cells with MA and dGuo; the salvage pathway from dGuo to GTP is not present in these cells while the pathway to dGTP is intact. At a level of GTP depletion that was associated with MA toxicity in wild type cells, toxicity was also observed in the MA+dGuo-treated mutant cells. Thus, MA toxicity appears to be caused by the depletion of GTP alone.

Pool size measurements confirm that GTP and dGTP were depleted independent of each other in the above experiments (Table IV-1). Also, the near normal level of other nucleotides in MA+dGuo-treated cells, while toxicity remained, demonstrate that the changes in the levels of other nucleotides in MA-treated wild type cells are not likely contributors to toxicity.

4.4- Effect of GTP Depletion on DNA Synthesis- Previous studies and our own results show that MA causes a drastic inhibition of DNA synthesis. Therefore, we wanted to determine the relationship of this inhibition to GTP depletion and to toxicity. The selective return of dGTP levels to normal by dGuo in MA-treated HGPRTase-deficient cells did not establish normal DNA synthesis. Only an alternate

supply of GTP, e.g. Guo or dGuo in wild type cells, was able to prevent this inhibition. Therefore, the selective depletion of GTP is associated with DNA synthesis inhibition; demonstrating for the first time in intact cells that the ribonucleotide, GTP, may be required for DNA synthesis. Also, the selective toxicity of MA on S-phase cells, that portion of the cell cycle during which DNA synthesis takes place, indicates that MA toxicity is related to the inhibition of DNA synthesis.

4.5- The Effect of RNA Synthesis on the Availability of GTP and DNA Synthesis- RNA synthesis is not severely inhibited by a toxic concentration of MA. It is possible that this continued RNA synthesis in the presence of MA critically depletes GTP which would normally serve a function in DNA synthesis. Therefore, actinomycin D was used to further inhibit RNA synthesis thereby reducing the demand for GTP. Indeed, this reduced demand for GTP for RNA synthesis allowed DNA synthesis to proceed normally in the presence of MA. These results show that the inhibition of DNA synthesis by MA does not result from an inability of the cells to enter S-phase because of a lack of RNA synthesis in G_1-phase (31), since DNA synthesis proceeded while RNA synthesis was inhibited more than that caused by MA alone.

4.6- Possible Links Between GTP Depletion and the Inhibition of DNA Synthesis- There are many cellular functions which might link the depletion of GTP to an inhibition of

DNA synthesis. Some posibilities can be evaluated using evidence already available. For example, GTP is necessary for both RNA and protein synthesis, and decreased RNA or protein synthesis can lead to an inhibition of DNA synthesis (31). However, incubation of L5178Y cells with MA did not severely inhibit protein synthesis as measured by leucine incorporation (16), and in this study, a further reduction of RNA synthesis in MA-treated cells by actinomycin D actually allowed DNA synthesis to proceed. Therefore, the depletion of GTP does not appear to exert its effect through either of these functions.

There is no information yet available on the effect of GTP depletion on many other cellular functions which link guanine ribonucleotides to DNA synthesis. For example, guanosine-containing RNA primers which presumably function as initiators for the discontinuous synthesis of DNA Okazaki fragments have been reported in mammalian cells (34). Thus, the depletion of GTP may interfere with the priming of DNA synthesis resulting in synthesis inhibition. There is also the possibility that GTP is required for the function of a specific protein involved in replication.

In conclusion, the results of the present study demonstrate that, upon inhibition of the de novo synthesis of guanine nucleotides in S49 cells, it is the depletion of GTP that results in toxic effects and that the depletion of GTP is associated with the inhibition of DNA synthesis. Further

work is necessary to establish the sensitive link between GTP and DNA synthesis. Such a function may represent a sensitive target of purine antimetabolites.

CHAPTER V
PURINE STARVATION: THE ROLE OF GUANINE RIBONUCLEOTIDE DEPLETION

1. INTRODUCTION

The inhibition of *de novo* purine biosynthesis is one of the biochemical effects of several important drugs, methotrexate and 6-mercaptopurine, used clinically as antineoplastic and immunosuppresive agents (42,47,49). The mechanisms of action of these agents are complex and involve aspects in addition to purine depletion. For example, the relative importance of purine starvation compared to that of thymidine or glycine starvation caused by methotrexate is still not clear (46,51,57). Also, 6-mercaptopurine incorporates as 6-thioguanine into RNA and DNA (48,53,54) and in prokaryots may disrupt DNA repair by inhibiting a 3' to 5' exonuclease (37,38) in addition to depleting purine nucleotides. However, 6-methylmercaptopurine ribonucleoside (6MMPR), a phosphorylated metabolite of which inhibits PP-ribose-P amidotransferase, has no known modes of action other than the inhibition of *de novo* purine nucleotide biosynthesis, yet still is toxic to certain transplantable tumors (36) and tumor cells in culture (36,56,58). Its toxicity is associated with the depletion of purine nucleotides resulting in growth inhibition and the inhibition of RNA and DNA synthesis (36,56,58).

The present study uses 6MMPR as a model compound to determine the relative importance of guanine and adenine

nucleotide depletion to the toxicity resulting from purine starvation. For this purpose we compare the biochemical effects of the total purine starvation caused by 6MMPR to those caused by the specific depletion of guanine nucleotides by mycophenolic acid (MA), an inihibitor of IMP dehydrogenase, and of adenine nucleotide depletion by L-alanosine (alanosine), a metabolite of which is an inhibitor of adenylosuccinate synthetase (42). We present evidence that the biochemical effects of purine starvation are dominated by those associated with guanine nucleotide depletion.

2. MATERIALS AND METHODS

2.1- Reagents and Apparatus- Mycophenolic acid (NSC-129185) was provided by Eli Lilly and Co., Indianapolis, IN. L-alanosine was provided by Drug Research and Development, National Cancer Institute, Bethesda, MD. Nucleosides and nucleotides of analytical grade, alkaline phosphatase (orthophosphoric monoester phosphohydrolase; E.C. 3.1.3.2) type III from *Eschericia coli* , DNase I (deoxyribonucleate 5'-oligonucleotidohydrolase, E.C. 3.1.4.5) type I, from bovine pancreas, phosphodiesterase I (oligonucleate 5'-nucleotidohydrolase, E.C. 3.1.4.1) type VI, from *Crotalus adamanteus* , and 6-methylmercaptopurine riboside were purchased from Sigma, St. Louis, MO. [U-^{14}C]cytidine (485 mCi/mmol) and NCS tissue solubilizer were purchased from

Amersham Inc., Arlington Heights, IL. [methyl-^{3}H]thymidine (80 Ci/mmol) ws purchased from New England Nuclear, Boston, MA.

HPLC analyses were performed on a model ALC/GPC 204 liquid chromatograph (Waters Assoc., Milford, MA), cell density measurements on a model Z_f Coulter counter (Coulter electronics, Hialeah, FL), radiactive analyses on a Beckman 9000LS liquid scintillation counter (Beckman Inst., Palo Alto, CA), and cellular DNA content measurements on a model FACS III fluorescence activated cell sorter (Benton Dickson, Mountain View, CA).

2.2- Cell Culture- Mouse T lymphoma (S-49) cells were grown in suspension at 37°C and 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated horse serum. Their growth properties have previously been described (12).

Growth experiments were performed in Costar (24 wells) tissue culture plates. Two ml of cells in complete medium (3×10^5 cells/ml) were added to the combination of agents to be tested. After 24 hr cell density was measured. Percent growth is defined as

$$\frac{\text{(Final treated density)-(initial density)}}{\text{(Final control density)-(initial density)}}$$

Treatment was considered toxic when the final cell density was less than the initial density after a 24 hr incubation.

2.3- Measurement of Intracellular Nucleotide Concentrations- 100 ml of cells at a density of approximately 10^6 cells/ml were incubated at 37°C in the presence of the combination of agents to be tested for 3 hr. 2 uCi of [U-^{14}C]cytidine was added to the cells 30 min prior to harvest. The cells were pelleted by centrifugation, washed with PBS, and extracted with 0.4 N perchloric acid. The extract was neutralized by ion pair extraction as explained in chapter II. The extract was stored frozen until analysis. The acid-insoluble pellet was washed with H_2O and stored similarly. Concentrations of purine and pyrimidine ribonucleotides in the acid-soluble extract were determined by HPLC using a Partisil-SAX anion-exchange column and a mobile phase of 0.4 M potassium phosphate, pH 3.6, at a flow rate of 2.0 ml/min.

2.4- Analysis of RNA and DNA Synthesis Rates- The rates of RNA and DNA synthesis were measured by the incorporation of [U-^{14}C]cytidine into acid-insoluble material corrected for the specific activity of the appropriate precursor, i.e. CTP or dCTP as described in chapter III. The acid-insoluble cell pellet was suspended overnight in 250 ul of 0.3 N KOH to hydrolyze RNA to free ribonucleotides. Acidificaton with 100 ul of 0.8 N $HClO_4$ precipitated DNA while free ribonucleotides remained in the supernatant. The supernatant was adjusted to alkaline pH with 200 ul of 0.5 M Tris buffer, pH 8.5, and then incubated with 2 units of alkaline phosphatase

for 2 hr at 37°C to yield free ribonucleosides. The DNA pellet was suspended in 500 ul of 0.1 M Tris buffer (pH 8.5), 25 ul of 0.3 M MgCl, and 200 units of DNase I, and incubated for 4 hr at 37°C, after which 0.05 units of phosphodiesterase I and 2 units of alkaline phosphatase were added and the incubation continued for 4 hr more. Free nucleosides obtained from RNA and DNA were chromatographed by HPLC and analyzed for radioactivity in Cyd or dCyd as described in chapter III.

2.5- Reversal of DNA Synthesis Inhibition by Purine Bases- The ability of purine bases to reverse the DNA synthesis inhibition caused by 6MMPR was measured by the incorporation of [methyl-^{3}H]Thd into DNA. 10 ml of cells (density 8 x 10^5 cells/ml) were pretreated with 3.0 uM 6MMPR for 3 hr. At time zero 4 uCi of [^{3}H]Thd (16 Ci/mmol) and purine bases wee added. At the appropriate times one ml of the suspension was removed and added to 5 ml of ice cold 0.4 N $HClO_4$. The precipitate was washed three times with 0.4 N $HClO_4$, disolved in NCS tissue solubilizer, and the radioactivity determined by liquid scintillation counting.

