AN ABSTRACT OF THE DISSERTATION OF

Mary Lynn Benka Tassotto for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on September 4, 2002.

Title: Deoxyguanosine Triphosphate, a Possible Target for Reactive Oxygen Species-Induced Mutagenesis.

Abstract approved: Redacted for Privacy

Christopher K. Mathews

Intracellular dNTP pool sizes are highly asymmetric, with dGTP usually comprising 5 to 10% of the sum of the dNTP pools. The work presented in this dissertation addresses the question of whether the underrepresentation of dGTP is related to its potential to be oxidized by reactive oxygen species. 8-oxo-guanine is important in oxidative mutagenesis, and current evidence indicates that this lesion arises in DNA partly through oxidation of dGTP, followed by incorporation of 8-oxo-dGTP into DNA. The bacterial MutT protein and its mammalian homolog catalyze the hydrolysis of 8-oxo-dGTP to 8-oxo-dGMP *in vitro*. It is a widely accepted premise that the primary function of these enzymes is to remove 8-oxo-dGTP from the nucleotide pool of cells so that it cannot be used as a substrate for DNA synthesis. However, this model has been called into question by observations that some *mutT* strains of *E. coli* display a mutator phenotype when grown anaerobically, and by kinetic studies that showed 8-oxo-dGTP to be a poor DNA polymerase substrate.

In this study, the dNTP pools of mammalian cells cultured in varying oxygen conditions were measured, with the expectation that the dGTP pool would expand under low oxygen conditions if it were a target for damage by reactive oxygen species. HeLa cells cultured in 2% O_2 showed no change in the dGTP pool when compared to

cells cultured in 20% O_2; however, in V79 cells, the dGTP pool did expand in 2% O_2. This result was not specific to the dGTP pool, as pools of dATP and dTTP also increased when V79 cells were cultured at 2% O_2. These results suggest that there may be increased turnover of the dGTP pool when cells are cultured in high oxygen, but these experiments did not address the reason for this oxygen-dependent change.

In order to determine whether 8-oxo-dGTP accumulates to levels that are sufficient to cause mutagenesis in cells, an analytical method for the measurement of 8-oxo-dGTP from cell extracts was developed. By use of this method, which involves reversed-phase high performance liquid chromatography coupled with electrochemical detection, no 8-oxo-dGTP was detected in *mutT E. coli* cells, even when they were cultured in the presence of H_2O_2. The estimated upper limit of 8-oxo-dGTP in these cells is about 240 molecules per cell, which corresponds to an intracellular concentration of approximately 0.34 μM. When 8-oxo-dGTP was added at this concentration to an *in vitro* DNA replication system in which replication errors could be scored as mutations, along with the four normal dNTPs at their estimated intracellular concentrations, there was no detectable effect on the frequency of mutation. Therefore, the presence of 8-oxo-dGTP at physiologically relevant concentrations does not appear to be significantly mutagenic. The results presented in this dissertation suggest that the mechanism by which the MutT enzyme counteracts mutagenesis should be reevaluated.

DEOXYGUANOSINE TRIPHOSPHATE, A POSSIBLE TARGET FOR REACTIVE OXYGEN SPECIES-INDUCED MUTAGENESIS

by
Mary Lynn Benka Tassotto

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment
of the requirements for the
degree of

Doctor of Philosophy

Presented September 4, 2002
Commencement June 2003

Doctor of Philosophy dissertation of Mary Lynn Benka Tassotto presented on September 4, 2002.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Christopher K. Mathews for his support of this research and for the encouragement, support and patience he has shown me during the course of this work. Thanks also go to specific members of the Mathews lab: Dr. Stella Martomo, who provided invaluable help with the *in vitro* DNA replication assays; Dr. Michael Olcott, who helped with the purification of 8-oxo-dGMP; and Dr. Indira Rajagopal and Linda Wheeler with whom I had many useful discussions. To all members of the Mathews lab, past and present, whose time overlapped with mine: it was an honor to consider you as my colleagues - I will always consider you among my friends.

Thanks go to specific members of the Biochemistry and Biophysics department: Dr. Dean Malencik, for sharing his HPLC expertise with me, and Dr.Wil Gamble, for use of rotovap in his laboratory. I would also like to thank the OSU Environmental Health Sciences Center, Cell and Tissue Analysis Facility staff, specifically Corwin Willard and Julie Oughton, for their assistance with flow cytometry.

The development of the HPLC-ECD assay for 8-oxo-dGTP could not have been completed without the assistance of many individuals. First of all, I thank the Center for Gene Research and Biotechnology for loaning me the Beckman HPLC system, and special thanks go to Dr. William Baird for generously allowing me to use his electrochemical detector for an extended time. I would also like to thank John Ancmon from LPS for his technical help with the HPLC system and Dr. Ray Weigand at Alltech Associates for his assistance, advice and generosity.

Finally, I would like to acknowledge and thank my family. Their support, encouragement and love for me have been steadfast throughout this process. They uplifted me in discouraging times and celebrated with me in joyful times. To simply say "thank you" seems so insufficient. I am grateful to my parents, Marian and John Benka, who were always willing to make sacrifices so that I could pursue my goals. The writing of this dissertation would have never been completed, if it were not for

their willingness to spend countless hours babysitting! To my son, Matthias: you are too young to understand this, but you also had to make sacrifices so that I could achieve this goal, and for that I thank you. Since you came into this world, you have given me more joy than I ever thought possible, and your smiling face has been just what I needed at the end of so many days while I was in the process of writing this dissertation. Finally, I especially want to acknowledge Michael, my best friend who also became my husband while we ventured through graduate school together. I cannot even begin to list all of the ways that you have helped me along this journey. Thank you for being my strength. You are and will always be the "best part of every day of my life". I love all of you very much.

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LIST OF ABBREVIATIONS

AGR 8-aminoguanosine
AP ..... apurinic / apyrimidinic
BrdUrd 5-bromodeoxyuridine
BrdUTP ..... 5-bromodeoxyuridine triphosphate
BSA ..... bovine serum albumin
cAMP ..... cyclic AMP
CPM ..... counts per minute
dGuo ..... deoxyguanosine
DHF ..... dihydrofolate
DMSO ..... dimethyl sulfoxide
DTT 1,4-dithio-DL-threitol
ECD ..... electrochemical detection
EDTA ..... ethylenediaminetetraacetic acid
FapyGua 2,6-diamino-4-hydroxy-5-formamidopyrimidine
FPG ..... formamidopyrimidine DNA glycosylase
Gua ..... guanine
Guo ..... guanosine
HGPRT ..... hypoxanthine-guanine phosphoribosyltransferase
HPLC ..... high performance liquid chromatography
IPTG ..... isopropyl thiogalactoside
kDa ..... kilodalton
MNNG ..... *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
MNU ..... *N*-methyl-*N*-nitrosourea
mtDNA ..... mitochondrial deoxyribonucleic acid
4-NQO 4-nitroquinoline-*N*-oxide
8-oxo-dG ..... 8-oxo-7,8-dihydro-2'-deoxyguanosine
8-oxo-dGDP ..... 8-oxo-7,8-dihydro-2'-deoxyguanosine diphosphate

LIST OF ABBREVIATIONS (Continued)

8-oxo-dGMP ..... 8-oxo-7,8-dihydro-2'-deoxyguanosine monophosphate

8-oxo-dGTP ..... 8-oxo-7,8-dihydro-2'-deoxyguanosine triphosphate

OH· ..... hydroxyl radical

$O_2^{\bullet -}$ ..... superoxide radical

PBS ..... phosphate-buffered saline

PEI ..... polyethyleneimine

PRPP 5-phosphoribosyl-α-pyrophosphate

RNR ..... ribonucleoside diphosphate reductase

ROS ..... reactive oxygen species

SDS ..... sodium dodecylsulfate

TBA-OH ..... tetrabutylammonium hydroxide

TBS ..... tris-buffered saline

TCA ..... trichloroacetic acid

TEAB ..... triethylammonium bicarbonate

THF ..... tetrahydrofolate

TOA ..... tri-*N*-octylamine

TPA 12-*O*-tetradecanoylphorbol-13-acetate

UV ..... ultraviolet

Xgal ..... 5-bromo-4-chloro-3-indoyl β-D-galactoside

DEDICATION

To my parents, Marian and John Benka.

DEOXYGUANOSINE TRIPHOSPHATE, A POSSIBLE TARGET FOR REACTIVE OXYGEN SPECIES-INDUCED MUTAGENESIS

Chapter 1. Introduction – DNA Precursor Pools and Mutagenesis

1.1. Deoxynucleoside Triphosphate Biosynthesis

One major factor involved in minimizing spontaneous and induced genetic instability in bacteria and eukaryotic cells is the careful regulation of DNA precursor biosynthesis. A balanced supply of the four deoxynucleoside triphosphate (dNTP) precursors is required for the accurate replication and repair of DNA. Consequently, endogenous or induced imbalances in the DNA precursor pool can elicit a range of genetic effects including elevated spontaneous mutagenesis, recombination, enhanced sensitivity to carcinogens and mutagens, chromosomal rearrangement, breakage and loss, stimulation of apoptosis, and oncogenic transformation. Thus, the relative sizes of intracellular dNTP pools are carefully regulated to maximize genetic stability [1].

As shown in Figure 1.1, the synthesis of deoxyribonucleoside triphosphates involves many enzymes, three of which are allosterically regulated to control the relative sizes of intracellular dNTP pools. Mutations affecting the allosteric control mechanisms of these enzymes lead to unbalanced dNTP pools and consequently to mutagenesis. Ribonucleotide reductase, RNR, is one enzyme that is allosterically controlled and is essential for regulation of the dNTP pool levels [2]. It catalyzes the reaction that reduces ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which are then phosphorylated by the action of nucleoside diphosphate kinase to form the dNTPs used in DNA replication and repair. RNR is a $\alpha 2\beta 2$ tetramer, and the $\alpha 2$ subunit has two independent allosteric sites that control both the enzyme's catalytic activity and its substrate specificity. The binding of ATP to the activity site increases the enzyme's ability to reduce all substrates, and the substrate to be reduced is determined by the nucleotide effector bound at the specificity site.

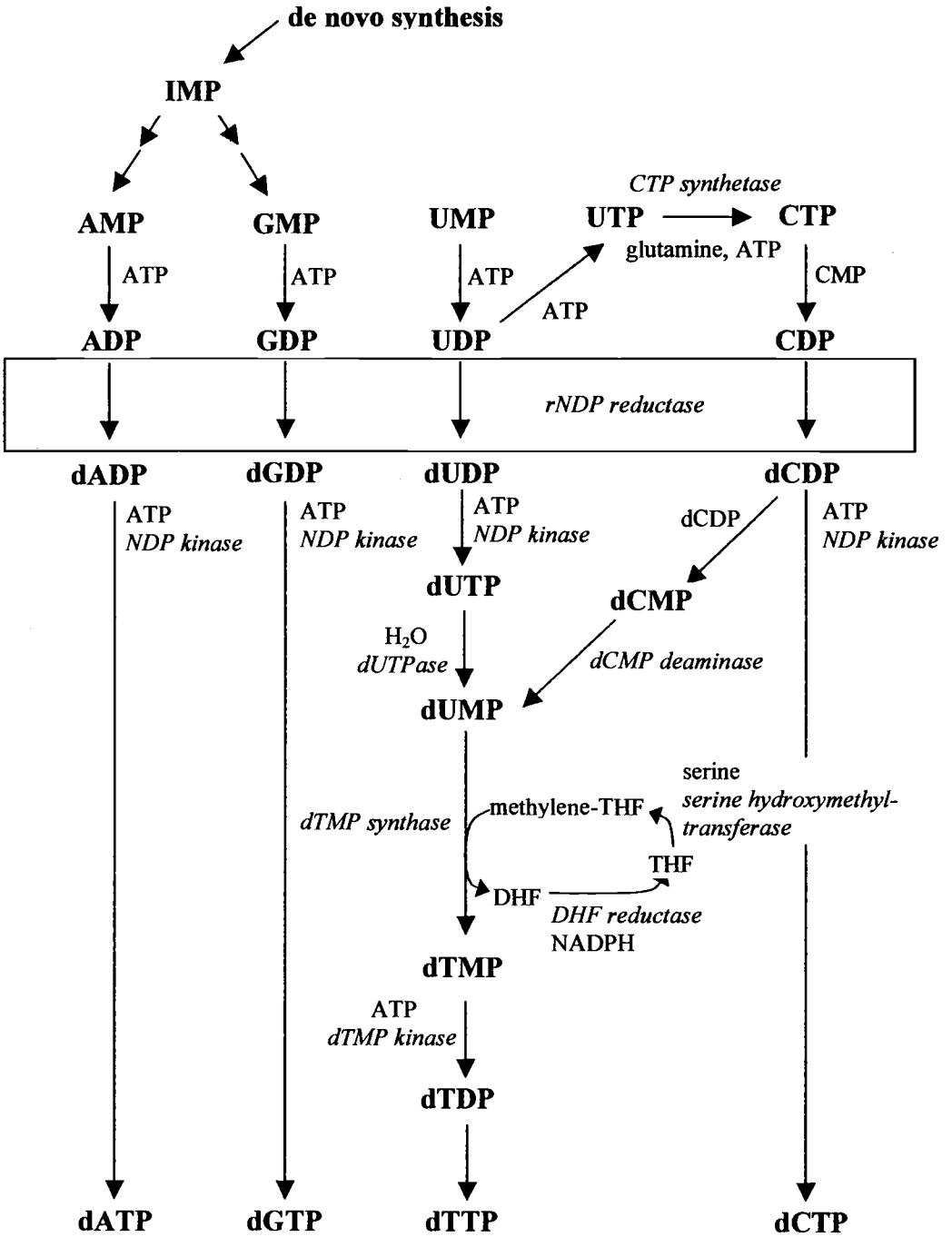


Figure 1.1 Pathways of dNTP synthesis.
Enzymes involved in the biosynthesis of dNTPs are shown in italics. Allosterically regulated enzymes include CTP synthetase, rNDP reductase, and dCMP deaminase.

At the specificity site, ATP or dATP binding stimulates CDP and UDP reduction, dTTP binding stimulates GDP reduction but inhibits CDP and UDP reduction, and dGTP binding stimulates ADP reduction but, in mammalian cells only, dGTP inhibits CDP and UDP reduction [2]. Cells expressing ribonucleotide reductase that has mutations leading to altered feedback inhibition by the dNTPs show marked changes in their spontaneous mutation rate [3]. Thus, this complex allosteric regulation by RNR is essential for the maintenance and production of balanced dNTP pools.

CTP synthetase is another enzyme that is allosterically regulated and involved in dNTP biosynthesis [1]. It controls the relative levels of cytidine or deoxycytidine nucleotides by converting UTP to CTP in the presence of glutamine and ATP. GTP stimulates the activity of the enzyme, while the enzyme is feedback inhibited by CTP. Cells deficient in this regulation have increased pools of CTP and dCTP, which leads to spontaneous mutation rates that are 5- to 300-fold higher than in CTP synthetase proficient cells. Analysis of mutations in such CTP synthetase-deficient cells reveals that the mutator phenotype is due to replication errors induced by the dNTP pool imbalance [3].

The third enzyme of dNTP biosynthesis that is allosterically regulated is dCMP deaminase. The regulation of ribonucleotide reductase does not distinguish between the pyrimidine rNDPs, UDP and CDP, so dCMP deaminase activity is necessary to control the relative levels of dCTP and dTTP [4]. This enzyme catalyzes the deamination of dCMP to form dUMP, and it is allosterically activated by dCTP and inhibited by dTTP [1]. As is seen in CTP synthetase-deficient cells, dCTP levels are elevated in the case of dCMP deaminase deficiency, and providing thymidine to the cells can restore the dCTP:dTTP ratio and thus prevent mutagenesis by dNTP pool imbalances.

1.2. Deoxynucleoside Triphosphate Levels and the Maintenance of Genetic Stability

In addition to the careful regulation of DNA precursor biosynthesis, DNA synthesis must proceed with very high fidelity in order for genetic stability to be maintained. During replication, polymerases catalyze template-directed polymerization of dNTPs onto DNA. At several points in this polymerization cycle, the dNTP concentrations are critical for determining the fidelity of DNA synthesis. The probability that either an incorrect or correct nucleotide will be inserted is partially determined by the ratio of concentrations of the competing nucleotides. Therefore, for each nucleotide to be incorporated, an increase in the correct nucleotide pool will increase the fidelity of synthesis, whereas fidelity would decrease by an increase in the incorrect nucleotide pool. The potential for insertion of an incorrect nucleotide also exists due to the "next nucleotide effect" as described by Zhang and Mathews [5], who hypothesized that insertion errors would be more common at template C sites where dGTP, the least abundant nucleotide, would be the correct nucleotide to be inserted. In this example, proofreading immediately 5′ to template C sites would be more efficient, because slower insertion of dGTP would give proofreading exonucleases more time to act. However, if the concentration of the next correct nucleotide to be incorporated increases, the rate of incorporation from a mismatch increases, therefore decreasing the fidelity of polymerization and causing mutagenesis.

Alterations in dNTP pools or conditions that modify DNA precursor levels have also been found to influence mutagenesis in other ways. This point is illustrated when one considers that exposure to alkylating agents or UV radiation can induce point mutations in DNA, but this effect can be modulated in the presence of altered dNTP pools. For example, the mutagenic lesion O^6-alkylguanine is produced in cells following treatment with the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). During DNA replication this adduct mispairs with thymine producing G·C→ A·T transition mutations [6,7]. This effect is increased by the elevation of

dTTP pools, but it could be modulated in a dCMP deaminase-deficient strain by elevation of the dCTP:dTTP ratio [7]. Therefore, one mechanism for modulation of mutagenesis by alteration of the dNTP pools is insertion of the dNTP present in excess across from template damage to prevent point mutations induced by mispairing. Another example of how dNTP levels influence mutagenesis is the induction of mutagenesis by BrdUTP incorporation across from template guanine, which occurs both *in vitro* and *in vivo*, when cells are incubated in the presence of BrdUrd. Elevating dTTP levels by incubating cells in the presence of deoxythymidine inhibits the reduction of CDP by ribonucleotide reductase, thus lowering dCTP levels and enhancing mutagenesis by favoring BrdUTP incorporation opposite template guanine [8]. So, mutagenesis can be both potentiated and modulated by alterations in dNTP pools.

Imbalances in the dNTP pools might also enhance mutagenesis if these nucleotide precursors were targets for DNA damaging conditions or agents. Topal and Baker showed that in synchronized S-phase cells treated with a nontoxic dose of *N*-methyl-*N*-nitrosourea (MNU), the nucleotide precursor pool was 190-13,000 times more susceptible to methylation than a deoxyribonucleotide residue in the DNA duplex [9]. This effect may be due to the fact that histones protect bases in DNA from modification, and the dNTP pool is more susceptible to modification because it lacks such protection. Arecco et al. [10] tested the hypothesis that if a free nucleotide is a target for a DNA damaging agent, then expansion of the pool of that nucleotide should increase the target size, and hence, augment the biological effect of the DNA damaging agent. Their experiments with two mammalian cell lines showed that conditions that expanded pools of dATP, dTTP and dGTP stimulated mutagenesis by MNU several fold. This result is consistent with the hypothesis that dNTP pools can be targets for the effects of DNA damaging agents, and emphasizes that the careful regulation of dNTP pool sizes is critical in order to prevent mutagenesis.

Some researchers have also suggested that dNTP pools can be modified by oxidation in cells (see below and Chapter 2) to produce abundant pools of potentially

mutagenic nucleotides [11,12]. While this may be an accepted concept in the scientific literature, no attempt has yet been made to quantitate intracellular pools of these oxidized nucleotides, and therefore their significance with respect to mutagenesis *in vivo* is still unclear. However, based on *in vitro* studies of the incorporation of oxidized nucleotides [11,13-15], if these altered dNTPs are not removed from the nucleotide pool, it is possible that they could then contribute directly to mutagenesis by mispairing upon incorporation into DNA during replication or repair synthesis. Additionally, modified nucleotides may alter the pool sizes of the normal nucleotides by interfering with the allosteric regulation of enzymes involved in dNTP biosynthesis and therefore indirectly produce mutagenic effects. However, it is important to note that all of these effects are expected to be seen only at nonphysiological concentrations of the modified precursors and may not be relevant under physiological conditions [16].