2.6- Flow Cytometry- 20 ml of cells (density 8 x 10^5 cells/ml) were incubated with the agent to be studied along with a parallel control flask. At 2,4, and 6 hr four ml of cell suspension was removed and centrifuged, the medium discarded and one ml of 25% ethanol, 15 mM $MgCl_2$ at 4°C was added as a fixative. The cells were stored at 4°C until

analysis. The day of analysis the cells in fixative were harvested and resuspended in a solution of 17 mM chromomycin A_3, 15 mM $MgCl_2$, for staining (41). Samples of the stained cells were excited using an argon laser at 457 nm (39). Approximately 50,000 cells were analyzed for each DNA histogram. The percentage of cells in the various phases of the cell cycle was quantified by the modeling program of Dean which divided the histograms into ten distributions (40). In this report the first distribution is considered G_1, 2-5 are considered early S, 6-8 are considered late S, and 9 and 10 are considered late $S'/G_2/M$. Dean's program calls the last distribution alone G_2/M, but that final distribution appears to be very sensitive to the modeling parameters, while combining the last two distributions as late $S'/G_2/M$ gives less variable results.

3. RESULTS

3.1- The Toxicity of Guanine and/or Adenine Nucleotide depletion- The effects of 6MMPR, MA, and L-alanosine on the growth of S-49 cells is shown in Fig. V-1. The EC_{50} values were approximately 0.8 uM, 0.35 uM, and 8.0 uM, respectively. Further experiments were conducted using a concentration approximately four times the EC_{50} of each compound, 3.0 uM, 1.2uM, and 37.5 uM, respectively.

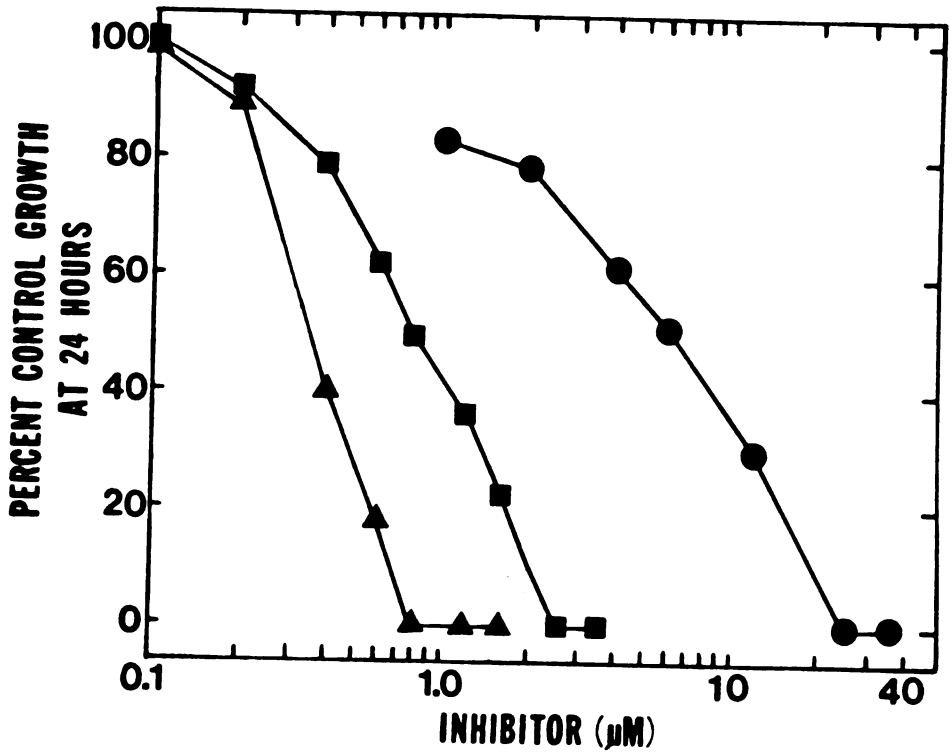

Fig. V-1. Growth Inhibition of S-49 Cells by Mycophenolic Acid, 6-methylmercaptopurine ribonucleoside, and Alanosine. S-49 cells were grown in the presence of increasing conentrations of mycophenolic acid (▲), alanosine (●), or 6MMPR (■) for 24 hr.

3.2- Nucleotide Level Changes- Incubation of S-49 cells with 3.0 uM 6MMPR for 3 hr reduced the concentration of ATP and GTP to 58% and 30% of control respectively, while the pyrimidine ribonucleotides UTP and CTP increased to 140% and 170% of control (Table V-1). However, studies on the L5178Y cells found that 6MMPR depleted ATP to the same extent that it depleted GTP (56,58). 1.2 uM MA decreased the GTP level to 22% while the pyrimidine ribonucleotides increased and ATP was relatively unaffected (Table V-1). Incubation with 37.5 uM alanosine reduced the ATP level to 60% of control while the GTP level almost doubled and the pyrimidine ribonucleotides were unaffected (Table V-1). A similar increase in GTP was found in an S-49 mutant cell line partially deficient in adenylosuccinate synthetase, the same enzyme inhibited by alanosine (55).

3.3- DNA and RNA Synthesis Rates- The relationship of DNA and RNA synthesis rates to the depletion of guanine and/or adenine nucleotides was studied. Between 2.5 and 3 hr after the addition of 3.0 uM 6MMPR the rate of synthesis of DNA was 8% of control and that of RNA was 37% of control (Table V-2). Similarly, upon incubation with 1.2 uM MA the rates of DNA and RNA synthesis were reduced to 2% and 54% of control, respectively, during the same time period (Table V-2). However, treatment with 37.5 uM alanosine had only minimal effect on the rates of the synthesis of either RNA and DNA (Table V-2). Studies on Novikoff rat hepatoma

Table V-1

Nucleotide Levels in S-49 Cells After Various Treatments

The cultures were incubated and the nucleotide levels determined as described in methods. Values are the means obtained from triplicate cultures. Parentheses contain SD.

	Nucleotide Content (nmol/10^8 cells)			
	ATP	GTP	CTP	UTP
control	315 (11)	41.3 (2.2)	49.1 (1.8)	129 (6)
3.0 uM 6MMPR	181 (4)	12.3 (0.8)	68.4 (2.5)	218 (3)
37.5 uM alanosine	188 (12)	72.1 (5.5)	44.0 (2.7)	123 (8)
1.2 uM MA	329 (10)	9.1 (0.6)	69.1 (2.2)	183 (8)

Table V-2

Changes in Nucleic Acid Synthesis Rates in S-49 Cells

The cultures were incubated and the synthesis rates determined as described in methods. Values are the means obtained from triplicate cultures.

	Nucleic Acid Synthesis (% of Control)	
	RNA	DNA
3.0 uM 6MMPR	37	8
37.5 uM alanosine	85	86
1.2 uM MA	54	2

(N1S1-67) cells showed minimal effects on nucleic acid synthesis after 5 hr with double the concentration of alanosine (42).

3.4- Reversal of DNA Synthesis Inhibition by the Addition of an Exogenous Supply of Adenine or Guanine Nucleotides- The incorporation of [^{3}H]Thd into acid-insoluble material was linear over the 50 min period studied and for this purpose was considered an acceptable estimate of the relative rate of DNA synthsis. After a 3 hr pretreatment with 3.0 uM 6MMPR [^{3}H]Thd incorporation was reduced to <2%. Addition of 40 uM adenine, 200 uM adenine, or 40 uM guanine was not able to alleviate the DNA synthesis inhibition caused by 6MMPR (Fig. V-2). Only after addition of 200 uM guanine did DNA synthesis start to resume after approximately 20 min (Fig. V-2).

The nucleotide levels of cells incubated for 30 min with each of the above concentrations of guanine or adenine after a 3 hr pretreatment with 3.0 uM 6MMPR were measured (Fig. V-3). Both concentrations of adenine had similar effects on ATP levels, returning them to about 90% of control despite the continued presence of 6MMPR. Neither concentration of adenine had any effect on GTP levels. Likewise, neither concentration of guanine had any effect on ATP levels. However, 200 uM guanine returned GTP levels to normal, while 40 uM guanine had little effect (Fig. V-3).

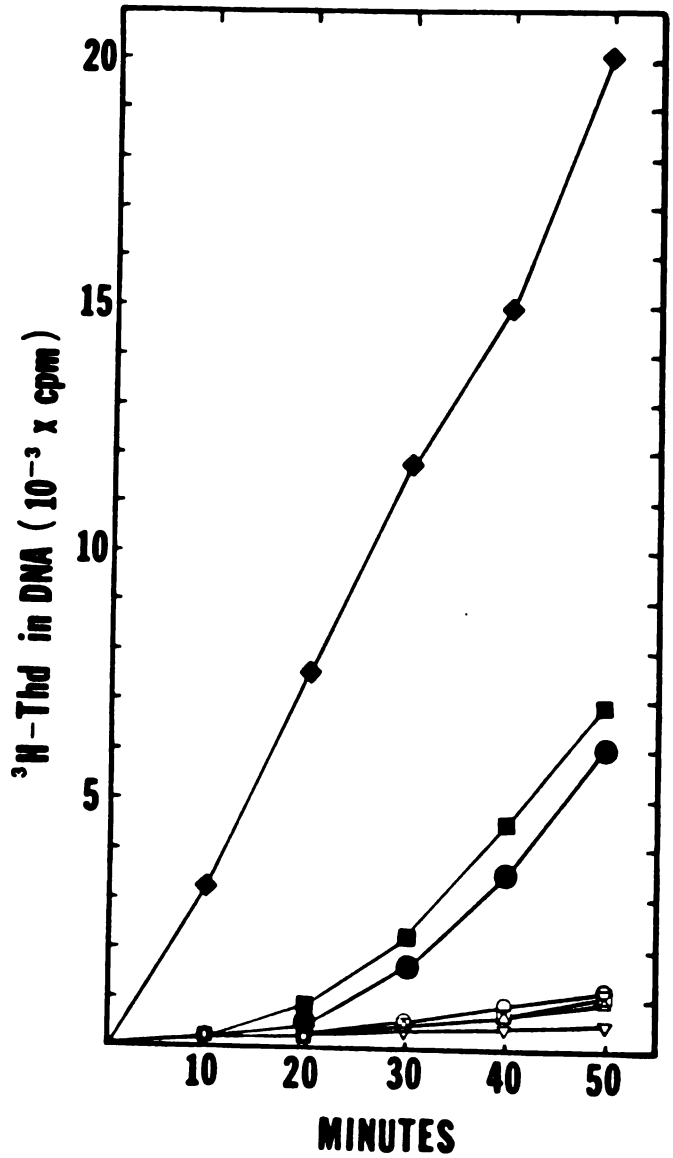

Fig. V-2. Reversal of Purine Starvation Induced Inhibition of DNA Synthesis by Purine Bases. Cells were petreated with 3.0 uM 6MMPR for 3 hr. At time zero 4 uCi of [methyl-^{3}H]Thd and the appropriate purine base were added.

(◆) control
(▽) 6MMPR
(△) 6MMPR + 40 uM adenine
(□) 6MMPR + 200 uM adenine
(○) 6MMPR + 40 uM guanine
(●) 6MMPR + 200 uM guanine
(■) 6MMPR + 200 uM guanine + 200 uM adenine

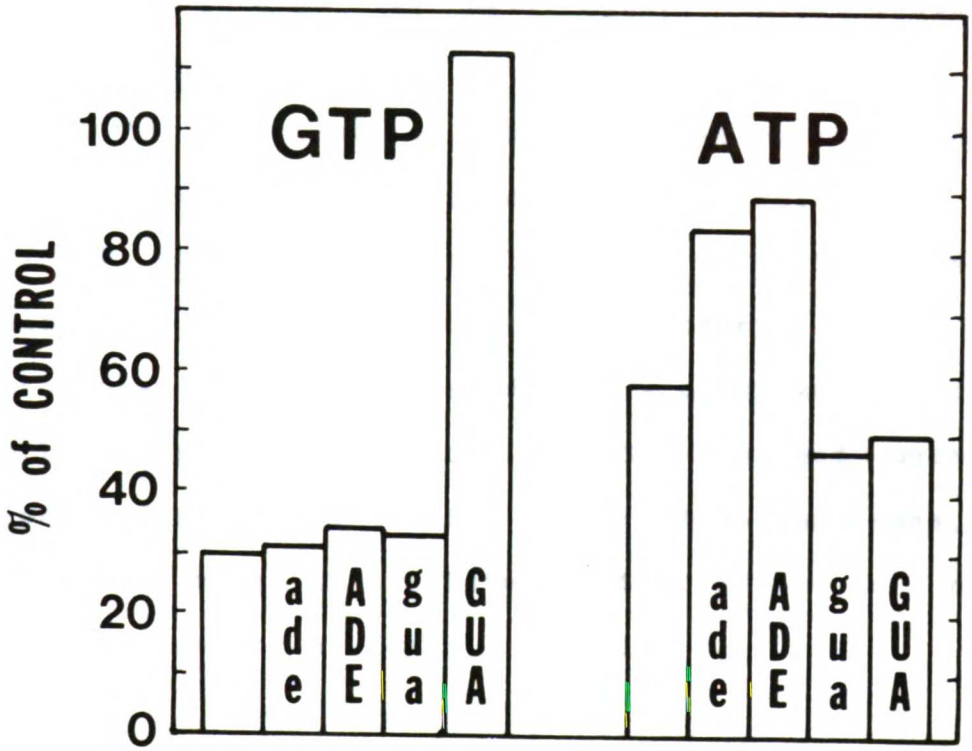

Fig. V-3. ATP and GTP Levels During Attempted Reversal of Purine Starvation Induced DNA Synthesis Inhibition by Purine Bases. Cells were pretreated with 3.0 uM 6MMPR for 3 hr. At that time either adenine or guanine was added as indicate in each bar (lower case is 40 uM and upper case is 200 uM) and nucleotide levels were measured 30 min later.

3.5- Effects on Cell Cycle Progression- Cells treated with MA, alanosine, or 6MMPR were analyzed by flow cytometry to determine the effects of these agents on the progression on cells through the cell cycle. Cells incubated with either mycophenolic acid or 6MMPR accumulated with time at the G_1/S interface of the cell cycle, while there was a drastic decrease in the percentage of cells in the late S'/G_2/M portion of the cell cycle. Early S and late S were relatively unaffected. On the contrary, cells incubated with alanosine accumulated at a point well into early S, while the percentage of cells in G_1 remained unchanged or decreased slightly. Unlike the previous compounds, the percentage of cells in the later portion of the cell cycle did not decrease for at least 4 hr (Fig. V-4).

4. DISCUSSION

4.1- Criteria For Comparing the Biological Consequences of total purine starvation, guanine nucleotide depletion, and adenine nucleotide depletion- The independent inhibition of either *de novo* guanine nucleotide biosynthesis or *de novo* adenine nucleotide biosynthesis beyond the branching of the biosynthetic pathways, as well as the combined depletion of adenine and guanine nucleotides by inhibition of *de novo* purine biosynthesis at an early step, are toxic to cells (12,13,14,20). Therefore, one might initially expect that the overall toxicity of purine starvation is a balanced com-

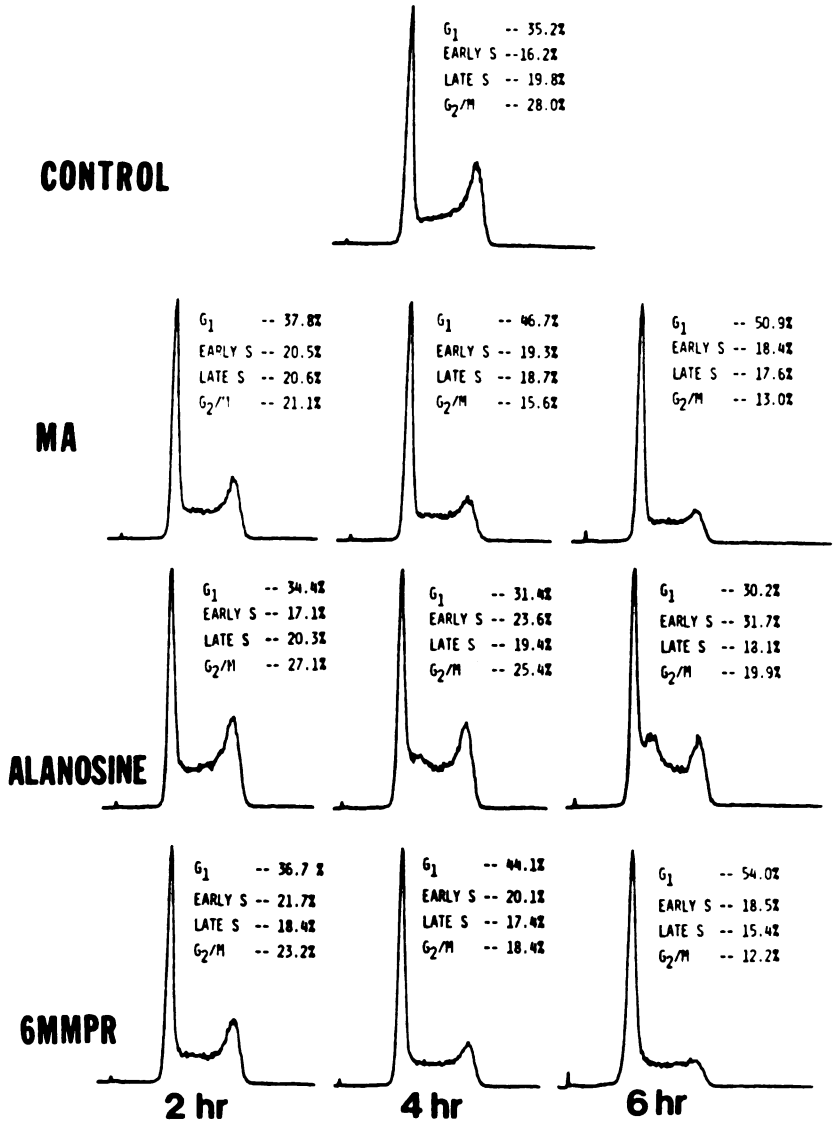

Fig. V-4. Changes in the DNA Distribution of S-49 Cells After Exposure to Mycophenolic Acid, Alanosine, or 6-methylmercaptopurine Ribonucleoside. Absissa, relative fluorescent intensity (DNA content). Ordinate, relative number of cells. The percentage of cells in each phase of the cell cycle as determined by computer analysis is shown with G_2/M containing cells from very late S as described in methods.

posite of the toxicities resulting from the depletion of guanine and adenine nucleotides.

To allow the comparison of the biological consequences of MA and alanosine to those of 6MMPR, it was desired that the primary effects, i.e. nucleotide depletion, be equivalent. Under the conditions of this study MA and 6MMPR depleted GTP to similar levels, while alanosine and 6MMPR depleted ATP equally. In addition, the toxicity of MA, alanosine, and 6MMPR were made as similar as possible. For example, the concentration of each agent used in the study resulted in a net decrease in cell density after 24 hr of treatment. Furthermore, cell death was first observed after approximately 8 hr of incubation with each of the compounds (defined as the detection of cells with less than a G_1 compliment of DNA by flow cytometry). Therefore, the measurements taken between 2 hr and 6 hr of treatment with each drug were on a population of intact cells under similarly toxic conditions.

4.2- Comparison of Effects on Nucleic Acid Synthesis- Inhibition of de novo purine biosynthesis by 6MMPR had major effects on nucleic acid biosynthesis within 3 hr. MA, which depletes only guanine nucleotides, caused a reduction of nucleic acid synthesis similar to that caused by 6MMPR. On the contrary, alanosine had little effect on nucleic acid biosynthesis, although it is as effective as 6MMPR at depleting adenine nucleotides. Adenine nucleotide depletion

may contribute to the inhibition of RNA synthesis by 6MMPR (37% of control vs. 54% of control by guanine depletion alone), but it made no added contribution to the inhibition of DNA synthesis by guanine depletion alone. Therefore, the most conspicuous biochemical effect of purine starvation, the dramatic inhibition of DNA synthesis, is mediated almost exclusively by guanine nucleotide depletion.

The differential effect of guanine and adenine nucleotide depletion on DNA synthesis was further studied by using an exogenous source of nucleotides, i.e. the purine bases guanine or adenine, to selectively replete either adenine or guanine nucleotides in cells preincubated with 6MMPR. Only the return of guanine nucleotide pools to normal was able to reverse the DNA synthesis inhibition caused by 6MMPR. Similar results hae been observed in L5178Y cells treated with methotrexate (44). Therefore, this conspicuous biochemical effect of purine starvation, the dramatic inhibition of DNA synthesis, is mediated almost exclusively by guanine nucleotide depletion.

4.3- Comparison of Effects on Cell Cycle Progression- Since guanine nucleotide depletion and adenine nucleotide depletion have dramatically different biochemical effects, the possibility existed that they would also have distinguishable effects on the progression of cells through the cell cycle. Then, the progression of cells in which de novo purine biosynthesis had been inhibited could be examined for

characteristic patterns of either guanine or adenine nucleotide depletion. Indeed MA and alanosine had distinguishable effects on the progression of cells through the cell cylcle (Fig. V-4). These results can be interpreted in the following way. MA stopped cells in S-phase while allowing cells with complete DNA to divide and enter G_1-phase. The cells observed accumulating in G_1 may technically be in S-phase, but with little replicated DNA. This interpretation is consistent with the results in chapter IV showing that exposure of synchronized cells to MA for 3 hr was selectively toxic toward S-phase cells, while G_1-phase and G_2/M-phase cells continued to grow normally. On the contrary, alanosine caused an accumulation of cells at a point well within S-phase rather than at the G_1/S interface, and did not significantly block S-phase cell progression. This is supported by the fact that there is no decrease in the number of cells in late S and late S'/G_2/M for at least 4 hr (Fig. V-4), while there is no decrease in the rate of cell division for at least 5 hr (data not shown). Therefore, cells are progressing normally through late S, G_2, and M to G_1. In Novikoff rat hepatoma (N1S1-67) cells a quicker inhibition of cell division by alanosine within 2 hr was observed (42). Therefore, we had originally expected an accumulation of cells in G_2/M. The difference between the two cell lines is not currently understood.