Modifications to the dNTP pools or alterations in the dNTP pool sizes can contribute to mutagenesis during both replicative and repair DNA synthesis. While replicative polymerases tend to catalyze DNA replication with very high accuracy, mammalian DNA repair polymerases beta and eta tend to have low fidelity when compared to replicative polymerases, and are thus considered to be error-prone [17,18]. This characteristic can lead to mutagenesis, especially when dNTP pools are imbalanced. The affinity of many DNA repair polymerases for modified nucleotides has not been studied in detail. However, it is clear that DNA polymerase β, the main polymerase responsible for catalyzing DNA repair in mammalian cells, has the ability to efficiently incorporate modified nucleotides, including the oxidized nucleotide 8-oxo-dGTP (see below), into DNA *in vitro* [17].

1.3. Deoxynucleoside Triphosphate Pool Asymmetry and the Underrepresentation of dGTP

Concentrations of deoxynucleoside triphosphates in cells are generally very low, compared to rNTP pools, and are sufficient to sustain chromosomal DNA replication

for only a few minutes [19]. The intracellular dNTP pools are compartmentalized, with the nuclear pools that supply DNA replication making up only 10-20% of the total cell pools [20]. The sizes of the whole cell dNTP pools in yeast (0.5-1.5 pmol/10^6 cells) are between those typically observed for bacterial cells (usually < 0.2 pmol/10^6 cells) and mammalian cells (usually >5 pmol/10^6 cells) [4]. Table 1.1 is a collection of data showing the results of deoxynucleoside triphosphate pool measurements from many mammalian cell lines. As shown in this table, there is considerable variation in the reported dNTP pools, even from the same cell line. This is to be expected considering that the sizes of dNTP pools are highly dependent on cell density. It has also been observed that the sizes of dNTP pools vary during the cell cycle [19,21] (also this work, see Figure 5.4).

The size of the dNTP pool depends both upon the rate of synthesis of the dNTP and on its turnover by incorporation into DNA or catabolism. Hence, measurements of the size of dNTP pools cannot give any information regarding the dynamic nature of dNTP turnover. However, the data shown in Table 1.1 and other measurements of dNTP pool sizes in many eukaryotic cell types and in bacteria are of interest because they show that the relative concentrations of the four dNTPs are not equal. Since an imbalance in the dNTP pools is mutagenic, it would be expected that the normal pool sizes reflect the relative abundance of each of the four deoxyribonucleotides in DNA. However, dNTP pools do not reflect the relative abundance of each of the deoxyribonucleotides in DNA. In fact, the intracellular pools of dNTPs are highly asymmetric, as can be seen in Table 1.1. The most notable observation regarding this natural dNTP pool asymmetry is the observation that dGTP is the least abundant nucleotide. In most cell lines, dGTP is consistently underrepresented, often comprising less than 5 percent of the total of the four dNTPs. This observation is not limited to mammalian cells grown in culture, as dGTP was also the least abundant of the four nucleotides when dNTP pools were analyzed from individual rat embryos [22,23].

Table 1.1 Deoxyribonucleoside 5′-triphosphate pool sizes in mammalian cells.

Cells or Nuclei	dNTP content, pmol/10^6 cells				Reference
	dATP	**dCTP**	**dGTP**	**dTTP**	
HUMAN					
HeLa Cells	122	25	11	131	[24]
HeLa Cells	96	55	20	153	[20]
HeLa Nuclei	42	20	7	43	[20]
HeLa cells (S phase)	26	31	22	45	[25]
Human fibroblasts	31	30	10	15	[26]
Human fibroblasts	24	12	5	47	[27]
Human lymphocytes	5	3	2	17	[28]
PHA-stim. lymphocytes	60	44	26	243	[29]
HL-60 Cells	50	16	19	58	[30]
H-9 Cells	71	44	29	150	[30]
K-562 Cells	57	24	15	120	[30]
MOUSE					
FM3A Cells	180	145	62	490	[31]
S49 Cells	20	20	4	60	[32]
S49 Cells	44	23	14	36	[33]
WEHI-7 Cells	23	20	10	25	[34]
HAMSTER					
CHO Cells	110	385	10	105	[20]
CHO Nuclei	31	46	3	25	[20]
V79 Cells	44	47	10	100	[10]

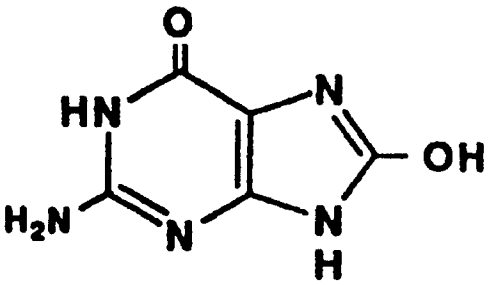


Figure 1.2 Structure of the oxidized base, 8-oxo-guanine.

What is the reason for this underrepresentation of dGTP in the nucleotide pool? This is the question that prompted the work that is presented in this dissertation. As mentioned above, the nucleotide precursors of DNA can be targets for the same agents known to damage DNA. The oxidized base, 8-oxo-guanine (see Figure 1.2), was first described as a major lesion of DNA after exposure to reducing agents [35]. 8-oxo-dG is biologically important due to its ability to cause mutagenesis by incorrectly base pairing with adenine, as first described by Shibutani et al. [36]. This oxidized base was soon implicated not only in mutagenesis, but also in carcinogenesis and the ageing process, and was quickly accepted as the most significant and abundant marker of damage to cells by reactive oxygen species [37]. In their 1992 publication, Maki and Sekiguchi [11] commented that "As the oxidation of guanine proceeds *in vitro* more rapidly in dGTP than in DNA (unpublished data), it is conceivable that the oxidative damage to the base would occur more frequently in the nucleotide pool of *E. coli* cells than in their chromosomal DNA". This observation, coupled with Maki and Sekiguchi's description of the MutT enzyme in *E. coli* that prevents replication errors by degrading 8-oxo-dGTP, a potentially mutagenic substrate for DNA synthesis [11], led to the accepted premise that "oxidative DNA lesions are formed through the incorporation of an oxidatively damaged DNA precursor by a DNA polymerase(s), as

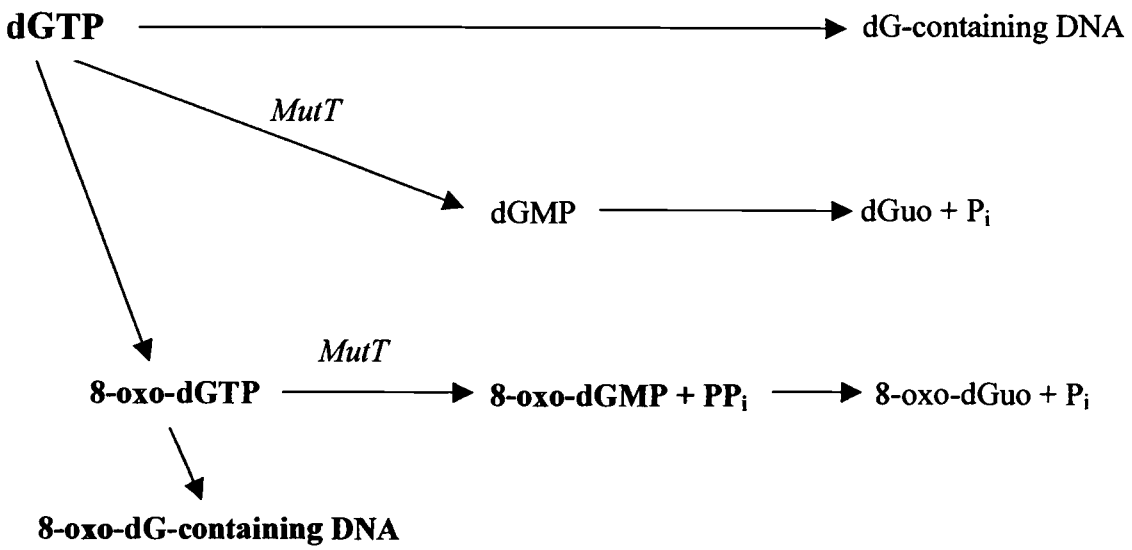


Figure 1.3 Possible metabolic fates of dGTP.
The pathways of interest with respect to the work presented in this dissertation are shown in bold type.

well as through the direct oxidation of a residue in DNA" [12]. This pathway and the other potential metabolic fates of dGTP are shown in Figure 1.3. A complete review of the published literature regarding the oxidized nucleotide, 8-oxo-dGTP, and its mutagenicity follows in Chapter 2.

This idea that the nucleotide, dGTP, could be a target for oxidation was of considerable interest with respect to the underrepresentation of the dGTP pool. If dGTP is susceptible to oxidation *in vivo*, then one reason for the very low pool size of dGTP could be that it is rapidly turned over upon oxidation by the action of the MutT enzyme. This would prevent accumulation of the mutagenic nucleotide 8-oxo-dGTP in cells and could account for the observation that dGTP pool sizes are so low. The identification and characterization of homologs of the MutT enzyme in mouse [38], rat [39], and human cells [40] supported the possibility that oxidation and degradation of the dGTP pool could be occurring in mammalian cells, which could account for their very low dGTP pools.

The initial goal of this work was to test the hypothesis that the oxidation of dGTP and its subsequent degradation by the action of the MutT enzyme are responsible for the underrepresentation of the dGTP pool in cells. However, during the course of this work, it became obvious that a more thorough study of the significance of dGTP as a target for oxidation and its mutagenic potential *in vivo* was necessary. While most of the published literature regarding the MutT enzyme (see Chapter 2) supports the premise that its primary role is elimination of 8-oxo-dGTP from the nucleotide pool, this function has recently been called into question [41,42] due to a number of observations. Fowler et al. [43] showed that in an *E. coli* strain lacking the MutT enzyme, the frequency of mutation was similar under both aerobic and anaerobic conditions, where little to no oxidation of dGTP should occur. Also, in *in vitro* studies of the incorporation of 8-oxo-dGTP into actively replicating DNA, the nucleotide was shown to be a poor substrate for many DNA polymerases tested [44], including the main replicative polymerase in mammalian cells, polymerase δ [41]. The results of these kinetic studies suggest that in order for 8-oxo-dGTP to substitute for dGTP and cause mutagenesis *in vivo*, intracellular levels of the oxidized nucleotide would have to be significant. While it is clear that cells lacking a functional MutT enzyme express a mutator phenotype [45,46] with spontaneous mutation frequencies ranging from 100- to 10,000-fold higher than normal, it is no longer clear that 8-oxo-dGTP is the critical substrate for this enzyme. This possibility is substantiated by the observation that the *E. coli* MutT enzyme and its human homolog, hMTH1, have multiple nucleotide substrates [47,48]. These observations suggest that the mechanism by which the MutT enzyme protects against the effects of oxidation and the role of 8-oxo-dGTP in mutagenesis need to be clarified. The work presented in this dissertation calls into question the widespread belief that dGTP oxidation induced by reactive oxygen species *in vivo* is a significant contributor to mutagenesis.

1.4. Dissertation Aims

The work presented in the following chapters addresses two questions regarding the underrepresentation of the dGTP pool and oxidative mutagenesis. First, is the oxidation of dGTP to 8-oxo-dGTP and its subsequent turnover by the MutT enzyme responsible for the very low dGTP pool found in cells? In order to address this question, a study of the dGTP pool sizes of mammalian cells cultured at varying oxygen concentrations was completed. If oxidation of dGTP occurs *in vivo* and the turnover of 8-oxo-dGTP is abundant, then one would expect pools of dGTP to be elevated when cells are grown in a low oxygen atmosphere. Secondly, does the incorporation of 8-oxo-dGTP into DNA cause mutagenesis *in vivo*? To evaluate the potential of dGTP as a target for oxidation, it was necessary to measure the concentration of 8-oxo-dGTP in cells. To date, no measurements of 8-oxo-dGTP pools have been reported in the literature. However, the potency of this nucleotide's mutagenic effect during *in vitro* DNA synthesis (see Chapter 2 and [15]) suggests that 8-oxo-dGTP may exert significant biological effects if it is present at even low concentrations *in vivo*. An analytical method for the detection and quantitation of 8-oxo-dGTP in cell extracts was developed to meet this goal. If the action of the MutT enzyme is significant in protecting against oxidative mutagenesis by 8-oxo-dGTP, then one would expect that pools of 8-oxo-dGTP would accumulate in extracts from cells lacking a functional MutT enzyme or in cells that were exposed to reactive oxygen species. Measurements of the intracellular concentration of 8-oxo-dGTP in a wild type *E. coli* strain and in MutT-deficient strains were conducted. Finally, once the intracellular concentration of 8-oxo-dGTP was determined, experiments were conducted to evaluate whether this "physiological" concentration of the oxidized nucleotide is sufficient to cause mutagenesis *in vivo*.

Chapter 2. Literature Review – Mutagenesis by Guanine Nucleotide Oxidation

2.1. Oxidative Stress and Damage to Macromolecules

The use of oxygen presents a unique problem for aerobic organisms. While oxygen is necessary for aerobic respiration, its utilization results in the formation of reactive oxygen species (ROS) within cells. Intracellular reactive oxygen species are also generated by exposure to drugs and toxic agents, by disease and tissue injury, and by exposure to environmental pollutants [49]. Many of these reactive oxygen species are free radicals that contain unpaired electrons, which can cause damage to most cellular components. The reactive oxygen species known to damage macromolecules are the superoxide anion ($O_2^{\bullet -}$), the hydroxyl radical ($OH\cdot$), hydrogen peroxide (H_2O_2), and peroxynitrite ($OONO^-$). These can nick DNA and modify bases found in DNA [50], can damage essential enzymes and structural proteins [51] and can also provoke uncontrolled chain reactions, such as lipid peroxidation [52]. The excessive production or insufficient disposal of reactive oxygen species within cells constitutes oxidative stress [49]. The damage resulting from exposure to these reactive oxygen species is collectively referred to as oxidative damage, and it has been implicated in many disease states, neurodegeneration, and ageing. In recent years, there has been considerable interest in the formation and the consequences of oxidative damage to DNA due to the potential cytotoxic, carcinogenic, and mutagenic effects of this type of damage.

2.2. *In vivo* Generation of Reactive Oxygen Species

The generation of reactive oxygen species *in vivo* occurs by two processes: 1) accidental generation as a byproduct of normal metabolic processes, and 2) deliberate generation of reactive oxygen species for cellular processes. One example of a metabolic process that generates ROS as byproducts is the mitochondrial electron

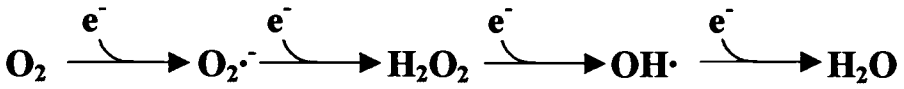


Figure 2.1 Generation of reactive oxygen species by the mitochondrial electron transport chain.
The formation of $O_2^{\cdot -}$, H_2O_2, and OH· occurs by successive one-electron (e^-) additions to O_2.

transport system, which consumes approximately 85% of the oxygen utilized by a cell. During aerobic respiration, mitochondria break down oxygen into water by a series of one-electron reductions, as shown in Figure 2.1. The leakage of electrons onto O_2 during aerobic metabolism in the mitochondria is one pathway by which ROS can be accidentally generated by cells [53]. Mitochondrial DNA has high levels of oxidative damage and mutation, which has been attributed to the location of mtDNA near the inner mitochondrial membrane where oxidants are formed, lack of protective histones on mtDNA, and limited DNA repair activity in mitochondria [54]. Another example of the production of ROS during normal metabolism is the β-oxidation of fatty acids that occurs in peroxisomes [53]. H_2O_2 is created as a byproduct of β-oxidation, which may leak into cells and mistakenly cause oxidative damage. Similarly, some cytochrome P450 isozymes in microsomes can directly reduce O_2 to $O_2^{\cdot -}$; or alternatively, substrates can accept single electrons from cytochrome P450 and transfer them to oxygen to generate reactive oxygen species in a process called redox cycling [53]. Although in these examples ROS are generated as a byproduct of normal metabolic functions, these biochemical activities can mistakenly cause oxidative stress.

In contrast, some cell types intentionally generate reactive oxygen species and employ their damaging characteristics for useful purposes. For instance, the immune system uses activated macrophages and neutrophils that produce H_2O_2 and $O_2^{\cdot -}$, to kill invading microorganisms in a process referred to as the oxidative burst [55]. Evidence suggests that H_2O_2 and $O_2^{\cdot -}$ are also produced by several other cell types, including lymphocytes, fibroblasts, and vascular endothelial cells [49]. Because of its ability to

easily diffuse within and between cells, a metabolic role for H_2O_2 in intracellular signaling has been proposed. It is known that H_2O_2 can induce the expression of genes controlled by NF-KB. H_2O_2 displaces the inhibitory subunit from NF-KB in the cytoplasm, so that the active transcription factor can migrate to the nucleus and bind to specific enhancer and promoter elements on DNA [56]. ROS generated during metabolic processes can also serve other necessary purposes in cells. A further example is the generation of H_2O_2 *in vivo* by several oxidase enzymes, such as glycolate oxidase and xanthine oxidase, and its use by the enzyme thyroid peroxidase to aid in the production of thyroid hormones [49]. Therefore, despite the harmful effects of ROS in cells, they also serve important biological functions.

2.3. Cellular Defenses Against Oxidative Damage

Cells have many universal defense mechanisms to minimize the damage produced by reactive oxygen species (reviewed in [52,53]). Cellular enzymes such as glucose-6-phosphate dehydrogenase, which regenerates NADPH, exist to maintain a reducing environment in the cell. Superoxide dismutases catalyze the conversion of $O_2^{\cdot -}$ to H_2O_2, while catalases and glutathione peroxidases convert H_2O_2 to H_2O. H_2O_2 can react with reduced transition metals, especially Fe^{2+} or Cu^{1+}, by the Fenton reaction [57] or by interaction with the $O_2^{\cdot -}$ radical in the Haber-Weiss reaction [58] to form the hydroxyl radical (OH·). These reactions are shown below:

$$\mathbf{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \ (Fenton)}$$

$$\mathbf{O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot \ (Haber\text{-}Weiss)}$$

Iron and copper are ubiquitous metals in cells, and are found abundantly in the structures of many enzymes and proteins. Because of their propensity to participate in one-electron transfer reactions, these transition metals can generate potentially damaging hydroxyl radicals [59], as shown in the above reactions. Thus, organisms utilize both transport and storage proteins to minimize the amount of free iron and

copper within cells and extracellular fluids. OH· is very energetic and short-lived, and it attacks biological molecules by abstracting hydrogen [60]. Due to these characteristics it is considered to be especially reactive, and it has been implicated in the damage of all classes of macromolecules. The toxicity of H_2O_2 and $O_2^{\cdot-}$ may be due to their conversion into OH· in cells. However, despite its reactivity, cells are protected from the damaging effects of OH· by the action of small antioxidant molecules such as the hydrophilic radical scavenger glutathione and the lipophilic radical scavenger α-tocopherol [61].

Cells not only have defenses that eliminate reactive oxygen species, but they also possess a wide array of repair activities to prevent the accumulation of oxidatively damaged macromolecules. For instance, phospholipase A_2 cleaves fatty acids that contain lipid peroxides [52], and proteases exist that preferentially degrade oxidized proteins [51]. Oxidative damage to bases and deoxyribose groups in DNA is repaired by the action of many DNA glycosylases and endonucleases, as well as by nucleotide excision and recombinational repair systems [61]. In order to adequately protect against the damaging effects of ROS in cells, the generation of ROS and the activity of antioxidant defenses and repair activities must be more or less balanced *in vivo*. However, despite the presence of such defense and repair mechanisms, there is some continuous low level of unrepaired oxidative damage in cells that accumulates with time and is thought to contribute to ageing and disease [55].