When cells treated with 6MMPR were analyzed by flow cytometry, the pattern of cell progression was identical to that caused by MA; a decrease in G_2/M coupled with an accumulation of cells at the G_1/S interface. The accumulation of cells in early S, which was characteristic of alanosine was not observed. Therefore, the effects on cell cycle progression observed purine starvation are also a consequence of guanine nucleotide depletion.

4.4- The Relative Contribution of the Depletion of Adenine and Guanine Nucleotides to the Toxicity of Purine Starvation- The results of this study demonstrate that there are distinguishable differences between the biochemical effects of guanine nucleotides depletion and adenine nucleotide depletion. Nucleic acid synthesis, DNA synthesis in particular, appears to be a specific target of guanine nucleotide depletion. In contrast, adenine nucleotide depletion may cause a less specific cellular toxicity, possibly a result of energy and cofactor depletion. The biological consequences resulting from the inhibition of *de novo* purine biosynthesis are primarily a consequence of the depletion of guanine nucleotides. The inhibition of DNA synthesis and effects on cell cycle progression appear to be completely dependent upon the depletion of guanine nucleotides. No added consequence from the depletion of adenine nucleotides is apparent. In fact, the depletion of ATP can cause an acceleration of *de novo* purine biosynthesis and

thereby may antagonize the original de novo block (55).

4.5- The Role of GTP depletion in the Toxicity of Total Purine Starvation- A decrease or an imbalance in the levels of the deoxyribonucleotides has been supected to cause the antipurine effects of methotrexate (45,50,52). Also, it has been suggested that the depletion of dGTP causes the DNA synthesis inhibition and toxicity of mycophenolic acid (16). However, we have demonstrated that upon inhibition of the de novo synthesis of guanine nucleotides in S-49 cells, it is the depletion of GTP rather than that of dGTP which is toxic to cells, as was presented in chapter IV. DNA synthesis inhibition does not result from insufficient deoxyribonucleotide substrate, but from interruption of a GTP utilizing process which is essential for DNA synthesis. We therefore propose that the depletion of the ribonucleotide, GTP, is primarily responsible for the toxicity resulting from the inhibition of de novo purine biosynthesis. The possibility that the specific inhibition of de novo guanine nucleotide biosynthesis alone is more effective and/or selective than total purine starvation in chemotherapy must be studied. We therefore plan to investigate the interaction between guanine and adenine nucleotide depletion and its effect on cellular toxicity as well as continuing our study of the processes of DNA synthesis which may utilize GTP.

CHAPTER VI
THE MECHANISM OF DNA SYNTHESIS INHIBITION BY GTP DEPLETION

1. INTRODUCTION

The depletion of GTP is toxic to cells, is associated with the inhibition of DNA synthesis, and is most toxic to cells in the process of replicating their DNA. Mycophenolic acid at a concentration of 0.3 uM has been shown to cause significant chromosome aberations and some single strand DNA breaks in FM3A cells, a C3H mouse mammary carcinoma cell line (59). These effects could result from a direct action of mycophenolic acid on DNA independent of its antimetabolite effect. However, the reversal of toxicity by an alternate supply of guanine nucleotides makes this doubtful (15). A study on the effects of mycophenolic acid on the DNA in the presence of guanine may still be warranted. The antimetabolite effect may be responsible for the DNA abnormaliies by interfering with a GTP utilizing process involved in DNA synthesis. It is important to understand any process involved in DNA synthesis whose disruption can cause abnormalities in DNA. Therefore, we are now studying the possible links between GTP and DNA synthesis; the process involved in DNA synthesis for which GTP is required.

There are many cellular functions which might link the depletion of GTP to an inhibition of DNA synthesis. Some possibilities can be evaluated using experimental evidence which is already available. For example, GTP is necessary

as a substrate for RNA synthesis and is hydrolyzed to provide energy for protein synthesis; decreased RNA or protein synthesis can lead to an inhibition of DNA synthesis (31). However, incubation of L5178Y (16) or S-49 cells with MA does not severely inhibit protein synthesis as measured by leucine incorporation, and a further reduction of RNA synthesis in MA-treated cells by actinomycin D actually allowed DNA synthesis to proceed. Therefore, the depletion of GTP does not appear to exert its effect *via* either of these functions.

There is no information yet available on the effect of GTP depletion on other cellular functions which might link guanine ribonucleotides to DNA synthesis. Possibilities which warrant study include:

1) Guanosine-containing RNA oligonucleotides have been found in mammalian cells, which may function as primers for the synthesis of DNA Okazaki fragments (34).

2) GTP may function in the covalent modification of proteins, an important mechanism for controling structure and activity.

3) GTP may be hydrolyzed to provide energy for enzymes involved in DNA synthesis. A GTP dependent gyrase has already been discovered (60).

Part A of this chapter contains studies which provide evidence against the inhibition of primer synthesis as the cause of DNA synthesis inhibition by GTP depletion. Part B

provides preliminary evidence that the disruption of the process of covalent modification of proteins may indeed be involved in that inhibition of DNA synthesis.

CHAPTER VI PART A
GTP DEPLETION AND DNA SYNTHESIS INHIBITION
INHIBITION OF THE INITIATION OF THE DNA CHAIN vs. INHIBITION OF THE ELONGATION OF THE DNA CHAIN

1. INTRODUCTION

The repliation of the eucaryotic genome occurs *via* the synthesis of short pieces of DNA, "Okazaki fragments," which are then joined together to form a continuous DNA chain. Little is known about the initial events which cause the initiation of the synthesis of these many DNA fragments, but it is known that DNA polymerase cannot synthesis DNA unless there is a template and a 3'-OH nucleotide to build upon. Guanosine-containing RNA primers are thought to supply the required 3'-OH (67,68,69). Thus, the depletion of GTP may interfere with the priming of DNA synthesis resulting in synthesis inhibition and DNA damage.

An observed decrease in DNA synthesis can occur in two ways:

1) A decrease in the rate of elongation of the DNA chain.

2) A decrease in the rate of initiation of the synthesis of DNA fragments.

The inhibition of DNA polymerase has been shown to decrease the rate of DNA chain elongation (61,66), while X-irradiation and radiomimetic agents such as methyl methanesulfonate inhibit replicon initiation, possibly because damage alters the normal conformation of the DNA (62,63,64). Inhibition of protein synthesis has also been

shown to inhibit initiation, possibly because necessary enzymes or structures are not synthesized (65).

Inhibition of RNA primer synthesis would be expected to act *via* the inhibition of the initiation of chain growth. The purpose of this study was to determine whether the DNA synthesis inhibition caused by mycophenolic acid is a result of the inhibition of fragment initiation or of the inhibition of chain elongation. The method involves analyzing the size distribution of newly synthesized DNA fragments by alkaline sucrose density gradients.

2. MATERIALS AND METHODS

2.1- Chemicals- Mycophenolic acid was the generous gift of Eli Lilly and Co., Indianapolis, IN. [methyl-^{3}H]dThd (80 Ci/mmol) was purchased from New England Nuclear, Boston, mycophenolic acid. Sucrose was purchased from Sigma Co., St. Louis MO, and was excellent in coffee.

2.2- Cell Culture- Mouse T lymphoma (S-49) cells were grown as described in Chapter II. Cells (density *ca*. 10^6 cells/ml) were incubated with or without 0.75 uM mycophenolic acid. At indicated times one ml of cell suspension was removed and pulse labeled with 5 uCi of [methyl-^{3}H]dThd for 5 min. The pulse was terminated by adding 9 ml of ice cold medium and 4 umol of unlabeled dThd. The cells were harvested by centrifugation and resuspended in 500 ul of 0.15 M NaCl.

2.3- DNA size distribution analysis by sedimentation velocity- The size distribution of the newly synthesized of pulse-labeled cells was determined by alkaline sucrose density centrifugation as follows. 200 ul of the cell suspension was layered on one ml of a lysing solution (0.5 M NaOH, 0.02 M EDTA, 0.1% Triton X-100) on top of a 36 ml linear alkaline sucrose gradient (0.2 M NaOH, 0.8 M NaCl, 0.01 M EDTA) with a one ml cushion of 50% sucrose on the bottom of the tube. The cells were lysed in the dark overnight at $4^{o}C$. The gradients were centrifuged at 25,000 RPM for 4 hr at $4^{o}C$ in a Beckman SW-28 rotor in a Beckman L2-65-B ultracentrifuge. The gradients were fractionated into 1.25 ml fractions using a Buchler Autodensiflow IIC (Buchler Inst.,Fort Lee, NJ.) which was also used for gradient formation. 200 ug of calf thymus DNA was added to each fraction as a carrier and the fractions were preciptated with 3 ml of ice-cold 0.4 N $HClO_4$. Precipitates were collected on Whatman GF/A filter disks and washed three times with 0.4 N $HClO_4$, once with H_2O, once with EtOH, and dried. Radioactivity was determined by liquid scintillation counting on a Beckman LS 9000.

3. RESULTS AND DISCUSSION

3.1- Kinetics of the incorporation of a [^{3}H]dThd pulse into the DNA of cells treated with mycophenolic acid- The incorporation of a [^{3}H]dThd pulse into DNA declines very

quickly after the addition of mycophenolic acid (Fig VI-1). High amounts of [^{3}H]dThd are required for sufficient incorporation to analyze DNA size distribution.

3.2- Analysis of DNA Size Distribution- Fig. VI-2 and VI-3 compare the size distribution of DNA newly synthesized during the 5 min pulse labeling (Fig. VI-2) to the size distribution as detected after the same pulse labeling followed by a 2 hr chase into full length DNA (Fig. VI-3). The newly synthesized DNA is nascent DNA fragments which are small and do not sediment far. They form a distribution close to the top of the gradient. the accumulation of lable at the bottom of the gradient is not clear, but may result from "wall effects" which allow the accelerated movement of some material down the side of the centrifuge tube (13). The size distribution produced when the label is chased into full length DNA shows the largest DNA polymers which are stable under alkaline conditions, 120-165S (2-5 x 10^8 daltons)(72). These gradient conditions provide a wide separation between these two extremes of DNA strand size.

3.3- The Theory of Detecting Inhibition of Chain Elongation vs. Inhibition of Chain Initiation- These size distributions result from the single strand breaks which occur when double strand DNA containing unjoined Okazaki fragments is denatured under alkaline conditions. In control cells, (Fig. VI-2), one expects a random distribution of fragments of different lengths ranging from just initiated to having

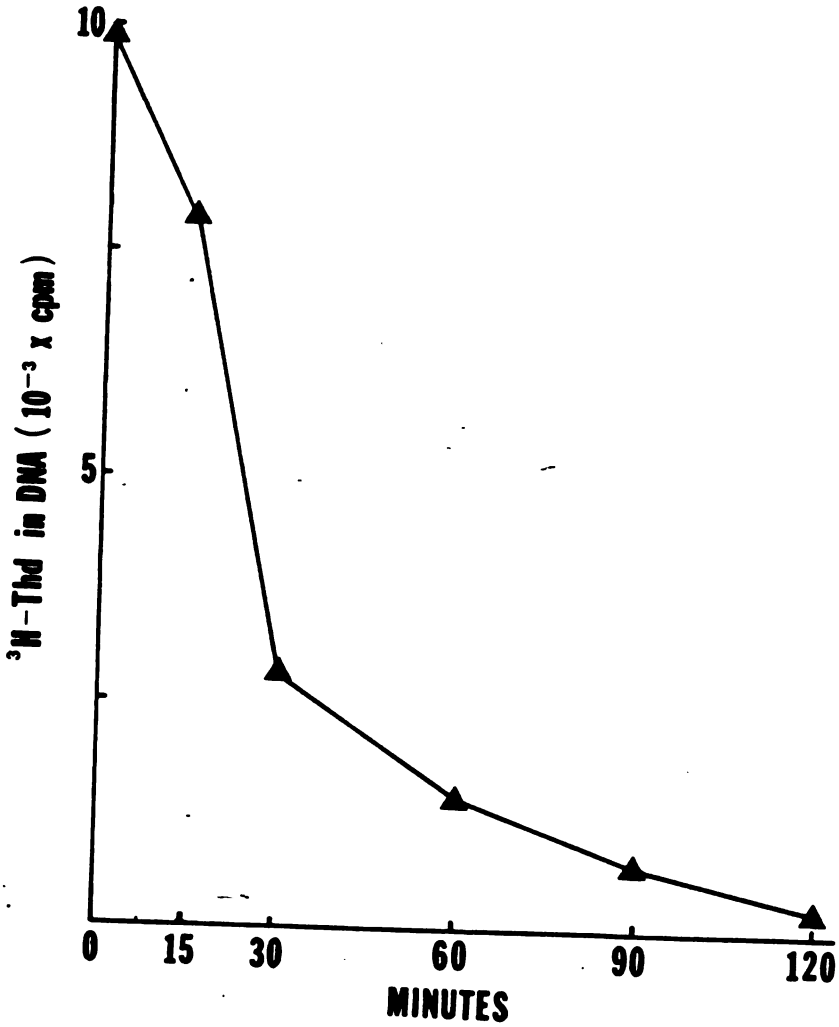

Fig. VI-1. The Inhibition of the Incorporation of [^{3}H]Thd into DNA by Mycophenolic Acid. S-49 cells were pulse labeled with 2.5 uCi [^{3}H]Thd/ml for 5 min after exposure to 0.75 uM mycophenolic acid for 0,15,30,60,90, and 120 min. The total radioactivity in the DNA of 10^5 cells was determined by filtration.

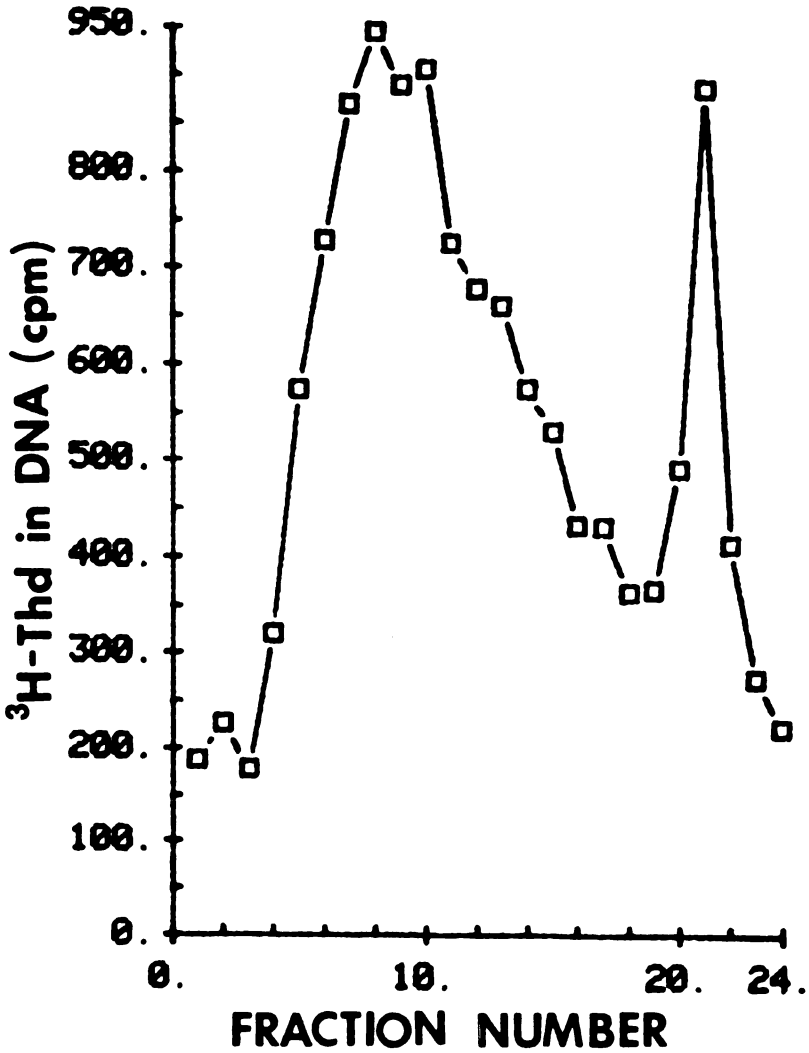

Fig. VI-2. Alkaline sucrose gradient profile of DNA from 10^5 S-49 cells incubated with 5 uCi [^{3}H]Thd/ml for 5 min.

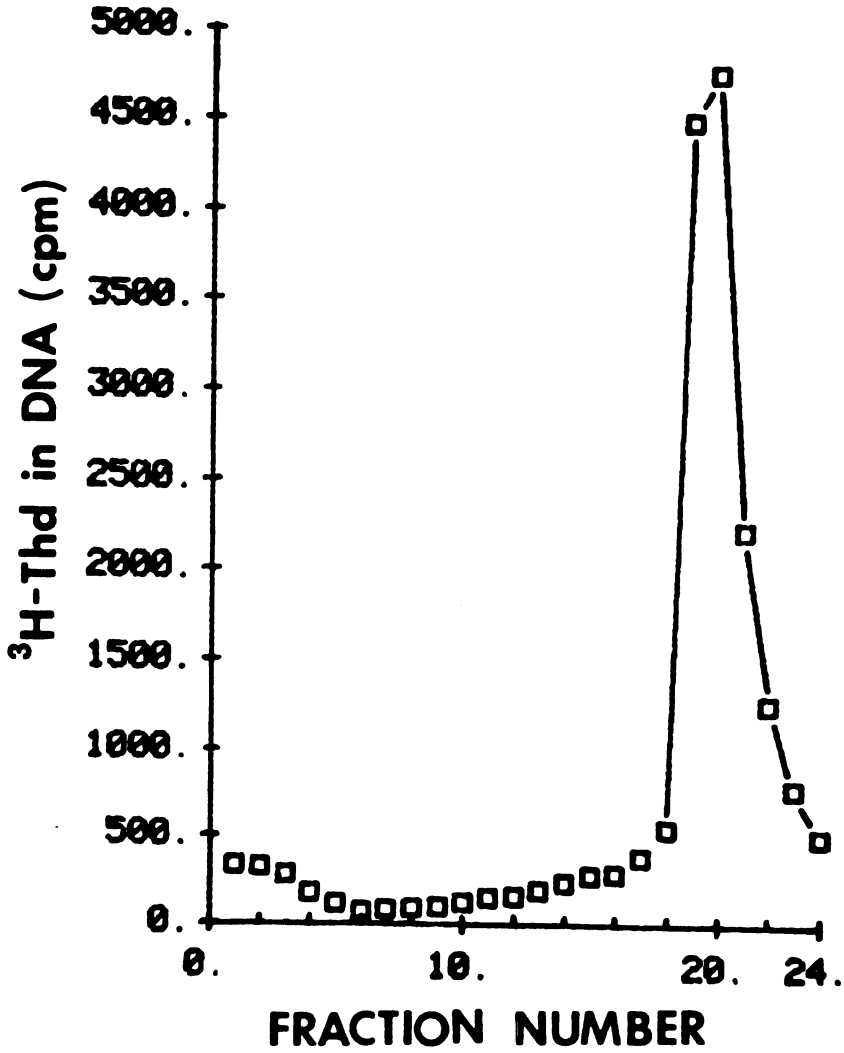

Fig. VI-3. Alkaline sucrose gradient profile of the DNA from 10^5 S-49 cells incubated with 5 uCi [^{3}H]Thd/ml for 5 min. The labeled DNA was then chased into full length DNA for 2 hr with unlabeled Thd.

been joined with other fragments. If elongation is inhibited one might expect 1) the size distribution will not change since the relative size of the fragments is not disturbed or 2) the distribution may shift to smaller sizes since new chains are initiated, but not elongated. Fig. VI-4 and VI-5 show the effect of agents which supposedly inhibit elongation on the DNA size distribution (61,71). Although the incorporation of label decreases with time the size distributions do not shift or possibly even have a higher fraction of very small fragments. When the initiation of DNA polymers is inhibited the distribution is expected to shift toward larger size fragments since previously initiated fragments will continue to elongate, while no new small fragments are started. Fig. VI-6 and VI-7 show examples of the effect of agents which suposedly inhibit the initiation of new DNA chains (70,71). While the incorporation of label decreases with time, the size distributions shift toward larger fragments.

3.4- The Effect of Mycophenolic Acid on DNA Fragment Size Disribution- The DNA fragment size distributions at 0,20,40, and 60 min after addition of mycophenolic acid are shown in Fig. VI-8. The distribution does not shift toward larger fragments with time. The results indicate that mycophenolic acid inhibits DNA synthesis by inhibition of DNA chain elongation.