2.4. Oxidative Damage to DNA Bases: 8-oxo-Guanine

DNA damage by ROS can cause multiple lesions, including single- and double-strand breaks, and apurinic/apyrimidinic sites. However, the following section will focus on DNA base modification by ROS, in particular the very abundant and highly mutagenic oxidized base, 8-oxo-guanine. Oxidized bases in DNA can arise by two distinct pathways: 1) direct oxidation of bases in DNA, and 2) oxidation of a nucleotide precursor and incorporation of the modified nucleotide into DNA. The specific pathways that lead to the presence of 8-oxo-guanine in DNA are discussed in

detail below. When bases in DNA are attacked by the hydroxyl radical (OH·), many modified bases can be formed. While damage can occur at many sites on the bases, attack by the hydroxyl radical results primarily in the addition of a hydroxyl group to the electron-rich double bonds, particularly the purine N7-C8 bond and the pyrimidine 5,6 bond [50]. The oxidized adducts in DNA occur at a frequency that is more than an order of magnitude higher than non-oxidative DNA adducts. But despite their abundance, oxidative DNA adducts still exist in a large background (10^5-10^6) of unaltered nucleotides [62]. Figure 2.2 shows just a fraction of the modified bases found in DNA after exposure to reactive oxygen species. The full spectrum of lesions produced by oxidative damage in endogenous DNA is estimated to be greater than 100 different types of lesions [63,64].

Of the many oxidized bases found in DNA, 8-oxo-guanine is thought to be the most abundant. The current estimate of the ratio of 8-oxo-dG/dG is approximately 0.25×10^{-5}, and 8-oxo-guanine is estimated to exist at a level of 7,500 8-oxo-guanine adducts per human cell [62]. This oxidized base was first described by Kasai et al. [65], who were studying the DNA damage induced by heating carbohydrates, a model for cooked foods. The following evidence suggested a mechanism for the formation of 8-oxo-guanine: (1) 8-methylguanosine was formed by the reaction between guanosine and methyl radical [66]; (2) deoxyguanosine and guanosine were hydroxylated at the C-8 position by the Fenton reaction using Fe^{2+}, EDTA, ascorbic acid and O_2 *in vitro* [35,67]. In addition, various oxygen radical-forming carcinogenic substances, such as cigarette smoke tar, asbestos plus H_2O_2, and ionizing radiation were found to be able to induce the formation of 8-oxo-guanine in DNA *in vitro* [66].

In vivo, 8-oxo-guanine is formed in mammalian cells by irradiation with X-rays and γ-rays [68], or exposure to 4-nitroquinoline-*N*-oxide (4-NQO) [69] or the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [70]. It is also formed in cellular DNA when bacterial cells are treated with H_2O_2 [66]. Thus, due to the highly reactive nature of the hydroxyl radical and its ability to oxidize the C8 position of guanine both *in vivo* and *in vitro*, it was expected that this oxidized adduct may be

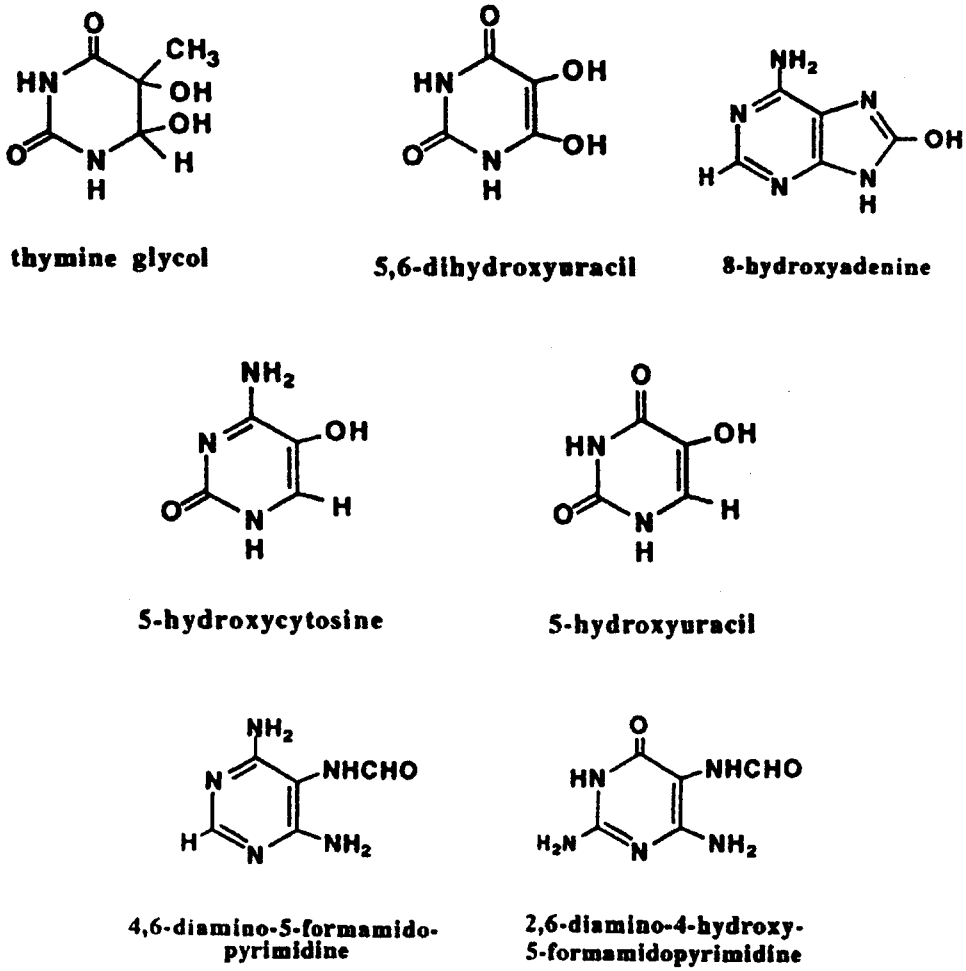


Figure 2.2 Examples of oxidized bases found in mammalian DNA.

important with respect to oxidative damage *in situ*.

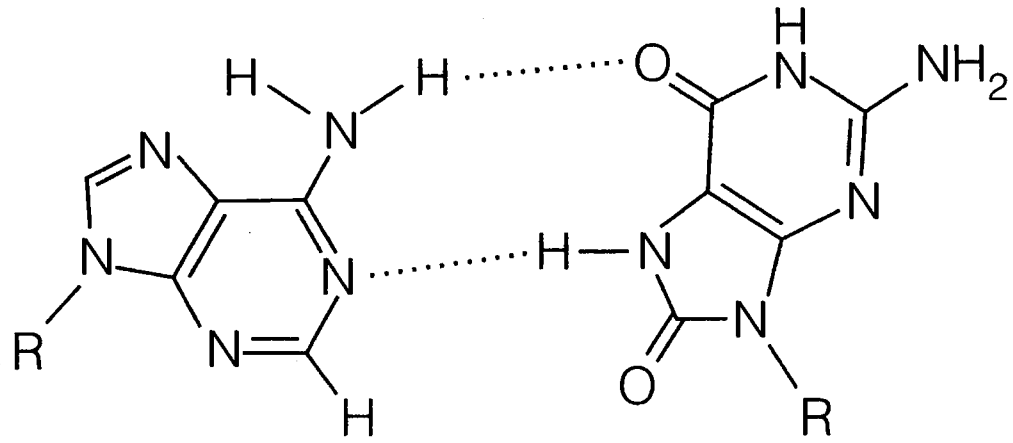
To study the correlation between oxidative DNA damage and carcinogenesis, the levels of 8-oxo-guanine in various organs were measured after oxygen radical-forming carcinogens were administered to rats [71]. As expected, a significant increase in 8-oxo-guanine was observed in target organs such as kidney. Similarly, when the peroxisome proliferator, ciprofibrate, a compound that induces hepatocellular carcinoma in rats and that is known to increase the release of H_2O_2 from liver peroxisomes, was orally administered to rats, a significant increase in the level of 8-

oxo-guanine was observed in liver DNA [72]. As a result of these and similar studies, the formation of 8-oxo-guanine in cellular DNA began to be used as a marker for monitoring cellular oxidative DNA damage, and in evaluating the carcinogenic potential of various oxygen radical-forming agents. The presence of 8-oxo-deoxyguanosine in urine has also been used as a biomarker of oxidative stress [73].

2.5. Mutagenesis By Guanine Nucleotide Oxidation

Among the oxidized bases, 8-oxo-guanine has received widespread study not only because of its abundance in cells, but also because of its highly mutagenic nature. Due to its benign appearance, a nondistorting keto group at the C8 position that is situated outside of the normal base-pairing region of guanine (see Figure 1.2), 8-oxo-guanine might not be suspected of being highly mutagenic. However, Shibutani et al. reported that both dCTP and dATP are incorporated preferentially opposite 8-oxo-guanine residues, in a DNA template that was replicated by *E. coli* DNA polymerase and the mammalian polymerases α and β [36]. Consequently, in addition to forming a Watson-Crick type base pair with cytosine [74], 8-oxo-guanine is also able to form a stable base pair with adenine, where adenine is in the *anti* orientation, and 8-oxo-guanine is in the *syn* conformation [75]. The 8-oxo-guanine:adenine base pair (see Figure 2.3) can lead to G·C→T·A transversion mutations during replication if it is not repaired [14]. This mispairing potential is responsible for the mutagenic nature of 8-oxo-guanine.

8-oxo-guanine is thought to be one of the most stable products of oxygen radical attack on DNA [76]. Although the presence of 8-oxo-guanine in DNA is highly mutagenic, an elaborate repair system is present in cells to prevent oxidative mutagenesis involving 8-oxo-guanine (see Figure 2.4). This repair system has been fully characterized in *E. coli*, and similar repair proteins have been identified in other prokaryotes and in higher eukaryotes (see below). It is composed of three proteins, known in *E. coli* as MutM, MutY and MutT. Michaels et al. [77] have shown that a



adenine 8-oxo-guanine

Figure 2.3 The 8-oxo-guanine:adenine base pair.
Adenine is in the *anti* orientation, and 8-oxo-guanine is in the *syn* conformation.

knockout of this repair system results in mutation rates that are of the same order of magnitude as those in mutants lacking the polymerase III proofreading subunit (*mutD*) and about an order of magnitude higher than those in mutants lacking the methyl-directed mismatch repair system (*dam, mutHLS*). These results provide convincing evidence that reactive oxygen species pose a significant threat to the cell and that the removal of 8-oxo-guanine from DNA is critical to the maintenance of replication fidelity. The role of the MutM, MutY, and MutT proteins in protecting against oxidative mutagenesis are characterized in detail in the following sections.

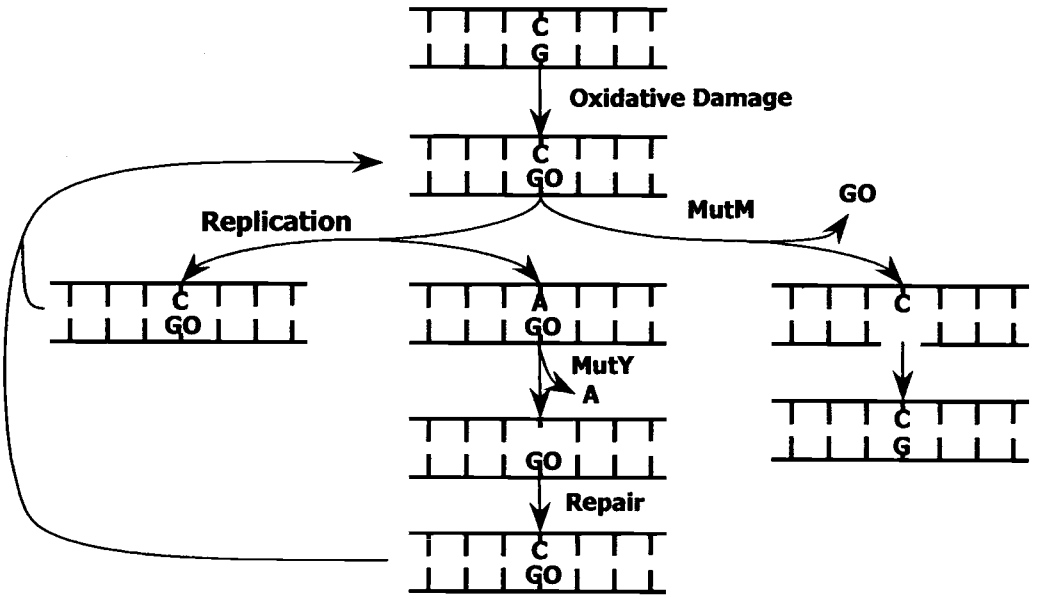


Figure 2.4 Involvement of the MutY and MutM proteins in the prevention of oxidative mutagenesis by guanine nucleotide oxidation in DNA. 8-oxo-guanine residues in DNA are shown schematically as GO. Oxidative damage to DNA can lead to the presence of 8-oxo-guanine in DNA, which is removed by the action of the MutM protein. Subsequent repair will restore the original C:G base pair. Mutations can arise when adenine is incorrectly incorporated opposite 8-oxo-guanine. The A:GO base pair is a substrate for the MutY protein which removes adenine. Repair polymerases can incorporate cytosine in the abasic site, resulting in C:GO base pair, which is a substrate for the MutM protein.

2.6. Pathways for the Removal and Repair of Oxidized Guanine Nucleotides in DNA

2.6.1. MutM: 8-oxo-Guanine Glycosylase

The *E. coli* formamidopyrimidine DNA glycosylase (also called FPG protein or Fapy DNA glycosylase) was first described as an enzyme that catalyzes the release of imidazole ring-opened forms of guanine and adenine from alkylated or irradiated DNA [78]. However, strains lacking the *fpg* gene have an elevated rate of G·C→T·A transversions [79], which cannot be attributed to the loss of an enzyme that removes ring-opened purines. These adducts could lead to cell death by blocking DNA

replication; however, they are not specific for induction of G·C→T·A transversions, suggesting that the Fapy DNA glycosylase enzyme could also act on a mutagenic substrate.

Chung et al. [80] later described the enzyme activity of the *E. coli mutM* gene product that cleaves 3′ and 5′ to the base, 8-oxo-G, when opposite cytosine, to produce a single nucleotide gap in the modified strand of DNA. This activity was further investigated by Tchou et al. [81], who compared the substrate specificities of the MutM enzyme and that of the FPG protein. Their results suggested that the two enzymes are identical, which was confirmed after cloning and sequencing of the two genes [82].

The mechanism by which MutM removes 8-oxo-guanine from DNA is shown schematically in Figure 2.4. MutM has DNA glycosylase activity, as well as apurinic/apyrimidinic (AP) endonuclease [83] and 5′ -terminal deoxyribose phosphatase [84] activities. The AP endonuclease reaction proceeds via a β-elimination reaction, resulting in a gap limited at the 3′ and 5′ ends by phosphoryl groups. Other enzymes excise the resulting 3′ end, and the gap is subsequently repaired. By this mechanism, the MutM protein can remove ring-opened purine lesions and the mutagenic 8-oxo-dG lesions, both of which can be generated by oxidative damage [85].

The MutM protein has a highly conserved function in prokaryotic and eukaryotic cells. In the gram-positive bacteria, *Lactococcus lactis,* the enzyme shares 59% similarity with the *E. coli* enzyme, and it also shares the same substrate specificity [86]. In the hyperthermophilic methanogen, *Methanococcus jannaschii*, a thermostable 8-oxo-guanine DNA glycosylase exists that shares little sequence homology with its counterparts [87]. This conservation of function in evolutionarily distant organisms reflects the importance of the role of 8-oxo-guanine DNA glycosylase in protecting bacterial DNA from the damaging effects of reactive oxygen species. MutM functional homologs have been identified and characterized in *Saccharomyces cerevisiae* [88], rat [89], mice [90,91], and human [91,92]. However, these proteins do

not share an obvious region of conservation with the *E. coli* enzyme [86]. The mammalian homologs contain a helix-hairpin-helix structural motif with conserved residues characteristic of a recently defined super-family of DNA glycosylases [91], which also includes the MutY adenine glycosylase of *E. coli* (see below).

The yeast MutM homolog is called Ogg1, or 8-oxo-guanine glycosylase. Compared to the to MutM protein from *E. coli*, which releases 8-oxo-dG and ring-opened purines at similar rates, the yeast Ogg1 protein releases 8-oxo-dG 12-fold faster than the Fapy lesion [88]. Unlike the situation in *E. coli*, two distinct glycosylases are expressed in yeast to remove Fapy and 8-oxo-dG residues from DNA. The yeast Fapy DNA glycosylase preferentially cleaves the 8-oxo-G:G duplex and Fapy residues, whereas the Ogg1 protein cleaves the 8-oxo-G:C duplex most efficiently [88].

Enzymes having 8-oxo-guanine glycosylase activity have been studied in detail in eukaryotic cells, and many functional homologs of the MutM protein exist. For example, rat liver mitochondria contain a novel enzyme that is involved in the removal of oxidatively damaged nucleotides [89]. This enzyme, mtODE, cleaves at 8-oxo-dG sites in double-stranded DNA, but it does not act on the ring-opened formamidopyrimidine adducts. This enzyme's preference for 8-oxo-G:C base pairs suggests that it functions independently of the DNA replication process and that it is functionally similar to yeast and mouse Ogg1. The localization of this enzyme in the mitochondria supports the idea that mitochondria are capable of repairing oxidative damage to DNA, which is reported to occur at high levels in the mitochondria due to the proximity of mtDNA to the free radical-producing electron transport chain [93].

In *Drosophila* and humans, it was reported that the ribosomal S3 protein possesses an N-glycosylase and AP lyase activity that specifically cleaves double-stranded DNA at sites containing 8-oxo-G:C base pairs and FapyGua with the same specificity [94]. Both of these adducts are formed as a result of hydroxyl radical attack on the C8-position of guanine, but FapyGua is formed under reductive conditions, whereas 8-oxo-guanine is formed under oxidative conditions [95]. The mutagenicity

of the Fapy adducts is not known; however, the specificity of the ribosomal S3 protein for the removal of both 8-oxo-guanine and FapyGua suggests that both are physiologically important substrates for the enzyme.

Ames et al. [55] have proposed that oxidative DNA damage may be a significant causative factor for cancer and ageing in mammals. Supporting this proposal is the observation that mutations in the *hOgg1* gene are found in human lung and kidney tumors [96]. Although overexpression of human Ogg1 protein had no effect on the levels of spontaneous oxidative DNA damage and mutation rates in cultured mammalian cells [97], it was still of great interest to evaluate the possible damaging effects of endogenously produced reactive oxygen species in mammals. To do this, Klungland et al. generated knockout mice deficient in DNA repair enzymes that counteract oxidative damage [90]. Homozygous *ogg1*$^{-/-}$ null mice were viable, but they accumulated high levels of 8-oxo-dG in their genomes. Despite this increase in oxidative DNA lesions, Ogg1-deficient mice exhibited only a moderately elevated spontaneous mutation rate in nonproliferative tissues, did not develop malignancies, and showed no marked pathological changes. Interestingly, this study showed that there was significant but slow removal of 8-oxo-dG from proliferating cells, indicating that another repair pathway functions in the prevention of oxidative mutagenesis in these cells and demonstrating the importance of the removal of this oxidized lesion.

2.6.2. MutY: A DNA Mismatch Glycosylase Involved in the Repair of Oxidative Damage

Mutations in the *mutY* gene of *E. coli* elevate the spontaneous frequency of G·C→T·A transversions considerably [98]. The MutY protein was originally described as an adenine-specific DNA glycosylase acting at A:G mismatches [99]. This work showed that A:G mispairs could be corrected to C:G base pairs by a mechanism that was independent of *dam* methylation and host *mutHLS* gene function. More recently it was shown that the MutY protein efficiently removes adenine from 8-oxo-G:A base pairs that result from error-prone replication past the 8-oxo-guanine

lesion [100], as shown in Figure 2.4. Such a mispairing can occur during replication of 8-oxo-G-containing DNA templates; therefore, it was concluded that the MutY protein is involved in the defense system of *E. coli* against oxidative mutagenesis.

The *mutY* gene of *E. coli* encodes a 350-amino acid protein with a mass of 39 kDa. The enzyme is a glycosylase that also has associated AP endonuclease activity [101]. The mechanism by which MutY recognizes 8-oxo-guanine is not well understood; however, it has recently been shown that the reaction catalyzed by the enzyme involves a double base-flipping mechanism [102]. Extrusion of the 8-oxo-guanine base occurs first, and is followed by extrusion of the adenine base for excision. After removal of the adenine, MutY remains bound to the DNA. This binding protects the remaining 8-oxo-dG lesion from removal by the MutM protein, thus preventing the occurrence of a double strand break and loss of one nucleotide from the DNA. It may also serve as a signal to other proteins to complete the repair process [103].

A eukaryotic homolog of the *E. coli* MutY protein has been identified and characterized in nuclear extracts of calf thymus and human HeLa cells [100]. This protein, MYH, reacts with antibodies generated against the MutY protein of *E. coli*, and it is a DNA glycosylase that removes the mispaired adenine from an A:G mismatch. An AP endonuclease activity copurifies with the glycosylase and cleaves the first phosphodiester bond 3′ to the generated apurinic site. The activity of MYH is inhibited by treatment with the oxidizing agent, potassium ferricyanide, indicating that it may contain an iron-sulfur cluster. MYH is a 65-kDa protein that has substrate specificity similar to the MutY protein. The calf thymus enzyme has two-fold higher activity at an A:G mispair over an A:8-oxo-dG mispair; however, the binding is seven-fold greater to the A:8-oxo-G-containing DNA. Therefore, it seems likely that the MYH protein has multiple roles in mammalian cells, including the repair of A:G or A:C mismatches which arise from replicative errors and the removal of adenines misincorporated opposite 8-oxo-guanine oxidative lesions in DNA.

Both *mutM* and *mutY* strains have increased rates of G·C→T·A transversions. This genetic evidence suggested that the two genes are involved in a common repair pathway. Michaels et al. [77] found that overexpression of MutM from a plasmid can completely complement a *mutY* strain, and they also isolated a chromosomal suppressor of *mutY* that overproduces the MutM protein by about 15-fold. Their observation that the *mutM mutY* double mutant has a G·C→T·A transversion rate that is 20-fold higher than the sum of the mutation rates of the mutators alone suggested that the two gene products are interdependent and together play a critical role in preventing oxidative mutagenesis. This interdependence of the two proteins is illustrated in Figure 2.4, which shows that MutM and MutY act together to minimize mutations caused by the presence of 8-oxo-guanine into DNA.

2.6.3. MutT: A Nucleotidase That Removes 8-oxo-dGTP from Nucleotide Pools

Treffers et al. [45] were the first to describe a mutator strain of *E. coli*, that they designated MutT1, which was characterized as having a spontaneous mutation frequency ranging from 10^2- to 10^4-fold higher than normal. Characterization of the *E. coli* MutT protein followed from studies designed to investigate the biochemical basis of the *mutT* mutator phenotype. Unlike typical defective mutator genes that cause a variety of mutational events in DNA, the *mutT* gene exclusively caused A·T→C·G transversions [46]. The *mutT* gene was cloned by complementation of the *mutT* mutator phenotype and the lesion in the original *mutT1* allele was identified as an IS1 insertion in the *mutT* gene [104]. This work established that *mutT* is directly involved in preventing the increase in the frequency of A·T→C·G transversions.

The cloned *mutT* gene product was expressed, purified, and characterized as a nucleoside triphosphate pyrophosphohydrolase, an enzyme that cleaves pyrophosphate from a nucleoside triphosphate [104]. The MutT protein is active on all eight nucleoside triphosphates, but has a preference for dGTP. The reaction catalyzed by the MutT enzyme using dGTP as a substrate is as follows:

$$dGTP + H_2O \rightarrow dGMP + PP_i$$

The observation that a 23-amino acid region of homology exists between the MutX protein of *Streptococcus pnuemoniae* and the MutT protein in *E. coli* [105], led to the discovery of an entire family of hydrolases that contain the motif that was originally identified as the active site of the MutT enzyme. Members of this family of proteins can be found in organisms ranging from viruses to humans. To date, the number of proteins belonging to this family and having a described enzymatic activity is 13 [106], and many more open reading frames exist, although their biochemical activities have yet to be identified. Although the enzymes of this family act upon a wide variety of substrates, they are, in fact, closely related to each other by their ability to hydrolyze a particular class of compounds: a **nu**cleoside **di**phosphate linked to some other moiety, **X**. Accordingly, they are collectively referred to as the "NuDiX" hydrolases. Each member of the NuDiX hydrolases described so far contains the consensus sequence ^{38}G·····E·····REU·EE··U^{60}, where U represents the bulky aliphatic amino acids, isoleucine, leucine, or valine [106]. This consensus sequence is commonly referred to as the NuDiX box.

The *E. coli mutT* gene encodes a 129-amino acid protein with a mass of 15 kDa [107]. The solution structure of the enzyme has been completed [108], and showed that the enzyme consists of a 5-stranded, mixed β-sheet sandwiched between two α-helices connected by long loops. The positions of the amino acids of the NuDiX signature sequence are situated mostly in loop I and in helix I, they are closely arranged spatially, and they are readily accessible to the external environment. There is little homology between members of the NuDiX hydrolases outside of the NuDiX box, and it is believed that the specificity of each enzyme is determined by other amino acids outside of the homologous sequence [106].

2.7. 8-oxo-dGTP and Oxidative Mutagenesis

The involvement of the *E. coli* MutT enzyme in the prevention of oxidative mutagenesis became apparent when Maki and Sekiguchi showed that the enzyme is three orders of magnitude more active on the oxidized nucleotide, 8-oxo-dGTP, than it is on dGTP [11]. Misincorporation of 8-oxo-dGTP across from template adenine during DNA replication leads to an 8-oxo-G:A mispair, and subsequent replication results in an A·T→C·G transversion, which is the characteristic phenotype of a *mutT* strain. MutT degrades 8-oxo-dGTP to 8-oxo-dGMP, thus eliminating 8-oxo-dGTP as a substrate for DNA synthesis, as shown in Figure 2.5.

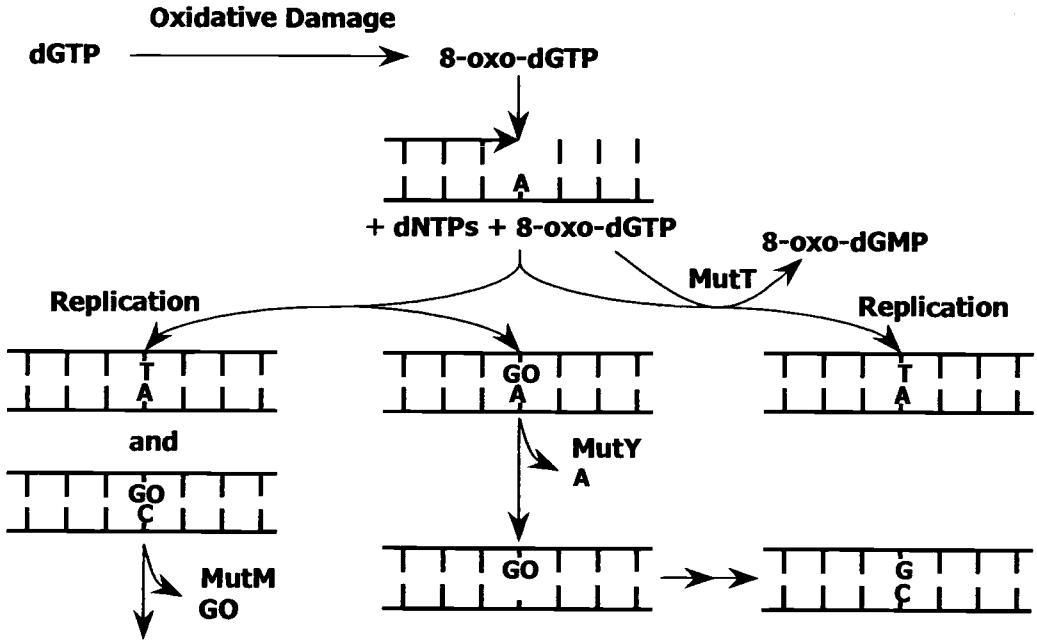


Figure 2.5 Involvement of the MutT protein in the repair system that protects against oxidative mutagenesis by guanine nucleotide oxidation. 8-oxo-guanine residues in DNA are shown schematically as GO. The oxidized nucleotide, 8-oxo-dGTP, is effectively hydrolyzed by the action of the MutT enzyme to remove it from the nucleotide pool. In the absence of MutT activity, replication with 8-oxo-dGTP could result in misincorporation of the damaged nucleotide opposite template adenine, a substrate for the MutY protein, or it could be incorporated opposite template cytosine, a substrate for the MutM protein.

Maki and Sekiguchi concluded that the MutT protein has a major role in preventing the A·T→C·G transversions caused by the incorporation of 8-oxo-dGTP into DNA. A key observation that these researchers had made was that dGTP is oxidized more rapidly than guanine bases in DNA *in vitro*. Their suggestion that 8-oxo-dGTP is formed spontaneously in the nucleotide pool of cells when reactive oxygen species are present and contributes to oxidative mutagenesis *in vivo* has since become an accepted premise in the scientific literature.

This premise was supported by a few studies that evaluated the ability of various DNA polymerases to use 8-oxo-dGTP as a substrate *in vitro*. Maki and Sekiguchi [11] were the first to show the mispairing potential of 8-oxo-dGTP opposite template adenine by the α subunit of *E. coli* DNA polymerase III lacking 3′→5′ exonuclease activity. Pavlov et al. [15] reported similar results using Klenow (exo$^-$), T4 polymerase (exo$^-$) and *Thermus thermophilus* DNA polymerase. The kinetics of incorporation of 8-oxo-dGTP opposite template C in both a DNA and RNA template by HIV-1 and murine leukemia virus reverse transcriptases and DNA polymerases α and β were reported by Kamath-Loeb et al. [109]. Most recently, the kinetics of 8-oxo-dGTP incorporation by *E. coli* polymerase I (exo$^-$), *E. coli* polymerase II (exo$^-$), HIV-1 reverse transcriptase, and bacteriophage T7 polymerase (exo$^-$) were examined [44], as was the ability of the mammalian enzyme, polymerase δ, to incorporate 8-oxo-dGTP [41].

Further studies of oxidized guanine nucleotides showed that 8-oxo-GDP can not be reduced by ribonucleotide reductase, that 8-oxo-dGTP can be generated not only by direct oxidation of dGTP but also by phosphorylation of 8-oxo-dGDP by nucleoside diphosphate kinase, that 8-oxo-dGMP cannot be rephosphorylated by guanylate kinase, and that 8-oxo-dGMP is further degraded to 8-oxo-deoxyguanosine by a nucleotidase so that it can be excreted from cells [110]. These results indicate that while MutT is involved in preventing the accumulation of 8-oxo-dGTP in cells, many other enzymes of dNTP biosynthesis are also involved in keeping the nucleotide pool free of 8-oxo-dGTP.

If this oxidized precursor is incorporated into DNA, results of *in vitro* DNA replication studies have shown that it would be strongly mutagenic. Cheng et al. [14] showed that misincorporation of 8-oxo-dGTP causes A to C substitutions as the result of replication of an 8-oxo-G:A mispair. Pavlov et al. [15] showed that when present during SV40 origin-dependent replication of double-stranded DNA in HeLa cell extracts at a concentration equal to the four normal dNTPs, 8-oxo-dGTP was at least 13-fold more mutagenic for A·T→C·G transversions than was a 100-fold excess of normal dGTP. Similarly, Minnick et al. [111] showed that the presence of 8-oxo-dGTP at equimolar concentration with each of the 4 normal dNTPs resulted in a > 46-fold increase in error rate for A·T→C·G transversion over that observed in the absence of 8-oxo-dGTP. Thus, all of these *in vitro* studies seem to support the concept first proposed by Maki and Sekiguchi [11] that the action of the MutT enzyme is to cleanse the nucleotide pool by removing from cells an oxidized nucleotide that, if incorporated into DNA, would be strongly mutagenic.

2.8. Is The MutT Story Complete?

Although the mechanism of action of the MutT enzyme appears to be clear, several observations have been made which call into question its role in preventing oxidative mutagenesis by the incorporation of modified guanine nucleotides into DNA. First, if the function of MutT is to remove 8-oxo-dGTP from nucleotide pools, it might be expected that *mutT* strains would show lower mutation frequencies in an anaerobic environment than in an aerobic environment. Fowler et al. tested this hypothesis [43], and they showed that *mutT E. coli* cultured in rich medium had similar mutation frequencies when grown in either aerobic or anaerobic conditions. Mutagenesis by guanine nucleotide oxidation during anaerobic growth could result only if reactive oxygen species were produced endogenously. Intracellular processes, such as the incomplete reduction of O_2 during electron transport, are known to generate intracellular reactive oxygen species; however, Fowler's observation is

puzzling because during conditions of anaerobic growth, this production should be minimal and no appreciable oxidation of guanine nucleotides should take place.

Additionally, the accepted premise that the degradation of 8-oxo-dGTP to prevent mutagenesis is the sole function of the MutT enzyme was proved to be incorrect when it was shown that the *E. coli* enzyme does not only hydrolyze 8-oxo-dGTP, but can also act on dGTP and on the RNA precursor, 8-oxo-rGTP [47]. This finding suggests that in *E. coli* MutT may function not only to decrease errors in DNA replication, but also to decrease transcription errors. The role of the MutT enzyme in counteracting mutagenesis induced solely by 8-oxo-dGTP has also been refined because of further studies of the substrate specificity of the enzyme and its homologs. Fujikawa et al. [48] determined that the human homolog of MutT, hMTH1, differs from the bacterial enzyme in its substrate specificity. hMTH1 can hydrolyze 8-hydroxy-dATP as efficiently as 8-oxo-dGTP and had even higher affinity for 2-hydroxy-dATP, while the *E. coli* enzyme did not act on oxidized adenine nucleotides. The finding that the human enzyme's ability to hydrolyze oxidized DNA precursors is not limited to the hydrolysis of 8-oxo-dGTP indicates that the role of hMTH1 and other MutT homologs in preventing oxidative mutagenesis may be far more important than previously expected.

Finally, the premise that 8-oxo-dGTP is the critical substrate for the MutT enzyme has been questioned because of the results of experiments that showed that the nucleotide is a poor substrate for many DNA polymerases. Einolf et al. [44] analyzed 8-oxo-dGTP incorporation and extension by replicative and repair polymerases and found that 8-oxo-dGTP was a poor substrate for the four polymerases tested, with insertion efficiencies $>10^4$-fold lower than for dGTP incorporation. In a later publication from the same laboratory [41], 8-oxo-dGTP was also shown to be a poor substrate for the main replicative polymerase in mammalian cells, polymerase δ, suggesting that in order for 8-oxo-dGTP to substitute for dGTP and cause mutagenesis *in vivo*, intracellular levels of the oxidized nucleotide would have to be significantly high. Although *in vitro* DNA replication assays with 8-oxo-dGTP have shown the

nucleotide to be strongly mutagenic [15,111], these studies were done with 8-oxo-dGTP concentrations that were equal to or greater than the dGTP concentration, experimental conditions that are not physiologically relevant.

To what extent does the oxidation of dGTP (see Figure 2.5) contribute to mutagenesis as compared to the direct oxidation of guanine bases in DNA (see Figure 2.4)? Is our present understanding of the role of the MutT enzyme complete? Observations in the published literature regarding 8-oxo-dGTP and the MutT enzyme suggest that a better understanding of the role of 8-oxo-dGTP in mutagenesis and the mechanism by which MutT minimizes the damaging effects of oxidation is needed. The results presented in this dissertation suggest that the currently accepted mechanism by which the MutT enzyme counteracts oxidative mutagenesis should be re-evaluated.

Chapter 3. Materials and Methods

3.1. Materials

1) 0.5 M TOA/Freon: 2.19 ml tri-n-octylamine (TOA) + 7.81 ml Freon, make fresh before use and keep on ice
2) 1.5X dNTP Assay Buffer: 7.5 mM Tris-HCl pH 8.3, 7.5 mM $MgCl_2$
3) RNAse Buffer: 10 mg RNAse A (95 Kunitz units/mg), 55.6 $Na_2HPO_4 \cdot 7H_2O$, 194 mg $NaH_2PO_4 \cdot H_2O$ per 10 ml
4) Luria Broth (LB): 20 mg/ml tryptone, 10mg/ml yeast extract, 10 mg/ml NaCl, 1 ml/l 2 M NaOH
5) 2X YT medium: 16 g tryptone, 10 g yeast, 5 g NaCl per litre
6) Top Agar: 4.5 g NaCl, 4 g agar per 500 ml
7) 5X salts: 52.5 g K_2HPO_4, 22.5 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 2.5 g Na-citrate$\cdot 2H_2O$
8) Minimal Medium plates: 12 g agar, 800 ml H_2O, 200 ml sterile 5X salts, 8 ml 50% glucose, 8 ml 1 M $MgSO_4$, 0.5 ml 10 mg/ml thiamine-HCl per 1000 ml
9) 10X SVRB: 0.3 M Hepes pH 7.8, 70 mM $MgCl_2$, 2 mM CTP, 2 mM GTP, 2 mM UTP, 40 mM ATP
10) Stop Mix: 2 mg/ml proteinase K, 2% w/v SDS, 50 mM EDTA
11) Precipitation Mix: 0.71 mg/ml yeast tRNA, 1.7 M ammonium acetate
12) TE buffer: 10 mM tris-HCl pH 8.0, 0.1 mM EDTA
13) PEB (Phenol Extraction Buffer): 100 mM tris-HCl pH 8.0, 1 mM EDTA, 300 mM NaCl

3.2. Methods

3.2.1. dNTP Pool Measurements from Cultured Cells Grown Under Varying Oxygen Conditions

3.2.1.1. Mammalian Cell Culture Conditions

HeLa S3 cells obtained from the American Type Culture Collection were grown in 250-ml culture flasks in 50% Dulbecco's Modified Eagle Medium (DMEM) and 50% Ham's F12 medium (purchased from Gibco BRL), supplemented with 10% Fetal Calf Serum and penicillin-streptomycin. Typically, four-250 ml flasks were used for each dNTP extract preparation. The flasks were equipped with sealable caps. For cultures grown at 2% oxygen, the culture medium was extensively degassed, and after seeding the cultures the flasks were flushed extensively with a gas mixture containing 93% N_2, 5% CO_2, and 2% O_2, and were capped tightly. Cells were cultured for two days, or until they reached approximately 70% confluency. Cultures grown at 20% (ambient) oxygen were cultured in unsealed flasks in a tissue culture incubator maintained at 5% CO_2, 95% air. As a control, additional cultures were grown in sealed flasks that had been flushed extensively with a gas mix containing 95% air and 5% CO_2. No differences were seen in the dNTP measurements from these control cells and those grown in unsealed flasks within the tissue culture incubator. As an alternative to using sealable flasks, cells could also be cultured in 100-mm tissue culture plates and placed into a sealed chamber within the tissue culture incubator that had been extensively flushed with a gas mix.

V79-4 cells obtained from the American Type Culture Collection were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4,500 mg/l D-glucose, supplemented with 5% Fetal Calf Serum and penicillin-streptomycin. Cells were grown in sealed flasks or in a sealed chamber flushed with gas mix, as described above for HeLa cells, in experiments involving growth under conditions of oxygen limitation.