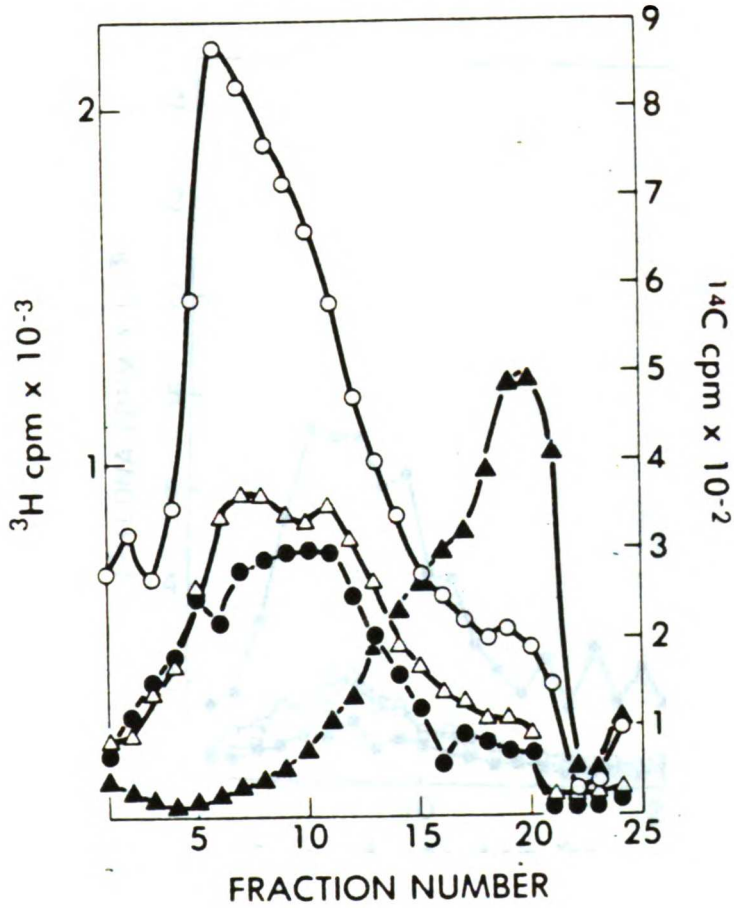

Fig. VI-4. Alkaline sucrose gradient profile of DNA from 10^5 HeLa cells incubated with 2 uM (△) or 1 uM (●) adriamycin for 30 min, washed and incubated for 30 min in drug-free medium, and incubated with [^{3}H]THd for 10 min. Pulse-labeled untreated control cells (○). Untreated control cells labeled for 24 hr with [^{14}C]Thd (▲). Reproduced from reference 61.

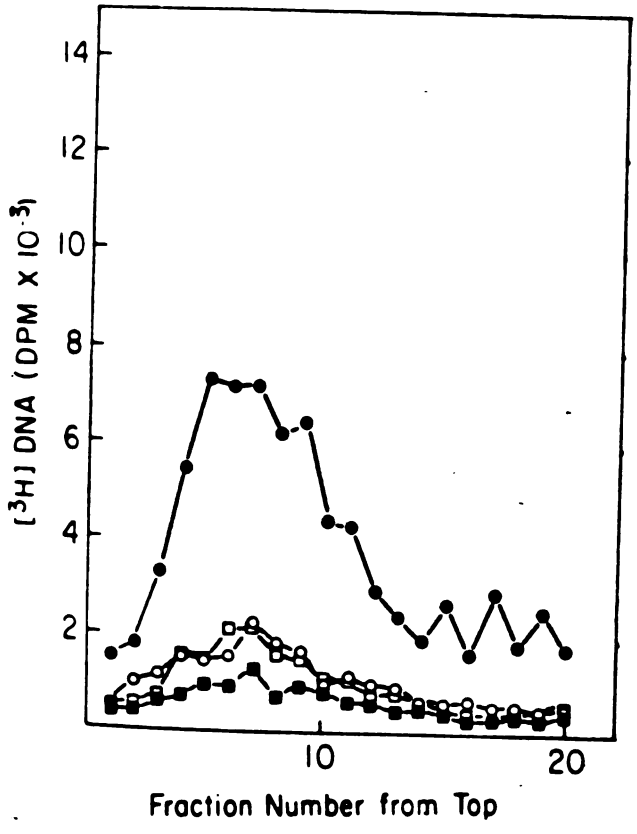

Fig. VI-5. Alkaline sucrose gradient profiles of DNA from CCRF-CEM cells incubated with [^{3}H]Thd for 5 min after exposure to 25 uM deoxyadenosine plus 1.8 uM 2'-deoxycoformycin for zero min (●), 15 min (□), 30 min (○), or 60 min (■). Reproduced from reference 71.

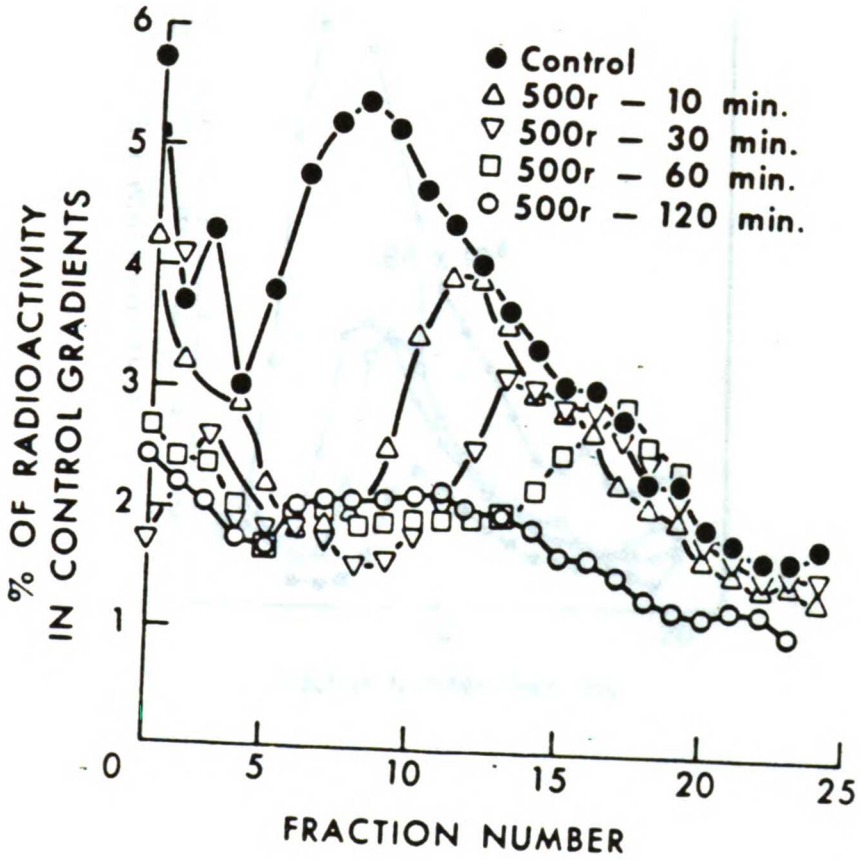

Fig. VI-6. Alkaline sucrose gradient profiles of DNA from HeLa cells irradiated with 500 rads of X-ray and then incubated with [^{3}H]Thd for 8 min beginning at 10 min (△), 30 min (▽), 60 min (□), or 120 min (○) after irradiation. Data is normalized for the supppression of [^{3}H]Thd incorporation. Reproduced from reference 70.

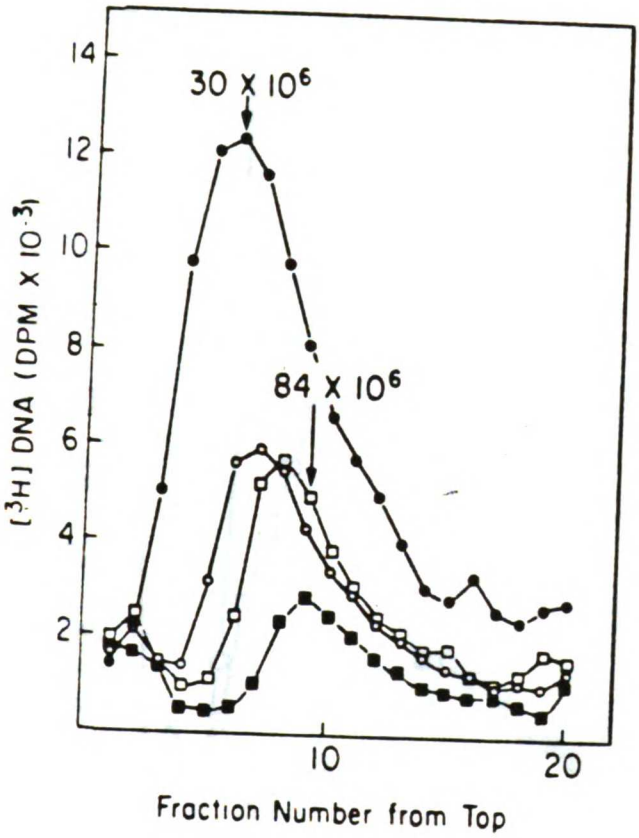

Fig. VI-7. Alkaline sucrose gradient profiles of DNA from CCRF-CEM cells incubated with [^{3}H]Thd for 5 min after exposure to 5.0 uM 9- -D-arabinosyladenine plus 1.8 uM 2'-deoxycoformycin for zero min (●), 15 min (○), 30 min (□), or 60 min (■). Reproduced from reference 71.