3.2.1.2. dNTP Extract Preparation from Mammalian Cells

Usually three or four 250-ml tissue culture flasks of cells were used for the preparation of a cell extract. One additional flask was trypsinized, and the cells were counted with a hemocytometer to gain an accurate count of the number of cells that contributed to each dNTP extract. When the cultured cells covered approximately 70% of the flask, the medium was aspirated and the cells were washed two times with ice-cold TBS. Then, ice-cold 60% methanol + 1% toluene was added to each flask, and the flasks were placed at -20°C for a minimum of 3 hours. At regular intervals the flasks would be gently rocked. At the end of the extraction time, the methanol + toluene was removed to 1000-µl microcentrifuge tubes, and each flask was washed with an additional 1 ml of methanol + toluene. This wash was also collected in microcentrifuge tubes. The methanol + toluene was centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatant was then transferred to fresh microcentrifuge tubes and dried under vacuum. After drying, the pellets were resuspended in 5% ice-cold TCA, and were immediately centrifuged at 10,000 rpm, 4°C for 30 minutes. It was important to minimize the time that cellular dNTP extracts were exposed to acidic conditions to prevent degradation of the dNTPs. The TCA extracts were neutralized by the addition of 1.5 volumes of ice-cold TOA/Freon. After addition of TOA/Freon, the extracts were vortexed briefly and then centrifuged briefly to separate the aqueous and organic phases. The pH of the aqueous phase was determined and neutralization with TOA/Freon was repeated if the pH was less than 5.5. 75% of the aqueous phase was recovered to fresh microcentrifuge tubes, and the extracts were dried in a speed vac. The final dNTP extracts were resuspended in 100-200 µl of H_2O and stored at –20°C until analysis. It was necessary to dilute each dNTP extract significantly before analysis, but the dilution factor had to be determined empirically. Results of the enzymatic assay routinely showed that the apparent dNTP pool size from a given extract increased with dilution of the final extract, indicating that some compound present in the final extract inhibits the DNA polymerase reaction (see Section 5.1.5).

To control for the incomplete recovery of each of the dNTPs during the extraction and preparation of cellular dNTP extracts, a control flask was prepared. This plate was treated identically to the plates that were used for extract preparation, except that in addition to methanol + toluene, a known concentration of each of the four dNTPs was added. After all steps of the extract preparation were completed, the dNTP pools in this control extract were measured by the dNTP enzymatic assay (see Section 3.2.1.4) to determine the recovery for each dNTP. Previously, the recovery of the dNTPs was routinely estimated by adding a known amount of a single radioactive dNTP to a control plate. The specific activity of the extract at the beginning and end of the protocol was measured and used to estimate yield. This method is inaccurate, since the recovery of each dNTP is variable, and using this method the recovery of only a single nucleotide was measured.

3.2.1.3. Preparation of Alternating Copolymer DNA Template

Poly (dA-dT) and poly (dI-dC) double stranded DNA templates were used in the dNTP pool enzymatic assay. These templates were supplied as lyophilized powder (Pharmacia), and it was necessary to purify the templates before use to ensure that no dNTPs were supplied to the dNTP enzymatic assay from the template preparation. To purify, the template DNA was resuspended in H_2O to a concentration of 2 mg/ml. $1/10^{th}$ volume of 5 M $NH_4CH_3COO^-$ and 2 to 3 volumes of ice-cold ethanol were added, and the DNA was placed on dry ice or at –20°C for several hours or overnight. The DNA was then centrifuged at 4°C, 14,000 rpm for 30 minutes to precipitate. The ethanol was decanted, and the pellet was washed with ice-cold 70% ethanol. Once again, the DNA was pelleted by centrifugation, and was dried completely in a speed vac. The final DNA pellet was resuspended in 0.02 M phosphate buffer, pH 7.0, and 0.1 M NaCl to a final concentration of 0.75 mg/ml. The final template DNA was used in a polymerase reaction to ensure proper function in the dNTP enzymatic assay.

3.2.1.4. dNTP Enzymatic Assay

Intracellular pools of dNTPs were analyzed by the method of North *et al.* [112] with the following modifications. The assay measures the incorporation of a limiting dNTP (supplied by the cell extract) onto an alternating copolymer DNA template by *E. coli* DNA polymerase I (Klenow exo$^-$ or exo$^+$) in the presence of an excess of the labeled complementary dNTP. Each reaction mixture (50 µl) contained 4.5 mM tris-HCl pH 8.3, 4.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.5 µl [^{3}H]-dNTP complementary to the dNTP being assayed (0.01 mCi/ml final concentration), 0.05 units DNA polymerase, 7.5 mg/ml template DNA, 0.2 mg/ml BSA, and 5 µl of the appropriately diluted cell dNTP extract. In reactions using exonuclease-proficient Klenow polymerase, 10 mM dAMP was added to the reaction mix to inhibit the enzyme's exonuclease activity. The reactions were initiated by addition of the reaction mix to the dNTP extract, after which the entire reaction mix was vortexed gently and incubated at 37°C for the determined time. The time course of the reaction had to be previously determined by adding known concentrations of the dNTP to be measured to the reaction mix and measuring the incorporation of [^{3}H]-dNTP over time. Reactions were stopped by spotting an aliquot of the reaction mix onto filter paper that had been soaked with 5% TCA and 2% Na-pyrophosphate. Unincorporated nucleotides were then washed from the filter paper by ascending chromatography using 5% TCA and 2% Na-pyrophosphate as the solvent. The filter paper containing the DNA sample was washed two times for 15 minutes per wash in 95% ethanol. The samples were dried under a heat lamp, placed into scintillation fluid and counted for 5 minutes per sample. Standard curves for each dNTP were previously completed using 0-10 pmol dGTP, 0-20 pmol dCTP, 0-20 pmol dATP, and 0-20 pmol dTTP.

3.2.1.5. Synchronization of Mammalian Cell Cultures

Cells were synchronized by aphidicolin treatment, using the method of Matherly *et al.* [113] for suspension cultures of murine L1210 leukemia cells, with the following modifications specific for the synchronization of V79 cells in monolayer cultures.

Exponentially growing cells were treated for 12 hours with 3 μM aphidicolin, which was prepared as a concentrated stock in DMSO. Following exposure to aphidicolin, cells were washed twice with PBS and supplied with drug-free fresh culture medium for 9 hours. The cells were once again treated with 3 μM aphidicolin for 12-14 hours. After this treatment, a highly synchronized population of cells in early S-phase was obtained, as shown by flow cytometric analysis (see below).

3.2.1.6. Preparation of Cells for Flow Cytometry

Samples of synchronized cells were analyzed for DNA fluorescence using flow cytometry. After decanting the medium, cells were washed twice with PBS, trypsinized, resuspended in culture medium, and transferred to centrifuge tubes. Cells were pelleted by centrifugation at 3,000 rpm for 3 minutes. The medium was aspirated, and cells were resuspended in cold PBS with gentle vortexing. Once again, the cells were pelleted by centrifugation and the PBS was aspirated. The cell pellet was resuspended in the residual PBS by gentle vortexing. Cells were fixed by the addition of 5 ml of ice-cold 70% ethanol, added dropwise during continuous agitation with a vortex mixer. The cell suspension was stored at 4°C until analysis. Prior to flow cytometric analysis, the cell suspension was centrifuged to pellet the fixed cells, and the ethanol was aspirated. Cells were resuspended in 1 ml of RNAse buffer and incubated for 15 minutes at 37°C. An aliquot of the cells was filtered through a 40 μm filter, and 200 μl of propidium iodide (300 μg/ml) was added to each sample. The stained cells were left at room temperature for 30 minutes before flow cytometric analysis. DNA content of the synchronized cells was analyzed by using a Coulter EPIVS V flow cytometer or a Coulter EPICS XL-MCL flow cytometer, and the data were analyzed using Multicycle software.

3.2.2. 8-oxo-dGTPase Assay

3.2.2.1. Preparation of 8-oxo-dGMP

A chromatographic standard of the nucleotide 8-oxo-dGMP was prepared by oxidizing dGMP with H_2O_2 and ascorbic acid, as described by Bialkowski and Kasprzak [114]. 50 mg of ascorbic acid and 25 mg of dGMP were dissolved in 40 ml of 20 mM phosphate buffer, pH 7.0, in a glass flask. The reaction was initiated by the addition of 250 µl of 30% H_2O_2. The reaction mixture was incubated in the dark at 25°C for 3 hours. The reaction was terminated by the addition of 60 µg of catalase, prepared as a 1 mg/ml stock in 20 mM phosphate buffer, pH 7.0. The entire mixture was then incubated for an additional 20 minutes at 37°C with vigorous stirring. The reaction mixture was vacuum filtered through a 0.2 µm nylon membrane and loaded to an equilibrated DEAE sephadex A25 column (2 x 15 cm) for purification.

3.2.2.2. Purification of 8-oxo-dGMP

The method of Bialkowski and Kasprzak [114] for the purification of 8-oxo-dGMP was used, with the following modifications: The product of the dGMP oxidation reaction was purified using a DEAE sephadex A25 column (2 x 15 cm), which had been equilibrated with 20 mM TEAB, pH 8.5. The chromatographic purification was performed at 4°C. To begin, the entire reaction mixture (40 ml) was loaded onto the column at a flow rate of 0.3 ml/min, and the column was then washed overnight at a flow rate of 0.1 ml/min. The oxidation products were eluted with 40 ml of 20 mM TEAB, followed by a 750-ml linear gradient of 20-110 mM TEAB, and 240 ml of 110 mM TEAB at a flow rate of 2 ml/min. The column effluent was monitored at a wavelength of 293 nm. 8-oxo-dGMP eluted after the large unoxidized dGMP peak, near the end of the TEAB gradient, and was collected in 27 fractions of 2 ml each. These fractions were pooled and evaporated to dryness under vacuum. TEAB was removed from the preparation by coevaporation (three times) with 100% methanol. The final 8-oxo-dGMP sample was resuspended in 500 µl H_2O and its concentration was determined spectrophotometrically using the molar absorbance

coefficient for 8-oxo-dG and its phosphate derivatives, ε_{293} = 10,300 [35]. The 8-oxo-dGMP solution was stored at –20°C.

3.2.2.3. Mammalian Cell Culture Conditions

V79 cells were grown in 10-cm culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) with 4,500 mg/l glucose and 5% Fetal Bovine Serum. Typically, three 10-cm plates were used for each extract preparation. For cultures grown at 2% oxygen, cells were grown in a sealed chamber within the tissue culture incubator that had been evacuated and flushed extensively with a gas mixture containing only 2% oxygen (93% N_2 : 5% CO_2 : 2% O_2) for two days, or until they reached approximately 70% confluency. For cultures grown under conditions of oxidative stress, plates that were approximately 50% confluent were treated for 8 consecutive treatments every 30 minutes with either a) 5 mM H_2O_2 or b) 5 mM H_2O_2 and 1.2 mM $FeSO_4$.

3.2.2.4. Bacterial Strains and Culture Conditions

Escherichia coli strains used in the 8-oxo-dGTPase activity assay experiments were obtained from the *E. coli* Genetic Stock Center are described in Table 3.1. Typically, 50-ml cultures of *E. coli* strains were grown in LB medium, with the addition of the appropriate antibiotic as necessary, from fresh overnight cultures. Anaerobic cultures were grown at 37°C in loosely capped 50-ml conical tubes within a Bactron anaerobic chamber (Sheldon Mfg.) that had been evacuated and thoroughly flushed with an anaerobic gas mix. Prior to growing the anaerobic cultures, the bacterial medium was left overnight in the anaerobic chamber to equilibrate. For bacterial cultures exposed to oxidative stress, cells were grown in a shaker incubator at 37°C until the cell density reached A_{600} = 0.3, after which the cells were treated with 5 mM H_2O_2 every 20 minutes until the cultures reached mid-log phase. For all bacterial cultures, cell extracts were prepared as outlined below after the cultures reached mid-log phase (A_{600} = 0.4-0.6).

Table 3.1 *Escherichia coli* strains used to study 8-oxo-dGTP pool sizes and 8-oxo-dGTPase activity.
All strains were obtained from the *E. coli* Genetic Stock Center.

***E. coli* Strain**	**Genotype**
E. coli B	wild type strain
ES-1580	*thr-1, ara-14, mutT1,* Δ*(gpt-proA)62, lacY1, tsx-33, glnV44*(AS), *galK2*(Oc), λ⁻, *Rac-0, hisG4*(Oc), *rfbD1, mgl-51, rpsL31*(strR), *kdgK51, xylA5, mtl-1, argE3*(Oc), *thi-1*
T-198	*thr-18, leu-29, mutT1*
58-278M	*mutT1, bio-1*(Unst), *spoT1, phe-1*

3.2.2.5. Preparation of Cell Ultrafiltrate

The method of Bialkowski and Kasprzak [114] was used for the preparation of cell ultrafiltrates from mammalian cells, and was modified as follows for the preparation of cell ultrafiltrates from bacteria. Cells were pelleted by centrifugation for 20 minutes at 3000 rpm. After decanting the culture medium, the cells were resuspended in 25 ml of 20 mM TBS, and pelleted again by centrifugation. After decanting the TBS, the cell pellets could be stored at –80°C or processed immediately. The cell pellet was resuspended in 250 µl of ice-cold 20 mM tris-Cl (pH 7.4) and transferred to a chilled 1.5-ml centrifuge tube. To lyse, the cells were sonicated 2 times for 30 seconds each time and were allowed to chill on ice for a few minutes between each sonication. The cell debris was pelleted by centrifugation at 10,000 rpm for 10 minutes, and the supernatant was transferred to chilled ultracentrifuge tubes. This supernatant was ultracentrifuged in a Beckman TL-100 ultracentrifuge for 1 hour at 49,000 rpm, 4°C. The protein concentration of the supernatant, termed "extract", was determined by using a BSA standard, with UV absorbance measured at 595 nm. To isolate the low-molecular-weight proteins, and to separate the 8-oxo-dGTP pyrophosphatase activity from 8-oxo-dGTP phosphatase and 8-oxo-dGMP

phosphatase activities, three aliquots of each extract were filtered through a 30-kDa NanoSep membrane by centrifugation at 6000 rpm until complete passage (usually 20 minutes). The resulting "ultrafiltrate" was used for determination of the 8-oxo-dGTPase activity either immediately, or after storage at –80°C.

3.2.2.6. 8-oxo-dGTPase Reaction

As described by Bialkowski and Kasprzak [114], the enzymatic reactions were run at 37°C in 500 µl capped microcentrifuge tubes in a reaction volume of 60 µl containing 5 mM $MgCl_2$, 100 mM tris-Cl (pH 8.5), 40 µM 8-oxo-dGTP, and 5 µl of ultrafiltrate. The concentration of ultrafiltrate protein in the final reaction mix was 7.9 µg/ml. The reactions were initiated by the addition of ultrafiltrate and were allowed to proceed for 3.75 hours. To determine the time course of the reaction, the volume of the reaction mixture was scaled up to 600 µl, and aliquots were analyzed by HPLC at 45-minute time intervals. The reactions were terminated by the addition of 20 µl of 50 mM Na_2EDTA. For each cell extract preparation, each of the three cell ultrafiltrates were used in separate enzymatic reactions, since it was shown by Bialkowski and Kasprzak [114] that the ultrafiltration step is the most critical factor for reproducibility of the whole analytical procedure.

3.2.2.7. 8-oxo-dGTPase Activity Assay

The 8-oxo-dGTPase reaction mixture was analyzed by HPLC using a Beckman 126 dual analytical pump system with a Beckman 166 UV detector, controlled by Beckman System Gold Chromatography software. The assay was performed using an Alltima C-18 column (250 x 4.6 mm, 5 µm) with a Phenomenex C18 guard cartridge. As described by Bialkowski and Kasprzak [114], the mobile phase consisted of 100 mM NaH_2PO_4, pH 5.5 (95%) and methanol (5%). The mobile phase was filtered and degassed before being used. The reaction mixtures (50 µl) were chromatographed isocratically at a flow rate of 1 ml/min. Solutions of known concentrations of 8-oxo-dGTP and 8-oxo-dGMP, ranging from 2 to 12 µM, were used for calibration. For

quantitative analysis of the reaction products chromatograms acquired at 293 nm were integrated by the Beckman System Gold chromatography software. The enzymatic activity of the 8-oxo-dGTPase in cell ultrafiltrates was defined as the amount of enzyme converting 1 pmol of 8-oxo-dGTP to 8-oxo-dGMP per minute.

3.2.3. Analysis of Replication Fidelity with 8-oxo-dGTP by In Vitro DNA Synthesis

3.2.3.1. Preparation of HeLa S3 Cell Extract

HeLa S3 cells were obtained from the American Type Culture Collection. The HeLa S3 cell cytoplasmic extract was prepared as described in Roberts and Kunkel [115] for these experiments by Dr. Stella Martomo and stored at –80°C until needed. Immediately before use, the extract was thawed on ice, centrifuged at 4°C for 4 minutes at 10,000 rpm, and the supernatant was used in the replication reactions.

3.2.3.2. *In vitro* DNA Replication Reactions

These experiments used a DNA construct of the phagemid M13mp2SV [115,116], which is an α-complementing derivative of M13 phage that contains an SV40 origin of replication and the first 45 codons of *E. coli* β-galactosidase plus 115 nucleotides of upstream sequence. The specific DNA construct used in these experiments is M13mp2SV/opal-7, which contains an opal (TGA) codon at position 7 of the *lacZα* peptide, and was originally prepared using site-directed mutagenesis by Zhang and Mathews [5]. Most base substitutions that occur at this site during *in vitro* replication of the DNA construct generate a peptide in which α-complementation is readily detected by plating in the presence of Xgal. *In vitro* DNA replication reactions contained 30 mM HEPES pH 7.8, 7 mM $MgCl_2$, 200 μM each of CTP, GTP and UTP, 4 mM ATP, 0.48 mM DTT, 100 μg/ml creatine kinase, 40 mM creatine phosphate, 80 ng M13mp2SV/opal-7 DNA, 1 μg SV40 T antigen, 75 μg protein from HeLa S3 cytoplasmic extract, 60 μM dATP, 60 μM dTTP, 30 μM dCTP, 10 μM dGTP, and 0-10 μM 8-oxo-dGTP. Additionally, *in vitro* DNA replication reactions were performed using exonuclease-deficient (3′→5′ exo-) Klenow polymerase (0.0025 units/reaction),

150 ng of gapped M13mp2SV/opal7 DNA and ligase. In these reactions, ATP, CTP, UTP, GTP, creatine kinase, creatine phosphate and T antigen were omitted from the reaction, and 25 µg of BSA was added to the reaction mix. The *in vitro* DNA replication reactions were carried out as described by Roberts and Kunkel [115] for 4 hours at 37°C. The reactions were terminated by the addition of 25 µl of Stop Mix, and were then incubated for an additional 30 minutes at 37°C. After the addition of 34 µl of Precipitation Mix and 100 µl of isopropanol, the DNA was allowed to precipitate overnight at –20°C. The DNA was collected by centrifugation for 30 minutes at 15,000 rpm, dried in a speed vac, resuspended in 100 µl TE, and further purified by extraction with PEB-equilibrated phenol (2x100µl). The DNA was precipitated at –20°C by the addition of ammonium acetate and isopropanol and once again collected by centrifugation, dried and resuspended in TE buffer. DNA replication reactions were then treated with *Dpn*I endonuclease to eliminate unreplicated DNA molecules [115].

Table 3.2 *Escherichia coli* strains used in the replication fidelity assays.
Strains were obtained from Dr. Thomas A. Kunkel, National Institute of Environmental Health Sciences.