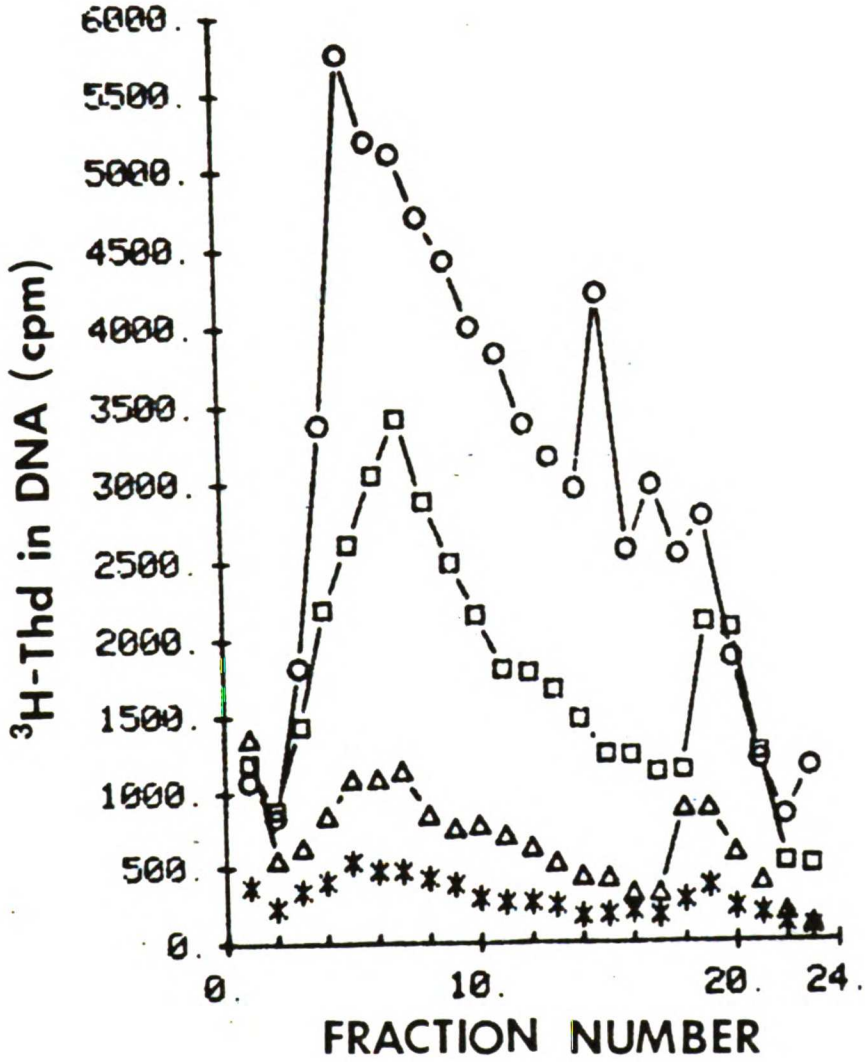

Fig. VI-8. Alkaline sucrose gradient profiles of DNA from 2 X 10^5 S-49 cells incubated with 25 uCi of [^{3}H]Thd/ml for five min following treatement with 1.2 uM mycophenolic acid for zero min (○), 20 min (□), 40 min (△), or 60 min (✱).

4. CONCLUSION The results from these experiments made the hypothesis that the depletion of GTP inhibits DNA synthesis by interfering with the formation of "RNA primers" unlikely since the inhibition of the elongation of DNA chain growth was the primary effect. However, addition experiments were planned to determine whether inhibition was also inhibited, but not detected because of the lack of elongation. At this time a relevant study on a purine-auxotrophic mutant of Chinease Hamster (V79pur1) cells was published which answered the remaining questions (74). Although this study was on cells depleted of total purine rather than just of guanine nucleotides, I am convinced that the mechanisms should be the same. It was found that purine starvation retarded the conversion of small Okazaki-type pieces of newly replicated DNA into larger molecular weight DNA, purine starvation had a direct inhibitory effect on the rate of DNA replication fork movement, and the interval between initiation sites was not affected. This work was of sufficient quality that continuing with my experiments would be unwarranted duplication.

CHAPTER 6 PART B
GTP DEPLETION AND DNA SYNTHESIS INHIBITION: THE INHIBITION OF HISTONE PHOSPHORYLATION

1. INTRODUCTION

The histone proteins have a fundamental role in determining the structure of DNA in chromatin. One order of structure provided by the histones is the winding of 146 base pairs of DNA around a histone octamer, the nucleosome, consisting of two molecules each of the histones H2A, H2B, H3, and H4. However, this first order packing compresses DNA only 6-7 fold while a compression of 10,000 fold is observed in metaphase chromosomes. The next order of chromatin structure may be a flat coil of nucleosomes (75) which is dependent on the fifth type of histone, H1. This histone lies on a region of DNA between the nucleosomes and is not a component of that octamer (76,77,78). This is supported by the observation of the relaxation of chromatin from a compact mass to a string of nucleosomes upon the removal of H1 (76,79).

The reversible chemical modification of histones by phosphorylation, methylation, and acetylation has been documented (80). In particular, the phosphorylation of H1 appears to be cell cycle dependent (80,81,82). It is therefore a popular hypothesis that the phosphorylation of H1 is a major mechanism for controlling the comformation of chromatin for such processes as DNA replication, RNA transcription, and chromosome condensation (80,83,84). Studies

on a temperature sensitive mutant cell strain (ts85) from a mouse mammary carcinoma (FM3A) defective in H1 phosphorylation have been reported which further support the hypothesis tha H1 phosphorylation regulates the structural changes of chromatin involved in both chromosome condensation during mitosis and DNA synthesis during S-phase (85).

In this report we examine the possible involvement of a GTP utilizing kinase in the phosphorylation of the histone proteins. The inhibition of such a kinase by the depletion of GTP could be the ultimate mechanism of the inhibition of DNA synthesis caused by mycophenolic acid and 6MMPR. The results presented are preliminary, yet very exciting.

2. MATERIALS AND METHODS

2.1- Reagents- Mycophenolic acid was provided by Eli Lilly and Co., Indianapolis, IN. [^{32}P]orthophosphate (carrier free), [γ-^{32}P]adenosine triphosphate (100 Ci/mmol), and [γ-^{32}P]guanosine triphosphate (100 Ci/mmol) were purchased from ICN Pharmaceuticals, Plainview, New York. Ampholines were purchased from LKB, Bromma 1, Sweden.

2.2- Whole Cell Incubations- Mouse T Lymphoma (S-49) cells were grown as described in chapter II. Prior to drug treatment cells were suspended at a density of 1.2 X 10^6 cells/ml in low phosphate medium. Two ml of cells were incubated at 37°C in the presence of drug for 1 hr and then for an additional 2 hr with 400 uCi of [^{32}P]orthophosphate.

Cells were centrifuged, washed with ice-cold PBS, and prepared for analysis by two-dimensional polyacrylamide gel electrophoresis.

2.3- Preparation of Isolated Nuclei- Nuclei were isolated by the method of Marzluff (86). S-49 cells at a density of approximately 10^6 cells/ml were harvested and the cell pellet washed twice with PBS. Cells were suspended in buffer I (0.32 M sucrose, 3 mM calcium chloride, 2 mM magnesium acetate, 10 mM tris (pH 7.5), 0.1 mM EDTA, 0.1% triton X-100, and 1 mM dithiothreitol) at a density of 2 X 10^7 cells/ml and homogenized 10 times in a dounce with a type B (tight) pestle. An equal volume of buffer II (2.0 m sucrose, 5 mM magnesium acetate, 10 mM tris (pH 7.5), and 1 mM dithiothreitol (added fresh)) was aded and mixed well. The suspension was layered over a 2 ml pad of buffer II in a Beckman SW-50 swinging bucket rotor and centrifuged for 60 min at 20K revolutions per minute. The result is a gentle pelleting of the nuclei at the bottom of the tuble separated from cytoplasmic material. The sucrose solution was gently poured off and the nuclei were resuspended in storage buffer (25% glycerol, 5 mM magnesium acetate, 50 mM tris (ph 8.0), 0.1 mM EDTA, and 5 mM dithiothreitol (added fresh)) at a density of 5 X 10^8 nuclei/ml and frozen in liquid nitrogen until needed.

2.4- Incubation of Isolated Nuclei- The buffer for the *in vitro* determination of protein kinase activity in iso-

lated nuclei consisted of 100 ul of 50 mM tris, pH 7.4, 1.0 mM dithiothreitol 5 mM magnesium chloride, and 0.5 mM of either [γ-^{32}P]ATP or [γ-^{32}P]GTP (100 mCi/mmol, total of 5 uCi). The reaction was initiated by adding 10 ul of nuclei suspension and incubaed at 37°C for 2 min. The reaction was halted by the addition of ice-cold PBS and centrifugation, and the sample was prepared for two-dimensional polyacrylamide gel electrophoresis.

2.5- Sample Preparation and Two-Dimensional Polyacrylamide Gel Electrophoresis- Basic proteins were separated using nonequilibrium (NEPHGE) two-dimensional gel elctrophoresis (87). Samples were prepared by dissolving in 100 ul of the lysis buffer of O'Farrell (87) containing 1.6 mg/ml protamine sulfate and 0.3 M NaCl which results in the complete extraction of histones and other basic proteins from chromatin (88).

NEPHGE was carried out out a modification of the procedure of O'Farrell (87) as described by Sanders (88). The first dimension was a 4% cylindrical polyacrylamide gel with 7.1% pH 5-8 and 1.8% pH 3-10 ampholines. The first dimension was electrophoresed at 350 volts constant voltage for 1.75 hr. Methyl green served as a tracking dye and migrated just ahead of the very basic histones. The second dimension was a 12.5% polyacrylamide-SDS slab gel electrophoresed at 15 mA/gel constant current for 4 hr (87).

The gels were dried against filter paper and autoradiographed using Kodak XR-5 X-ray film. Proteins of interest were cut out and counted by liquid scintillation counting.

3. RESULTS

3.1- Protein Phosphorylation in Isolated Nuclei- In this system the label from [γ-^{32}P]nucleotide was found to incorporate into six basic proteins as seen in Fig. VI-9. Three of these proteins are identified as the hisone proteins H1, H2A, and H3. GTP was much more effective than ATP at labeling H1. On the contrary, ATP was much more effective than GTP at labeling H3, a phosphorylation thought to be performed by a cyclic AMP-dependent ATP utilizing kinase (80). ATP and GTP were equally effective at labeling H2A. The identities of the three high molecular weight proteins are not presently known, but GTP is also quite effective at labeling them.

Isolated nuclei were also incubated in the presence of cyclic AMP (data not shown). An increase in the labeling of histone H3 by [γ-^{32}P]ATP relative to the other proteins was the only significant effect.

Control incubations included 50 mM EDTA to inhibit kinase reactions by complexing metal ions. Neither [γ-^{32}P]ATP or [γ-^{32}P]GTP labeled any proteins with EDTA present in this system.