E. coli Strain	**Genotype**
CSH50	[Δ(*pro-lac*)], *thi*, *ara*, *strA* / F' (*proAB*, *lacI*$_q$ZΔM15, traD36)
NR9162	*mutS*, *hsdR*, *hsdM*$^+$, *araD*, Δ(*ara*, *leu*), Δ(*lacIPOZY*), *galU*, *galK*, *strA*

3.2.3.3. Preparation of Competent Cells for Electroporation

Escherichia coli strains used in the replication fidelity assays are described in Table 3.2. Competent cells were prepared as described by Roberts and Kunkel[115], with the following modifications: Two 100-ml cultures of the strain NR9162, which is deficient in mismatch repair (*mutS*), were used in the preparation of competent cells. The cells were grown in 2X YT medium inoculated with 1/100 volumes of fresh

overnight culture at 37°C with vigorous shaking to an optical density at 600 nm of 0.5-0.8. Each flask was chilled on ice for 10 minutes, and the contents were transferred to six chilled 50-ml centrifuge tubes. The cells were pelleted by centrifugation at 3,750 rpm for 10 minutes at 4°C, after which time the medium was decanted. The NR9162 cells were then completely resuspended in 30 ml of ice-cold distilled water and pelleted again by centrifugation at 3,750 rpm for 10 minutes at 4°C. The cell pellet was then resuspended in 10 ml of ice-cold distilled water, and the contents of 3 tubes were transferred to one. Once again the cells were pelleted by centrifugation at 3,750 rpm for 10 minutes at 4°C, after which the cell pellet was resuspended in 25 ml of ice-cold 10% (v/v) glycerol. Cells were pelleted once again at 3,750 rpm for 10 minutes at 4°C, and the volume of the final cell pellet was estimated. To this pellet, an equal volume of ice-cold 10% glycerol was added, the cell suspension was divided into 50 µl aliquots in 500 µl microcentrifuge tubes, and these were frozen rapidly by immersion in liquid N_2 and stored at –80°C until needed. The efficiency of the competent cells was estimated by electroporating the cells for 30 seconds at 1,500 V with 0.08 ng of control DNA (M13mp2SV), plating aliquots of the cells on minimal agar plates with 2.5 ml top agar, 0.72 µg IPTG, 2.5 µg of Xgal, and 300 µl of a log-phase culture of CSH50 cells (the α-complementation strain), and counting the number of colorless plaques that are formed after incubation overnight at 37°C. The efficiency of the NR9162 cells should be $1x10^9 - 10x10^{10}$ pfu/µg DNA.

3.2.3.4. Electroporation and Plating of Cells

The electroporation and plating of cells was carried out as described by Roberts and Kunkel [115], with the following modifications: A portion of the replicated DNA (M13mp2SV/opal-7) sufficient to give about 2000 plaques/plate was added to 50 µl of the competent cells (NR9162), which was then put into a chilled electroporation cuvette and left on ice for 1 minute. The electroporation was then carried out for 30 seconds at 1,500 V, after which the cells were diluted in LB medium. Within 30 minutes of electroporation, an aliquot of the electroporated cells was added to a tube

containing 2.5 ml top agar, 0.72 μg IPTG, and 2.5 μg of Xgal, which was kept at 42°C. To this, 300 μl of a log-phase culture of CSH50 cells was added, and the entire mixture was poured onto minimal medium plates, which were inverted and incubated overnight at 37°C. The following day, mutant (clear) and revertant (blue) plaques were counted.

Chapter 4. 8-oxo-dGTP Pool Assay

4.1. Purpose

This chapter describes the development of an analytical method for the measurement of 8-oxo-dGTP pools in cells. To investigate the contribution to oxidative mutagenesis of damage by reactive oxygen species at the nucleotide level, as compared to damage produced by the direct oxidation of guanine residues in DNA, it was necessary to quantitate intracellular pools of 8-oxo-dGTP. To date, the level of 8-oxo-dGTP in cells has not been reported in the literature. Many researchers have published reports of the potency of 8-oxo-dGTP as a mutagenic substrate for DNA synthesis *in vitro* [14,15,111]. In each of these studies, 8-oxo-dGTP was present in the DNA replication reactions at equimolar concentrations to the normal dNTPs, or was present at a 10- to 100-fold excess. However, the lack of data regarding intracellular 8-oxo-dGTP pools makes it unclear whether or not the nucleotide accumulates to levels in cells that are sufficient to cause mutagenesis. To establish the relevance of 8-oxo-dGTP as a mutagenic DNA substrate when it is present at physiological levels, the actual intracellular concentration of the nucleotide must first be determined. These measurements were ultimately correlated with mutation frequency to determine the contribution of 8-oxo-dGTP to oxidative mutagenesis *in vivo*.

4.2. Experimental Approach

It was expected that pools of 8-oxo-dGTP are small; therefore, a very sensitive analytical method was needed for the detection of this nucleotide. High performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD) is routinely used for the measurement of 8-oxo-dG residues in DNA [117]. Deoxyguanosine is electrochemically active, as are most deoxyguanosine adducts; therefore, it was expected that 8-oxo-dGTP would also show electrochemical activity [118]. When using ECD, only the compounds capable of being oxidized at the specific

applied potential will give a response, making this analytical method extremely specific. The HPLC-ECD method combines the high resolution of HPLC with the specificity and sensitivity of ECD, and was therefore chosen as a means to detect and quantitate 8-oxo-dGTP in the nucleotide pools of cells. Another advantage of using the HPLC-ECD method to quantitate 8-oxo-dGTP pools was that it would allow the simultaneous measurement of the other dNTP pools from nucleotide extracts of cells.

The pool size of 8-oxo-dGTP was initially measured in *E. coli mutT* mutants. These mutant strains are readily available from the *E. coli* Genetic Stock Center. *E. coli* strains lacking the MutT 8-oxo-dGTPase activity should accumulate relatively high levels of the nucleotide, if in fact, 8-oxo-dGTP is a substrate for the MutT enzyme *in vivo* as stated in the current literature. In addition, these cells can be grown in large quantities, supplying adequate amounts of nucleotide extracts for analysis by HPLC-ECD.

4.3. Development of an HPLC Method for the Separation of Nucleotides from Cell Extracts

Originally, a linear gradient of ammonium phosphate buffers (0.075 M – 1 M, pH 3.7) was used to separate standard nucleotides using ion exchange HPLC, a modification of the method of Shewach [119]. This method did not sufficiently separate dNTPs and rNTPs, so periodate oxidation was used to selectively destroy the ribonucleotides [120]. Control experiments showed that after periodate oxidation, only 16-24% of the 8-oxo-dGTP standard was recovered. Alternatively, boronate column chromatography was used to remove ribonucleotides from the standard mixture [119]. However, control experiments showed that the boronate resin retained greater than 50% of the 8-oxo-dGTP present in the standard nucleotide mix. In addition, a chromatographic standard of 8-oxo-rGTP (see below) eluted at the same retention time as an authentic 8-oxo-dGTP standard when using this separation method, and was electrochemically active; therefore, ion exchange HPLC separation could not be used. The method of Arezzo [121], which separates dNTPs and rNTPs by isocratic elution

with potassium phosphate buffers on an ion-exchange column, was also attempted and failed to adequately separate the deoxy- and ribonucleotides. Next, the method of Cross *et al.* [122] which uses reversed-phase chromatography was attempted. dNTPs separated by this method each co-eluted from the column with a ribonucleotide, and because boronate chromatography or periodate oxidation could not be used to first remove the ribonucleotides, another method was necessary for the separation of nucleotides from cell extracts to detect and quantitate 8-oxo-dGTP.

The HPLC method eventually used for the detection and quantitation of dNTPs, including 8-oxo-dGTP, from cell extracts was based on the method of Di Pierro *et al.* [123]. This method uses reversed-phase chromatography coupled with ion-pairing to separate acid-soluble compounds, including nucleotides and deoxynucleotides. The ion-pairing reagent used was tetrabutylammonium hydroxide (TBA-OH), which is very hydrophobic and forms a neutral ion-pair with negatively charged nucleoside triphosphates. This interaction with the ion-pairing reagent changes the polarity of the molecules and increases their interaction with the non-polar, hydrophobic stationary phase of the C18 reversed-phase HPLC column. An ionic and polar (organic) mobile phase was then used to elute the sample from the column by altering the pH and ionic strength of the mobile phase during the separation.

The original method published by Di Pierro *et al.* involved a 100-minute chromatographic run that simultaneously detected 38 individual compounds, including nucleotides, deoxynucleotides, cAMP, nicotinamide coenzymes, oxypurines, nucleosides, and bases. The gradient program used by Di Pierro *et al.* is shown in Figure 4.1, upper panel. This method was modified extensively so that the column would not retain the early-eluting compounds. The result was that high resolution of the late-eluting deoxynucleoside triphosphates could be achieved in a much shorter length of time. Initially, separation was attempted using an Econosphere C18 column (Alltima); however, this column did not adequately resolve all of the nucleotides. A base-deactivated C18 column with higher carbon load (such as the Alltima C18 (Alltech) or the Discovery C18 (Supelco)) gave much higher resolution of the

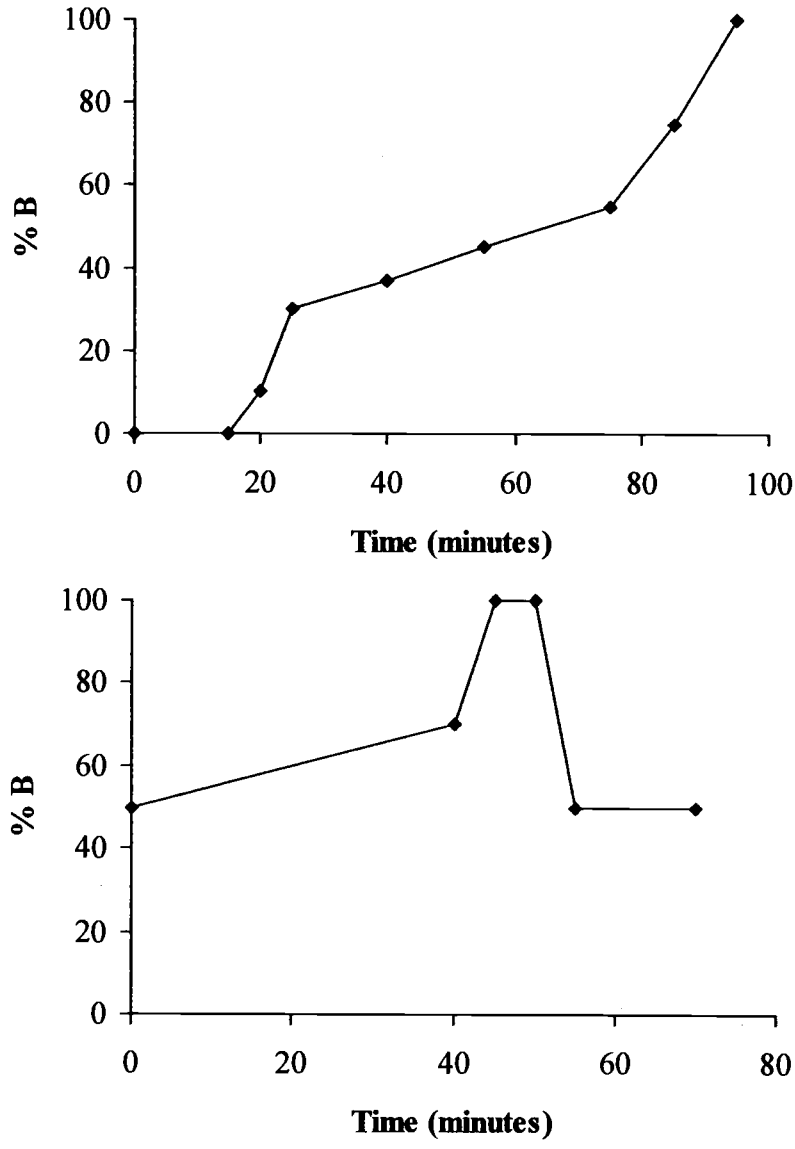


Figure 4.1 HPLC gradient programs for the separation of nucleotides from cell extracts.
Buffer A consists of 10 mM tetrabutylammonium hydroxide, 10 mM KH_2PO_4, 0.25% methanol, pH 7.0; and buffer B consists of 2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol, pH 5.5. The gradient program used by DiPierro *et al.* [123] is shown in the upper panel, and the lower panel shows the modified gradient used in this work (for use with the Alltech Alltima C18 column), which includes the wash program that was run after each injection.

nucleotides of interest, in a much shorter time. Details of the final method used for the separation of the four normal dNTPs and 8-oxo-dGTP from cell extracts follow, and the final gradient program used for the separation is shown in Figure 4.1, lower panel.

4.4. Separation of dNTPs by Reversed-Phase HPLC with Ion-Pairing

The chromatographic system consisted of a Beckman 126 analytical pump system with a Beckman 166 UV detector. Each of the four normal dNTPs was detected at a wavelength of 267 nm. Chromatographic separation of nucleotides was achieved using an Alltima C18 (Alltech Industies) with a Phenomenex C18 guard cartridge or a Discovery C18 (Supelco) column with C18 guard column (Supelco). All analytical columns were 250 X 4.6 mm, with a 5 μm particle size. The mobile phase consisted of buffer A: 10 mM tetrabutylammonium hydroxide, 10 mM KH_2PO_4, 0.25% methanol, pH 7.0 and buffer B: 2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol, pH 5.5. All mobile phase buffers were filtered and degassed, and were prepared fresh daily. Separation was achieved with the Alltima C18 column using a 40-minute linear gradient of 50% buffer A, 50% buffer B to 30% buffer A, 70% buffer B, followed by a wash with 100% buffer B. It was necessary to re-equilibrate the column in 50% buffer A, 50% buffer B for a minimum of 30 minutes after each chromatographic run to get reproducible retention times for nucleotides in subsequent runs. Using the Discovery C18 column, separation of all nucleotides could be achieved in a 25-minute linear gradient of 50% buffer A, 50% buffer B to 40% buffer A, 60% buffer B, when 6% acetonitrile was added to buffer B. All separations were performed at a flow rate of 1.5 ml/min.

4.5. Electrochemical Detection of 8-oxo-dGTP

A Beckman analog interface (AI406) was used to collect data from the electrochemical detector. The electrochemical detector used was a Bioanalytical Systems LC-4B amperometric detector with a CC4 thin-layer flow cell, which was connected in series to the UV detector. 8-oxo-dGTP was detected by using a glassy

carbon electrode set at a potential of + 900 mV versus the Ag/AgCl reference electrode. An offset current was applied to diminish the background current resulting from the oxidation of electrochemically active impurities in the mobile phase. An authentic standard of 8-oxo-dGTP was used to develop a calibration curve for the nucleotide.

4.6. HPLC Column Regeneration

After a series of injections, it was necessary to regenerate the HPLC column to avoid non-reproducibility of injections. This was done by washing the column with at least 70 ml of H_2O to remove salts, followed by a wash of 50 ml of 100% methanol to regenerate the column packing material. This procedure was completed at the end of each day, and the column was left in 30% methanol overnight. Prior to running the nucleotide separation, the column was re-equilibrated in 50% buffer A, 50% buffer B.

4.7. Bacterial Culture Conditions

E. coli strains used in the analysis of 8-oxo-dGTP pools are shown in Table 3.1. The three *mutT* mutant strains were obtained from the *E. coli* Genetic Stock Center. All bacteria were grown in LB medium with the addition of appropriate antibiotics, and 50- or 100-ml cultures were grown to mid-logarithmic phase before extraction of the dNTPs. For bacterial cultures exposed to oxidative stress, cells were grown in a shaker incubator at 37°C until the cell density reached approximately 3-6 x 10^8 cells/ml, after which the cells were treated with 2.5 mM H_2O_2 for 15 minutes prior to nucleotide extraction. The number of cells in each culture was determined by plating serial dilutions of each culture onto LB plates. The bacterial colonies were counted after overnight growth at 37°C.

4.8. Extraction of Nucleotides

Bacterial cultures were extracted for nucleotide analysis by a modification of the dual extraction procedure, using methanol and acid as described by Sargent and

Mathews [124]. Bacteria were collected by rapid filtration on a glass microfibre filter, and the filter was placed in a culture dish. 5 ml of a solution of ice-cold 60% methanol, 1% toluene, and 0.1 mM desferrioxamine mesylate (desferal) was added to the filter, and the culture dish was placed at –20°C for a minimum of two hours. The desferal was added during extract preparation to prevent oxidation during the procedure [117]. After incubation, the methanol extract was removed to microcentrifuge tubes, and the filter was rinsed with an additional 1 ml of the methanol solution. Following centrifugation at 14,000 rpm for 30 minutes at 4°C, the supernatant was transferred to fresh microcentrifuge tubes and dried under vacuum. The extracts were resuspended in 5% TCA and centrifuged for an additional 30 minutes at 10,000 rpm, 4°C. The supernatant was neutralized by the addition of 1.5 volumes of 0.5 M TOA/Freon (see section 3.1), which was vortexed for 90 seconds and then centrifuged briefly to separate the aqueous and organic phases. 80% of the aqueous phase was collected, and after verification that the pH was 5.5-6.0, was dried under vacuum. The final extract was resuspended after drying in 100 µl of HPLC buffer for analysis by HPLC-ECD.

To control for the incomplete recovery of each of the dNTPs during the extraction and preparation of bacterial dNTP extracts, a control culture was prepared. This culture was collected by filtration, and to the filter a 45-µl sample containing a known concentration of the four standard dNTPs and 8-oxo-dGTP was added. The extract from this filter was treated identically to the other extracts throughout the remainder of the nucleotide extract preparation, and was then subjected to HPLC analysis to determine the recovery of the dNTPs.

4.9. H_2O_2 Measurement

H_2O_2 in the bacterial cultures was measured according to the protocol of Nowak [125]. This reaction measures the oxidation of the oxidizable substrate, o-dianisidine, by horseradish peroxidase in the presence of H_2O_2. The mixture (total volume = 1 ml) contained 737 µl of horseradish type II peroxidase (14.2 U/ml, freshly prepared in

PBS), 63 µl of freshly prepared o-dianisidine (0.08 mg/ml), and 200 µl of bacterial culture medium. Absorbance was read at 470 nm. A 30% commercial solution of H_2O_2 was freshly diluted in H_2O to generate H_2O_2 stock solutions. The actual concentration of each H_2O_2 stock solution was calculated from a measurement of its absorbance at 230 nm ($\varepsilon = 81 \cdot cm^{-1} \cdot mol^{-1}$). H_2O_2 was diluted in LB medium (final concentration = 0-1000µM) to generate a standard curve for the assay.

4.10. Bacterial Mutagenesis

Mutagenesis in bacterial strains exposed to H_2O_2 was monitored by plating a 100-µl aliquot of the culture on each of three replicate plates containing rifampicin (150 µg/ml). Additionally, an aliquot of each culture was plated to determine the actual titer of the cells.

4.11. HPLC-ECD Analysis of dNTP Pool Sizes from Bacterial Cultures

Pure standards of dNTPs, rNTPs and 8-oxo-dGTP were used to determine the elution profile and develop calibration curves for all nucleotides. Bacterial dNTP extracts were prepared as described above. Injections (50 µl each) of bacterial dNTP extracts were performed in duplicate, and an internal standard of the 4 normal dNTPs and 8-oxo-dGTP was added to the second injection to verify the retention time for each nucleotide.

4.12. Oxidation of Nucleotides

Each of the standard dNTPs and rNTPs was exposed to oxidizing conditions according to the method of Mo *et al.* [40]. Each reaction mixture (1000 µl) contained 100 mM Na_2PO_4 pH 6.8, 6 mM dNTP or rNTP, 30 mM ascorbic acid, and 100 mM H_2O_2. The reaction mixtures were incubated in the dark at 37° C for 2 hours and 15 minutes. The nucleotides were purified following the method of Kasai and Nishimura [35] with the following modifications: After incubation, approximately 0.1 g of activated charcoal was added to each reaction mixture, which was then vortexed. The

charcoal was recovered by filtration and washed with 1.5 ml of H_2O. Nucleotides were eluted from the charcoal by the addition of 1.5 ml of acetone:H_2O (1:1, v/v) and evaporated to dryness under vacuum. The resulting pellet was resuspended in 1000 µl H_2O and analyzed by HPLC with electrochemical detection at + 900 mV.

Additionally, dCTP and dTTP were oxidized by dissolving 5 mg of each nucleotide in 1 ml of 50 mM sodium phosphate buffer, pH 7.4, and then adding 40 mM EDTA and 40 mM $FeSO_4$. The reaction mix was then shaken vigorously under air at room temperature for 30 minutes [126]. dATP was oxidized by dissolving 5 mg in 1 ml of 100 mM sodium phosphate buffer, pH 7.4, and then adding 25 mM EDTA and 25 mM $FeSO_4$. The reaction mix was shaken vigorously in an open tube for 120 minutes at 37°C, according to the method of Kamiya and Kasai [127]. After oxidation, the nucleotides were purified as described above and analyzed by HPLC-ECD.

Chapter 5. Results

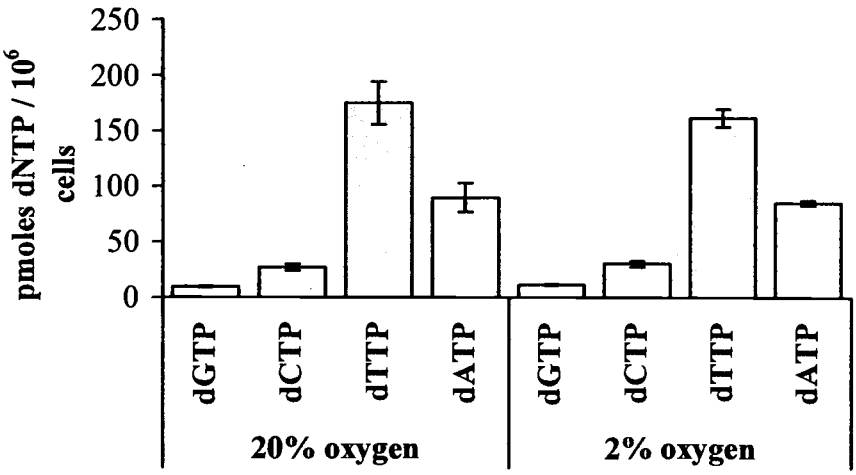
5.1. Oxygen Effects on Mammalian dNTP Pools

5.1.1. dNTP Pool Measurements and Varying O_2 – HeLa Cells

dNTPs, and specifically dGTP, are suspected to be targets for reactive oxygen species in cells, and their modification by oxidation produces potentially mutagenic substrates for DNA synthesis. If dGTP is oxidized to 8-oxo-dGTP, it can be degraded in cells by the action of the MutT enzyme and its mammalian homologs. If the dGTP pool is susceptible to oxidation *in vivo*, then one would expect the pool of dGTP to expand if cells are grown in conditions where reactive oxygen species are present in low amounts. This hypothesis was tested by growing HeLa S3 cells in an atmosphere containing approximately 20% oxygen (ambient oxygen conditions) and in an atmosphere containing only 2% oxygen, and then measuring the levels of dNTPs in the cells. *In vivo*, the partial pressure of O_2 is approximately 5% [53]. So, cells grown in typical culture conditions (~20% oxygen) are thought to experience higher levels of oxidative stress than they would *in situ*. However, at 2% oxygen the production of reactive oxygen species in cells and their exposure to these potentially damaging free radical species is expected to be low. If the turnover of dGTP to 8-oxo-dGTP and then to 8-oxo-dGMP is significant (see Figure 1.3), then dGTP should accumulate under low oxygen growth conditions.

Figure 5.1 shows the results of measurements of dNTP pools in HeLa cells using the dNTP enzymatic assay. As shown in this figure, HeLa cells grown in a low-oxygen atmosphere for two days showed no changes in the dGTP pool size, as compared to cells that had been grown in a normal atmosphere. In fact, pool sizes of each of the four dNTPs from the two culture conditions were identical, within experimental error. The HeLa cell line used in these experiments is derived from a human cervical adenocarcinoma. Tumor cells are thought to rely fairly heavily on

anaerobic metabolism when compared to non-tumor-derived cells, and consequently their dNTP pools may not be impacted by growth under low oxygen conditions. Therefore, it was of interest to also measure dNTP pool sizes in a non-tumor-derived cell line (see below). V79 cells derived from the lung tissue of a Chinese hamster were used in these experiments to further test the hypothesis that growth in low-oxygen



conditions will cause an accumulation of dGTP.

Figure 5.1 HeLa S3 cell dNTP pool sizes.
dNTP concentrations were measured by using extracts of HeLa S3 cells grown under control conditions (20% oxygen) and oxygen-limited conditions (2% oxygen). Data shown are the average of triplicate samples and error bars represent the standard error for n=3 similar experiments for the dGTP pool, and n=2 experiments for the dCTP, dTTP, and dATP pools.

In addition, experiments using the V79 cell line were designed to ask whether the low dGTP pool in mammalian cells is a consequence of rapid dGTP oxidation, followed by the enzymatic degradation of 8-oxo-dGTP. If this is the case, then culturing cells at low oxygen tension may diminish the turnover of the dGTP pool and allow the nucleotide to accumulate. In order to conduct these experiments, cells were labeled with a [^{3}H]-nucleotide precursor according to the methods of Bianchi *et al.* [128], and the loss of label from the nucleotide pool was followed during a non-

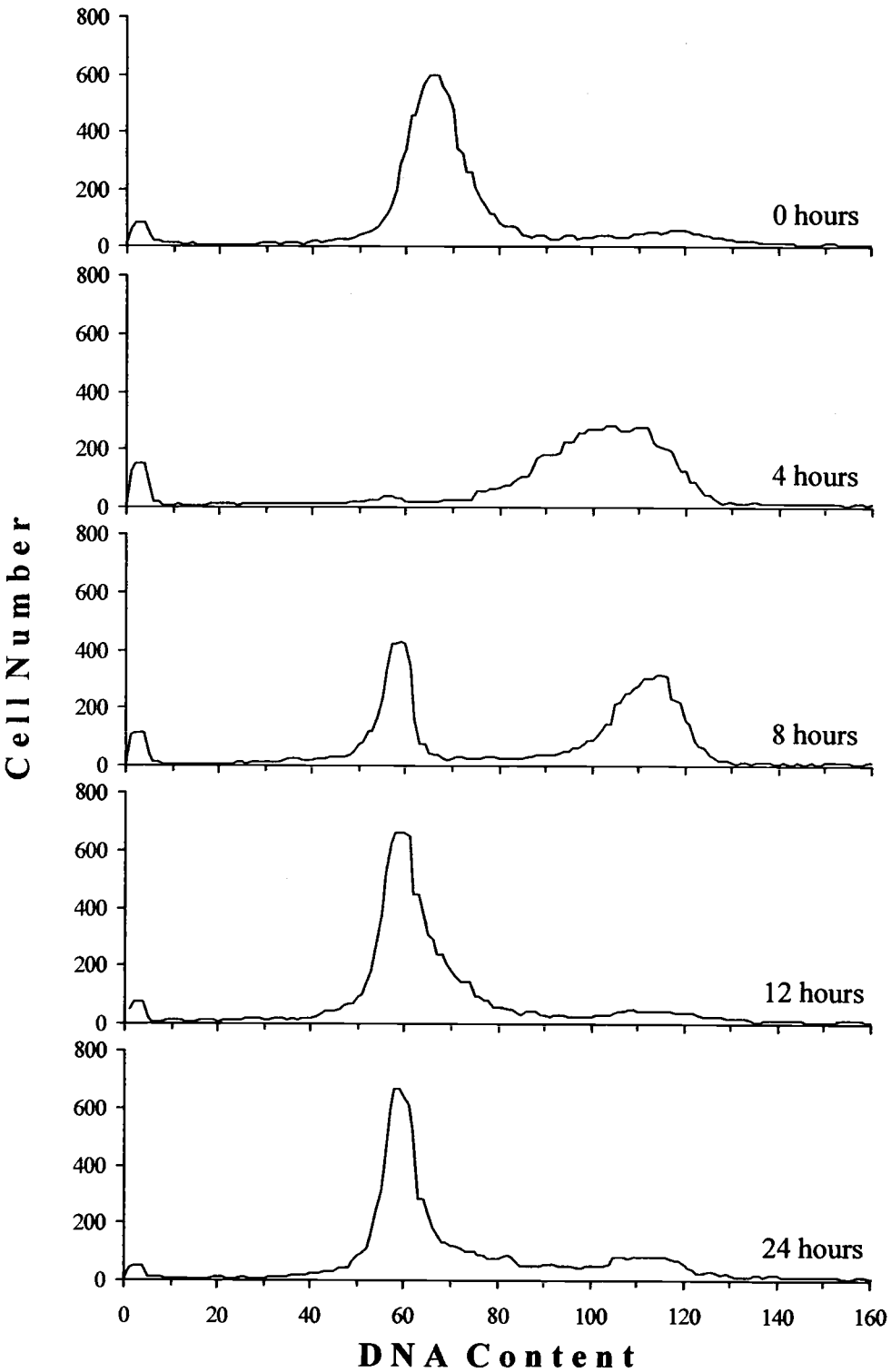
radioactive chase using the dNTP enzymatic assay to measure pool specific radioactivities. The incorporation of label into DNA was also measured. For these experiments it was necessary to synchronize the cells and label them while in S-phase, since dGTP can either be 1) incorporated into DNA, or 2) undergo catabolism or enzymatic degradation (see Figure 1.3). If dGTP is a significant target for oxidation, then aerobic cultures might show that the dGTP pool decays more rapidly than its utilization for DNA synthesis, while in an oxygen-limited culture, its turnover would equal the rate of incorporation of dGTP into DNA. If the turnover of dGTP varies with the oxygen content of the atmosphere in which the cells were cultured, this would identify dGTP as a significant oxidation target, even if large pool size changes were not observed as a function of changes in oxygen tension.

5.1.2. Cell Synchronization with Aphidicolin

The method of Matherly *et al.*[113] was adapted for the synchronization of V79 cells. This method utilizes the tetracyclic diterpenoid, aphidicolin, which inhibits DNA polymerases α, δ and ε in a reversible manner [129,130]. The use of aphidicolin to synchronize cells was important with respect to the goal of measuring the dNTP pools of cells, because the synthesis of nucleotides is largely unaffected by treatment of cells with aphidicolin [129]. The original protocol was developed for the synchronization of suspension cultures of murine L1210 leukemia cells, and its development was based on the doubling time of the cells. To adapt the method for V79 cells, the timing of the aphidicolin treatments was changed to account for the doubling time of V79 cells, which is 12-14 hours, slightly longer than the 11.5 ± 0.6 hours [113] required for the L1210 cells to double.

As shown in Figure 5.2, a homogeneous G_1/early S-phase population of V79 cells could be obtained using a two-step synchronization protocol, involving an initial 12-hour aphidicolin exposure, followed by drug removal for 9 hours, and re-addition of aphidicolin for 12-14 hours. The period of time that cells were allowed to cycle in drug-free medium after the initial aphidicolin exposure was increased from 6 hours for

Figure 5.2 DNA content in aphidicolin-synchronized V79 cells.
Cells were synchronized by two consecutive treatments with 3 μM aphidicolin, separated by a 9 hour drug-free period. At the times indicated in each figure, cells released from the second aphidicolin exposure were stained with propidium iodide and analyzed for DNA content.


800
600
400
200
0
0 hours
4 hours
8 hours
12 hours
24 hours
Cell Number
0
20
40
60
80
100
120
140
160
DNA Content

L1210 cells to 9 hours for V79 cells. The 9-hour release from the effects of aphidicolin was sufficient to allow the cells arrested initially at various stages throughout S-phase, to complete DNA synthesis and move through mitosis and into G_1. Readdition of aphidicolin after the 9-hour release for 12-14 additional hours prevented cell cycle passage beyond early S-phase.

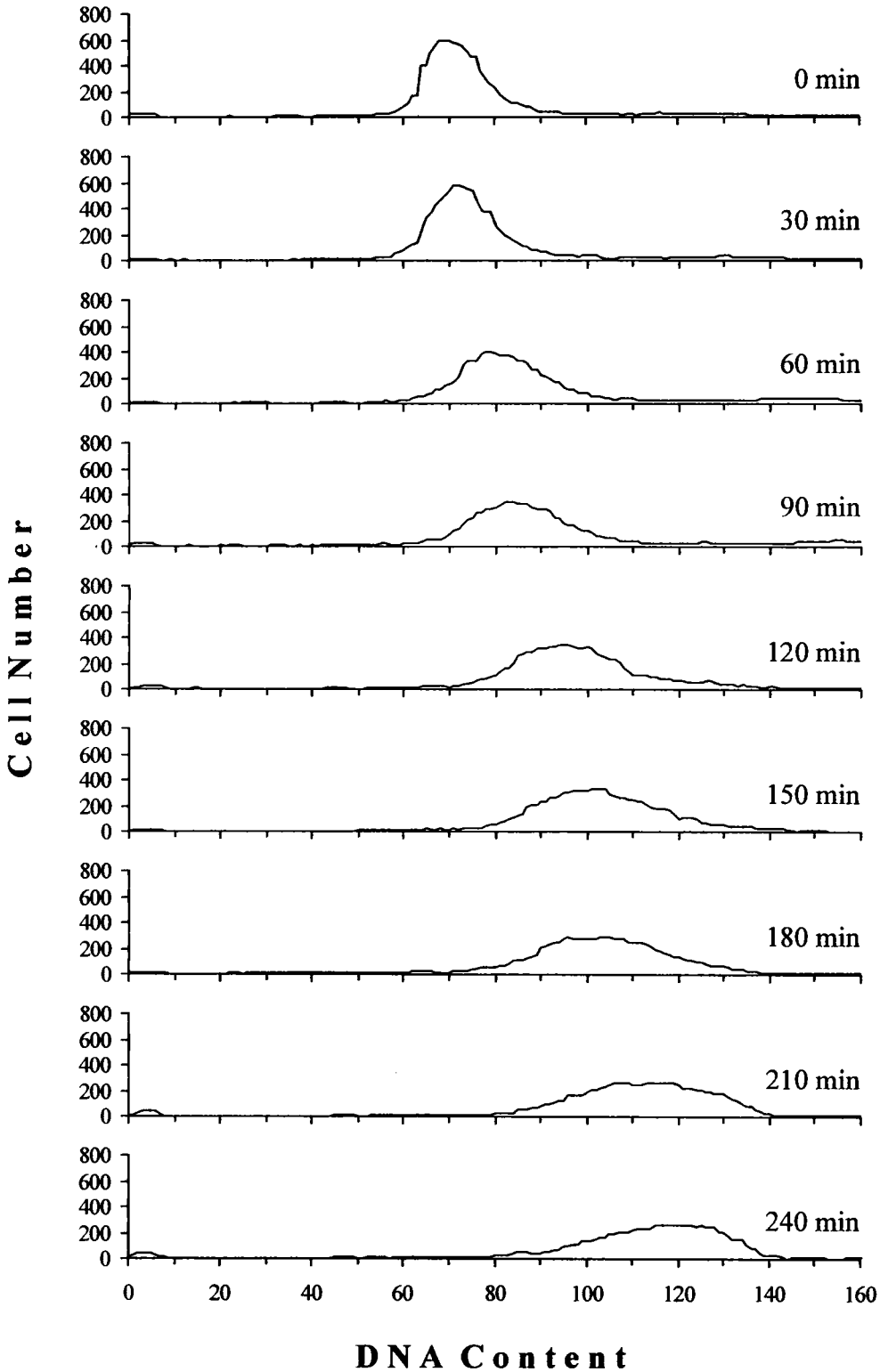
The cells began to cycle within two hours of their release from aphidicolin, showing that the effects of the inhibitor are fully reversible. Figure 5.3 shows that 4 hours after release from aphidicolin exposure, 100% of the cells were in S-phase, as determined by multicycle analysis. By 12 hours post-release, the majority of cells had cycled back to G_1. Synchronization by aphidicolin treatment is only short-term, as the results of flow cytometric analysis showed that by 24 hours the cell population was once again asynchronous (see Figure 5.2).

5.1.3. Cell Cycle Changes in the dNTP Pools of V79 Cells

dNTP pools are known to vary with the cell cycle [21]. There are conflicting reports in the literature regarding the sizes of dNTP pools during different phases of the cell cycle. It has been reported by some researchers that the largest pool sizes occur during S- and G_2-phases and the smallest in G_0 [19,131,132], whereas others report that non-S-phase cells have more substantial pools of dNTPs [133]. As mentioned (see Table 1.1), dGTP is the least abundant of the four dNTPs in asynchronous cultures, and when measured by the dNTP enzymatic assay, the dGTP pool is often near the lower limit of sensitivity of the assay. Before attempting to measure the turnover of the dGTP pool during S-phase, it was of interest to examine the pool sizes of the dNTPs of synchronized V79 cells growing under normal oxygen tensions.

To do this, dNTP extracts were prepared from aphidicolin-synchronized V79 cells at 45-minute intervals following the final release from aphidicolin. Changes in the dNTP pool sizes were followed for the next 4 hours, as the cells progressed from early S-phase into the G_2-phase of the cell cycle. The results of this experiment are

Figure 5.3 Re-entry of aphidicolin-synchronized V79 cells into the cell cycle following release from drug exposure.
Cells were synchronized as described in Materials and Methods, and their re-entry into S-phase was determined by analyzing the DNA content of the cells at 30-minute intervals.


0 min
30 min
60 min
90 min
120 min
150 min
180 min
210 min
240 min
800
600
400
200
0
0
20
40
60
80
100
120
140
160
Cell Number
DNA Content

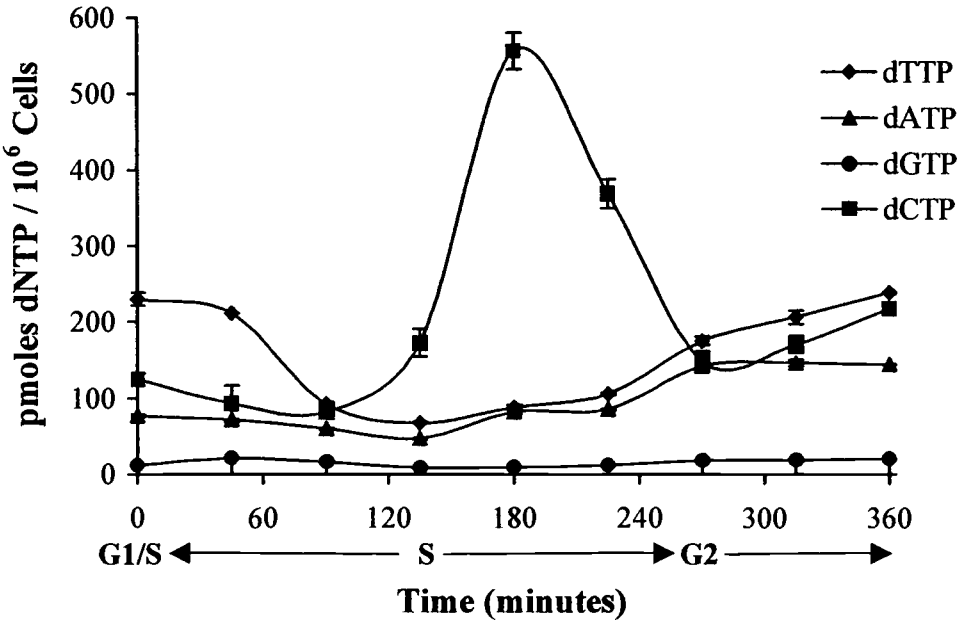


Figure 5.4 Cell cycle changes in the dNTP pools of V79 cells.
The time indicated is the time following release of the cells from aphidicolin block.

shown in Figure 5.4. Pools of dATP and dTTP decreased in late S-phase, and then increased to early S-phase levels as the cells cycled into G_2. Interestingly, the pool of dCTP expanded during late S-phase and decreased rapidly as the cells progressed to G_2. This same elevation of the S-phase dCTP pool has been observed in the isolated nuclei of S-phase CHO cells synchronized by isoleucine starvation [20].