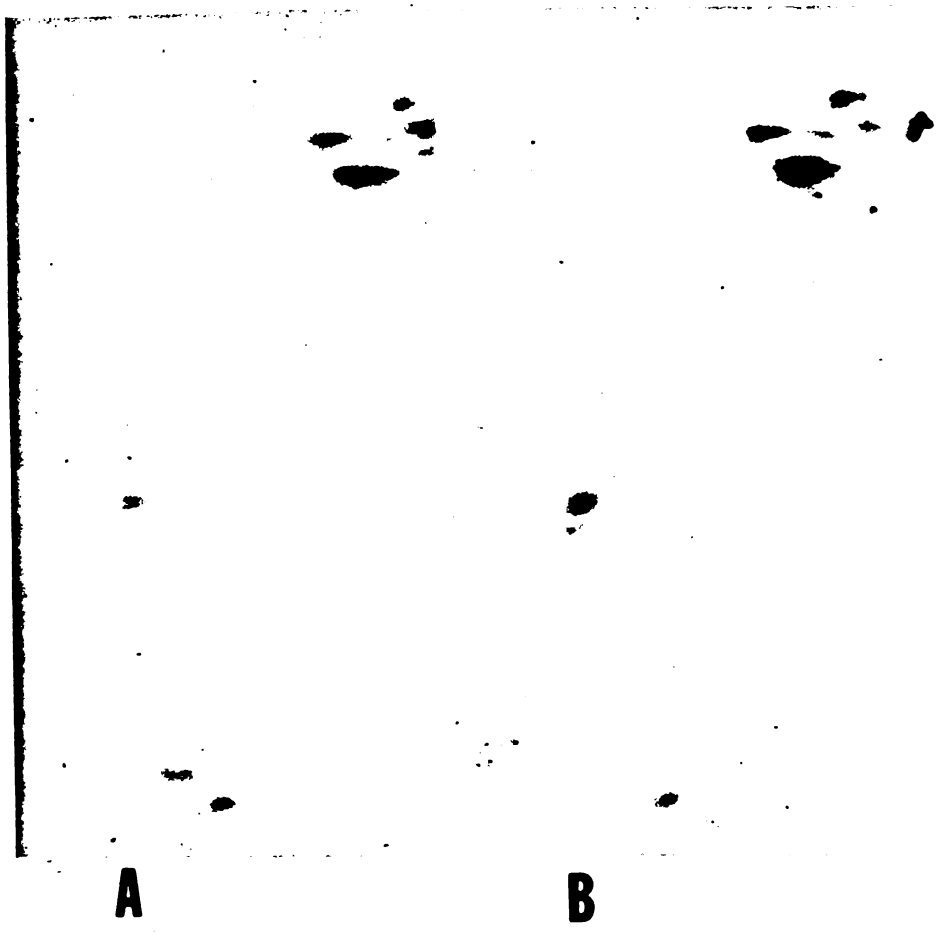

Fig. VI-9. Phosphorylation of Basic Proteins in Isolated Nuclei. Autoradiograms of nuclear proteins which were phosphorylated when nuclei were incubated with A) [γ-^{32}P]ATP or B) [γ-^{32}P]GTP as described in methods.

3.2- Effect of Mycophenolic Acid on Protein Phosphorylation in Cells- The separation of histones as well as other basic proteins labeled by [^{32}P]orthophosphate in whole-cell incubations is shown in figure VI-10. Proteins which were analyzed by scintillation counting are labeled. They include the histones H1, H2A, H3, and H4, as well as two other proteins, Y and Z, analyzed to indicate the general effect on the phosphorylation of nonhistone proteins.

Upon depletion of GTP by mycophenolic acid the phosphorylation of H1 dropped to 34% of control while that of the other histones was unaffected. The phosphorylation of the analyzed nonhistone proteins was approximately 75% of control (Fig. VI-10,Table VI-1). The effect on H1 phosphorylation is dramatically specific considering the lack of effect on the other histone proteins. The 25% drop in phosphorylation of proteins Y and Z is significant, but does not obscure that observed for H1. The specific activity of the .-phosphate of ATP and GTP will be measured in future experiments to determine whether any portion of the inhibition of phosphorylation can be attributed to a drop in nucleotide specific activity. In another experiment addition of guanosine as an exogenous source of guanine nucleotides totally prevented the inhibition of H1 phosphorylation by mycophenolic acid (Table VI-1)

3.3- Effect of DNA Synthesis Inhibition on Protein Phosphorylation- To test whether the observed decease in

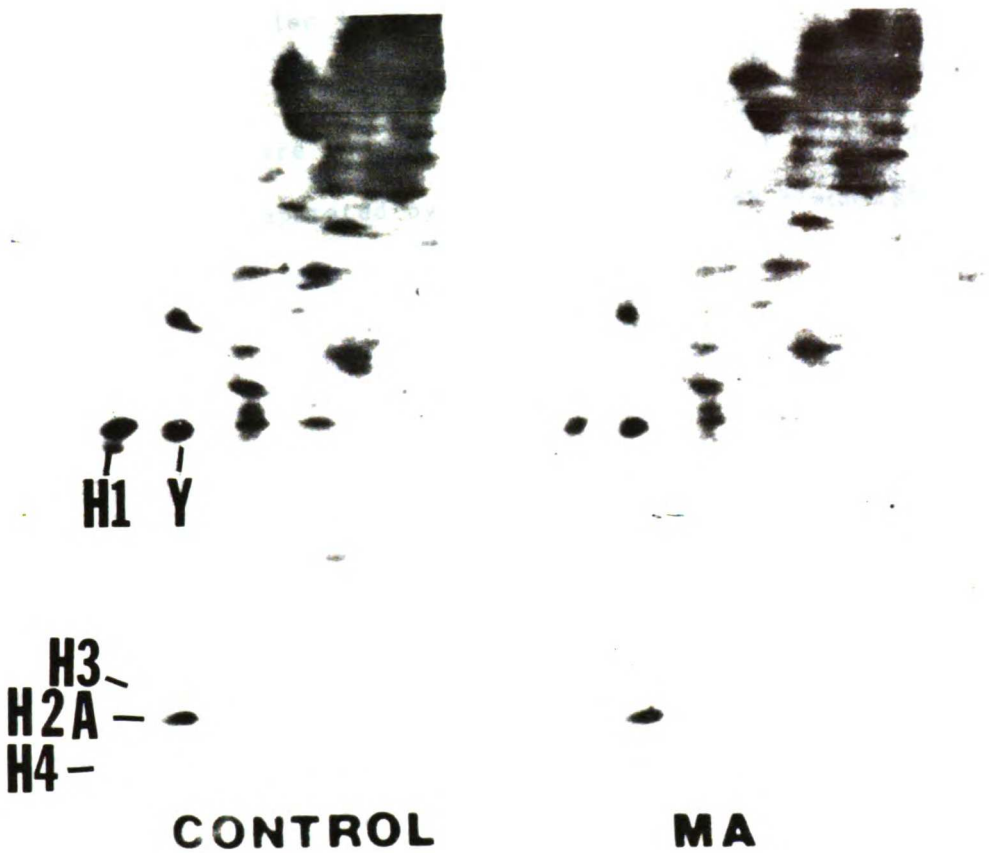

Fig. VI-10. Effect of Mycophenolic Acid on the Phosphorylation of Basic Proteins in Whole S-49 Cells. Autoradiograms of the proteins when control cells or cells treated with mycophenolic acid were incubated with [^{32}P] orthophosphate.

Table VI-1

Protein Phosphorylation in Whole S-49 Cells.

After a one hour incubation with the combination of compounds listed cellular proteins were labeled for two hours with [^{32}P]orthophosphate. The proteins were separated by two-dimensional gel electrophoresis as described in methods.

	Incorporation of [^{32}P]phosphate (% of control)					
	H1	Y	Z	H2A	H3	H4
MA	34	78	75	91	97	100
dbcAMP	91	54	64	47	329	62
MA + dbcAMP	53	52	56	34	323	51
MA + guanosine	155	181	130	193	109	162
aphidocolin	92	94	74	99	115	118

histone phosphorylation is simply a general effect of DNA synthesis inhibition we stopped DNA synthesis with a compound whose mechanism does not involve GTP depletion. Incubation of cells with aphidocolin, an inhibitor of DNA polymerase and ribonucleotide reductase, had no effect on histone phosphorylation. However, a decrease similar to that observed with mycophenolic acid was seen in protein Z. Therefore, the decrease in the phosphorylation of H1 is not simply a result of the inhibition of DNA synthesis. On the contrary, some of the decrease in proteins Y and Z which we considered as background may simply be a byproduct of DNA synthesis inhibition rather than a result of specific radioactivity effects.

3.4- Stimulation of Phosphorylation by cyclic AMP- Cells were incubated with dibutyl cyclic AMP, a transportable analog of cyclic AMP, to insure that putative cyclic AMP-dependent kinases react properly in this system. H3, the phosphorylation of which is likely by a cyclic AMP-dependent kinase (78) showed a three-fold increase in phosphorylation (Table VI-1). However, H1 was unaffected and all other proteins actually showed a decrease in phosphorylation of approximately 40%. Possibly, any phosphorylations not actually stimulated by cyclic AMP were decreased because of dilution of the adenine nucleotide specific radioactivity from breakdown products of dibutyl cyclic AMP or as a result of cyclic AMP cell arrest. Under this assumption it appears

that the phosphorylation of H3 is strongly stimulated by cyclic AMP, while that of H1 is weakly stimulated and that of other proteins are not stimulated. When cells were incubated with both mycophenolic acid and dibutyl cyclic AMP the slight stimulation of H1 phosphorylation by dibutyl cyclic AMP was more pronounced, possibly because non cyclic nucleotide dependent phosphorylations as a background were reduced.

4. DISCUSSION

These results suggest that a GTP utilizing kinase has a major function in the phosphorylation of histone H1 and that the mechanism by which mycophenolic acid inhibits DNA synthesis may involve the disruption of this function. The effectiveness of [γ-^{32}P]GTP at labeling H1 explains the previously unexplained observation by others that the pattern of protein phosphorylation, particularly of H1, by [γ-^{32}P]ATP is substantially different in isolated nuclei compared to whole cells (84). GTP rather than ATP may be the predominate phosphate donor for the phosphorylation of H1. These results in addition to those from experiments in whole cells indicate that H1 is phosphorylated both by a cyclic AMP-independent protein kinase, probably utilizing GTP as a phosphate donor, and a cyclic AMP-dependent protein kinase, probably utilizing ATP as a phosphate donor.

We have proposed that the drastic reduction in DNA synthesis observed upon inhibition of de novo purine biosynthesis by 6MMPR or de novo guanine nucleotide biosynthesis by mycophenolic acid is a result of the depletion of the ribonucleotide, GTP. The drastic effect of mycophenolic acid on histone phosphorylation, the reversibility of the effect by an exogenous source of guanine nucleotides, and the labeling of H1 by [γ-^{32}P]GTP in isolated nuclei support a role for GTP in H1 phosphorylation. Although a causal relationship between the inhibition of H1 phosphorylation and the inhibition of DNA synthesis has not yet been proven, the results do show that the inhibition of H1 phosphorylation is not a result of the inhibition of DNA synthesis. The widely believed function of H1 phosphorylation in regulating the conformation of chromatin makes H1 phosphorylation by a GTP utilizing kinase a prime candidate for the link between GTP and DNA synthesis. Submicroscopic changes in chromatin structure during interphase may be involved (83).

Establishing a causal relationship between the inhibition of H1 phosphorylation and the inhibition of DNA synthesis would have major impact. First, the mechanism of action of many of the purine antimetabolites used in anticancer, antiviral, and immunosuppressive chemotherapy would be solved. Also, and of great potential, an expanded understanding and new techniques for studying the basic process of DNA replication would result.

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