As observed with asynchronous cultures (see below, and [10]), the dGTP pool is the least abundant of the dNTP pools during S-phase. Levels of dGTP dropped to less than 10 pmoles / 10^6 cells during S-phase. This result was expected; since DNA synthesis draws substrates from the four dNTP pools and because dGTP is the least abundant nucleotide, it is not surprising that it would be substantially depleted during S-phase. This very low pool size of dGTP in S-phase V79 cells was of concern when considering the dGTP turnover experiments, because the success of such experiments

depends upon the accumulation of a radiolabeled pool of dGTP and an examination of its utilization for DNA synthesis or catabolism (see the Appendix).

5.1.4. dNTP Pool Measurements and Varying O_2 – V79 Cells

Measurements of the dNTP pool sizes of HeLa cells grown under varying oxygen conditions showed that oxygen did not have any effect on the dNTP pools (see Figure 5.1). In order to see if the same result is true with non-tumor-derived cells, similar experiments were conducted using V79 cells. The V79 cells used (V79-4) are derived from the lung tissue of a Chinese hamster, which is exposed to higher levels of oxygen *in situ*. Consequently, if the dGTP pool is affected by changes in oxygen tension, then culturing this particular cell line at low oxygen tensions was expected to impact the dGTP pool.

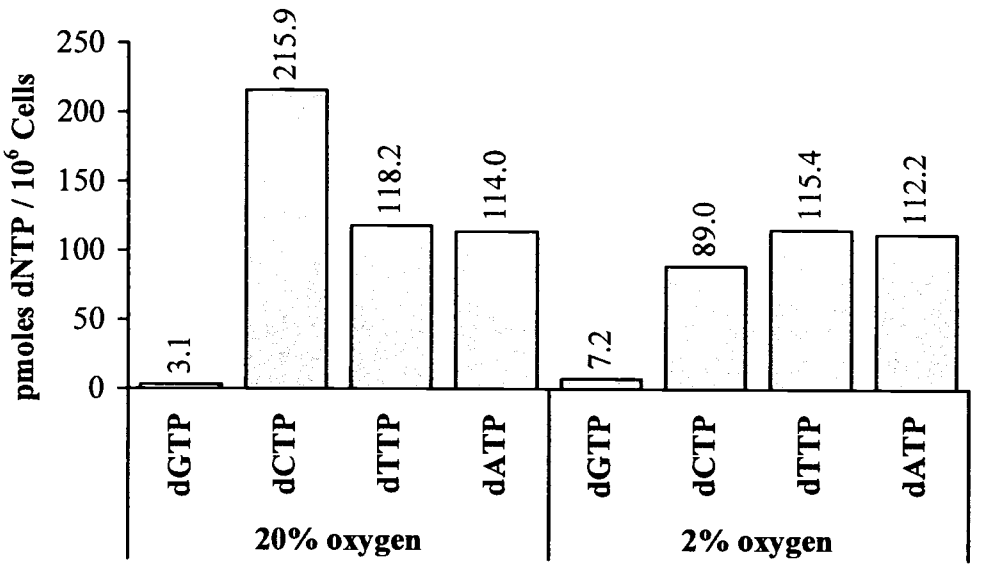


Figure 5.5 dNTP pool sizes in an asynchronous population of V79 cells.
Cells were grown in low and high oxygen conditions, dNTPs were extracted, and the pool sizes were measured using the dNTP enzymatic assay. Data shown are the average of triplicate samples and are representative of one of n=3 similar experiments for the dGTP pool, and n=2 experiments for the dCTP, dTTP, and dATP pools.

Preliminary studies of the dNTP pool sizes in V79 cells growing under varying oxygen conditions were done using asynchronous cultures. dNTPs were extracted from these cultures and measured by the dNTP enzymatic assay, as described for HeLa cells. The results of these dNTP measurements are shown in Figure 5.5. There was no change to the dATP and dTTP pools when cells were grown in 2% oxygen, as compared to 20% oxygen conditions. However, the dCTP pool decreased by more than two-fold when the cells were grown in low oxygen. Measurements of the dGTP pool of asynchronously growing V79 cells showed that the pool size of dGTP doubled when the cells were grown in low oxygen conditions, a result that supports the hypothesis that dGTP is a target for the damaging effects of reactive oxygen species.

To study the dNTP pool sizes of a synchronized population of V79 cells, cells growing under varying oxygen conditions were treated with aphidicolin, as described in Chapter 3, and 100 minutes post-release from the second aphidicolin block, the cells were shown by flow cytometry to be in S-phase of the cell cycle (see Figure 5.3). These S-phase cultures were originally intended for use in the measurement of dGTP pool turnover as described above; however, many attempts to label the dGTP pool with the dGTP precursors, [^{3}H]-hypoxanthine, [^{3}H]-guanosine, and [^{3}H]-deoxyguanosine were unsuccessful. The procedures used and the results of these attempts are described in detail in the Appendix. Despite the fact that the labeling of the dGTP pool was insufficient to measure turnover, the dNTP extracts obtained from these S-phase V79 cells were still useful for the measurement of dNTP pool sizes of cells grown in different oxygen tensions.

Preliminary measurements of the dGTP pool of these synchronized S-phase V79 cells showed that the dGTP pool from cells grown at 20% oxygen was extremely low, with the pool sizes measured to be less than 2 pmoles dGTP / 10^6 cells (see Figure 5.6). However, the pool size of dGTP in cells grown at 2% oxygen were 3-fold higher and approached the expected pool size reported for dGTP in asynchronous V79 cell cultures, approximately 10 pmoles / 10^6 cells [10]. As was true with the results of dGTP measurements from asynchronously growing V79 cells, this result also showed

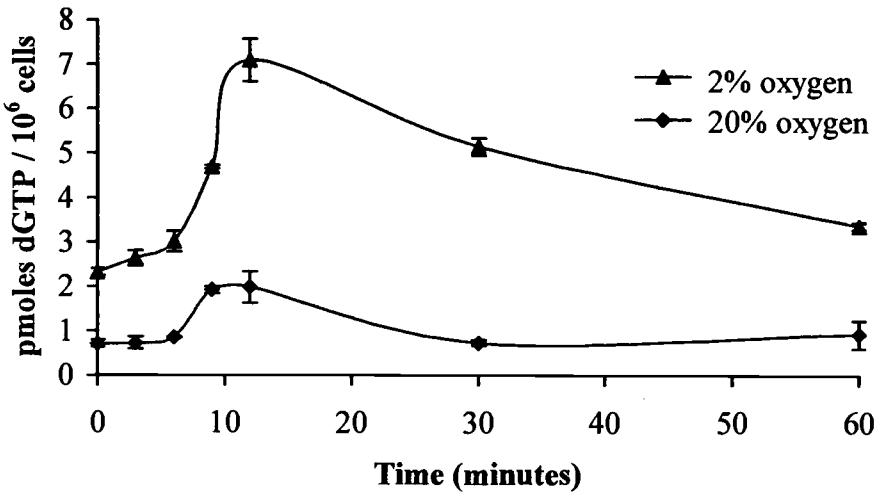


Figure 5.6 dGTP pool sizes in S-phase V79 cells synchronized by aphidicolin treatment and cultured in 2% or 20% oxygen.
0 minutes in the figure is 100 minutes after release from the aphidicolin block. Error bars represent the standard error from three independent measurements of the dGTP pool from one sample taken from an individual dNTP extract.

that the dGTP pool is lower in cells grown at higher oxygen tensions, and supported the hypothesis that dGTP may be a target for oxidative damage.

However, the cause of this change in pool size, specifically, whether or not it was due to increased turnover of dGTP at high oxygen tensions, was not made clear by these experiments. In order to determine whether other pools of dNTPs were affected by changes in oxygen tension, the pool sizes of dATP, dCTP and dTTP were measured from the same extracts. The results of these dNTP pool assays are summarized in Figure 5.7. This figure shows the ratio of the respective dNTP pool size in the culture grown at 2% oxygen compared with that of the culture grown at 20% oxygen. This representation of the data clearly indicates that the dGTP pool was not the only pool that expanded in the low oxygen culture conditions, relative to the control condition. The pools of dATP and dTTP also increased in low oxygen. In contrast, the dCTP pool decreased by two-fold under these conditions. A similar result

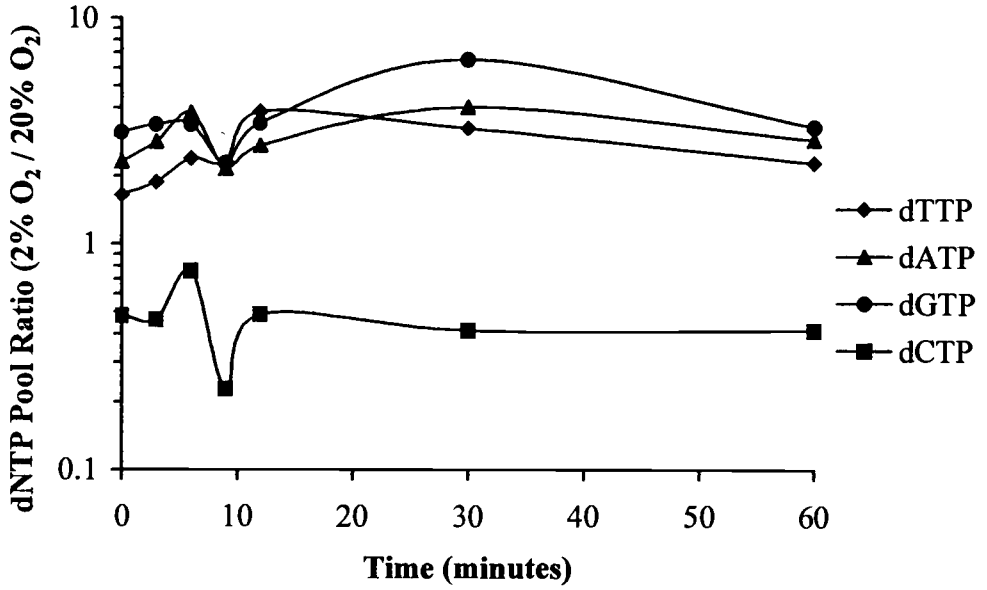


Figure 5.7 Effects of varying oxygen tension on dNTP pools in V79 cells.
Cells were cultured in 2% or 20% oxygen and were synchronized by aphidicolin treatment. Time zero on the figure corresponds to 100 minutes post-release from the aphidicolin block. Each data point represents the ratio of the respective dNTP pool size in the culture grown at 2% oxygen compared with that of the culture grown at 20% oxygen.

was seen for the dCTP pool of an asynchronous population of V79 cells grown at 2% oxygen (see Figure 5.5). These results indicate that growth in low oxygen does not specifically cause the dGTP pool of V79 cells to expand. Each of the four dNTPs are thought to be susceptible to damage by reactive oxygen species, since each of the bases can undergo oxidation in DNA (see Chapter 2). It is possible that the dATP and dTTP pools are experiencing turnover due to their oxidation and subsequent degradation to prevent errors in DNA synthesis by the incorporation of an oxidatively damaged nucleotide, as was hypothesized to occur with the dGTP pool. However, cytosine is also susceptible to oxidation, particularly at the C-5 position, to form 5-hydroxy-cytosine (see Figure 2.2). Therefore, it is difficult to attribute these results to

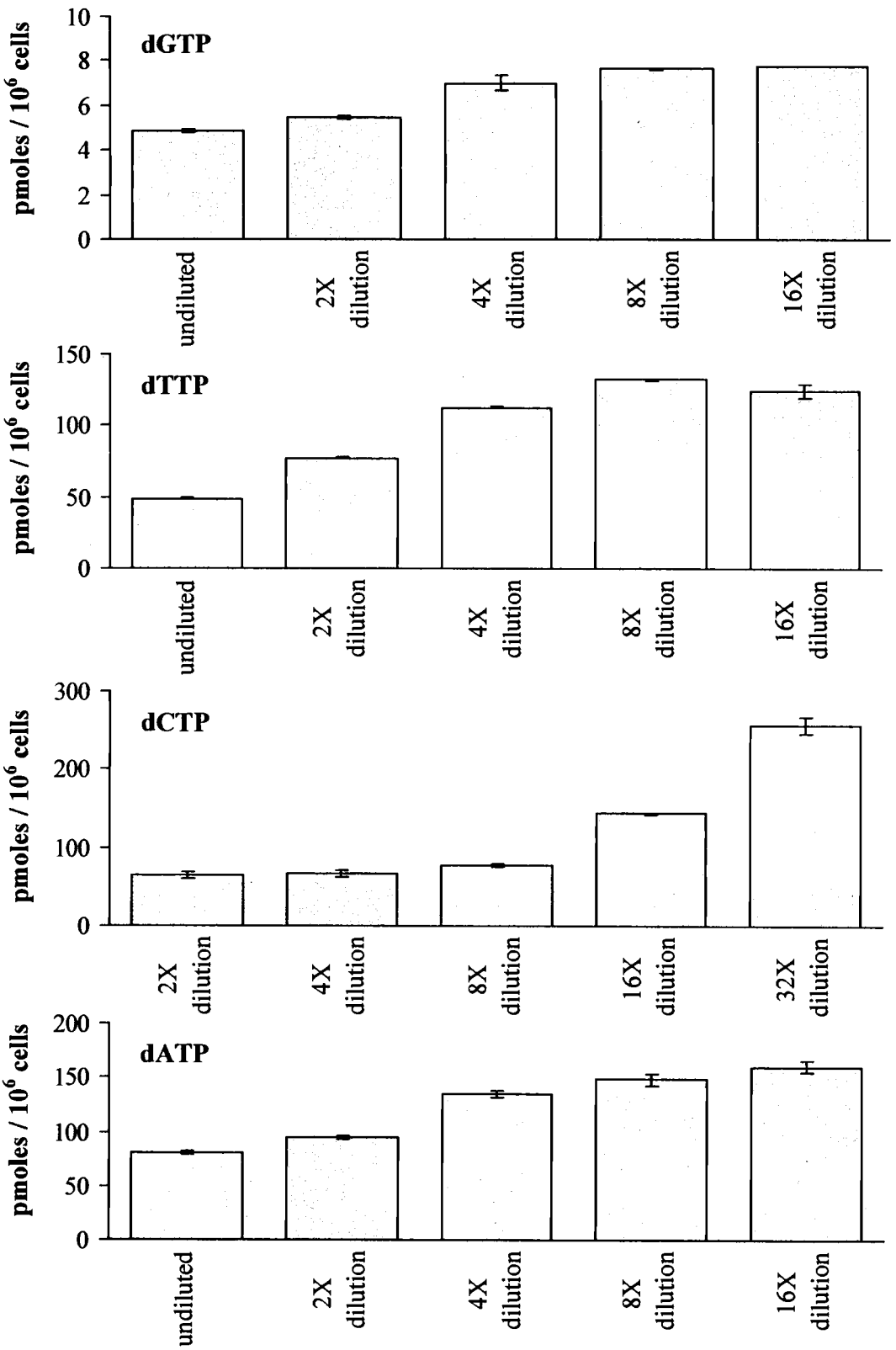
dNTP turnover due to damage caused by reactive oxygen species. While the results do suggest that there may be increased turnover of the dGTP pool under high oxygen conditions, the reason for this oxygen-dependent change in the dGTP pool could not be addressed by these experiments. Since attempts to measure dGTP pool turnover were unsuccessful (see the Appendix), a more direct approach was needed to test the hypothesis that 8-oxo-dG residues in DNA are formed as the result of oxidation of dGTP and incorporation of 8-oxo-dGTP into DNA. A direct measurement of the pool size of 8-oxo-dGTP in cells would determine whether this pathway is biologically significant and if it is involved in oxidative mutagenesis *in vivo*. The approach used to directly measure 8-oxo-dGTP pools is described in Chapter 4, and the results obtained using that approach are described below.

5.1.5. Notes on the DNA Polymerase-Based dNTP Enzymatic Assay

During the course of experiments using the DNA polymerase-based dNTP enzymatic assay it became evident that using an appropriate dilution of the dNTP extract from cells was necessary to obtain an accurate measurement of the dNTP pools. Previously, dNTP extracts prepared by methanol/toluene extraction of cells were routinely resuspended in 100-200 µl of H_2O and used undiluted in the enzymatic assay. Data obtained during the course of experiments described in this dissertation show that this practice leads to an underestimation of the dNTP pools.

Figure 5.8 shows the effect of dilution of the dNTP extract on apparent pool size results obtained using the DNA polymerase-based dNTP enzymatic assay. For these experiments, serial dilutions of nucleotide extracts from early S-phase V79 cells were made and were used in the dNTP enzymatic assay. With increasing dilution factor, the apparent pool size of each dNTP increased, until it reached a plateau. Further dilution of the extract would lead to a decrease in the pool size being measured, which can be attributed to a depletion of the dNTP being measured in the reaction mix due to the dilution of the extract. The same effect of dilution of the dNTP extract was seen for each of the dNTP pools, as indicated in Figure 5.8. This effect of dilution of the

Figure 5.8 dNTP extract dilution effects on apparent dNTP pool size measurements using the dNTP enzymatic assay.
Serial dilutions of the dNTP extract were made and used in the dNTP enzymatic assay to measure the pool sizes. Error bars represent the standard deviation of three independent measurements of the dNTP pools using the same dNTP extract.


dGTP
pmoles / 10^6 cells
10
8
6
4
2
0
undiluted
2X dilution
4X dilution
8X dilution
16X dilution
dTTP
pmoles / 10^6 cells
150
100
50
0
undiluted
2X dilution
4X dilution
8X dilution
16X dilution
dCTP
pmoles / 10^6 cells
300
200
100
0
2X dilution
4X dilution
8X dilution
16X dilution
32X dilution
dATP
pmoles / 10^6 cells
200
150
100
50
0
undiluted
2X dilution
4X dilution
8X dilution
16X dilution

extract on the outcome of the dNTP pool measurement can possibly be ascribed to the presence of a DNA polymerase inhibitor in the dNTP extract. In some cases it was necessary to dilute the dNTP extract 32-fold before the inhibitory effect was no longer observed. The identity of the inhibitor has not been determined, but the results of these experiments clearly indicate the need to empirically determine the dilution factor for each extract before analysis of the extracts by the dNTP enzymatic assay.

5.2. HPLC-ECD Assay of 8-oxo-dGTP Pools

5.2.1. Development of an HPLC Method for the Separation of Nucleotides from Cell Extracts

Reversed-phase chromatography coupled with ion-pairing was the method chosen for the separation of nucleotides from cells extracts. This method allowed for the separation of deoxy- and ribonucleotides in a single chromatographic run. Ionic compounds, such as nucleoside triphosphates, do not easily partition into the hydrophobic layer of reversed-phase columns due to the charge on the molecules. By adding a counter-ion of opposite charge (the ion-pairing reagent, in this case tetrabutylammonium hydroxide) to the mobile phase, the sample and ion-pairing reagent form a neutral ion pair that will readily partition into the reversed-phase column packing. The reversed-phase ion-pairing method that was developed allowed for the separation of the standard nucleoside triphosphates and their detection by UV absorbance, and the separation of 8-oxo-dGTP from the standard NTPs and its measurement by EC detection.

Figure 5.9 shows the elution profile of a mixture of 250 pmoles of each of the standard ribo- and deoxyribonucleoside triphosphates, along with dADP and dTDP that eluted with the triphosphates. Each of the standard dNTPs is well separated from the other nucleotides by using this HPLC method, allowing for the measurement of their pool sizes from a cellular dNTP extract. The calibration curves for each of the four normal dNTPs are shown in Figure 5.10.

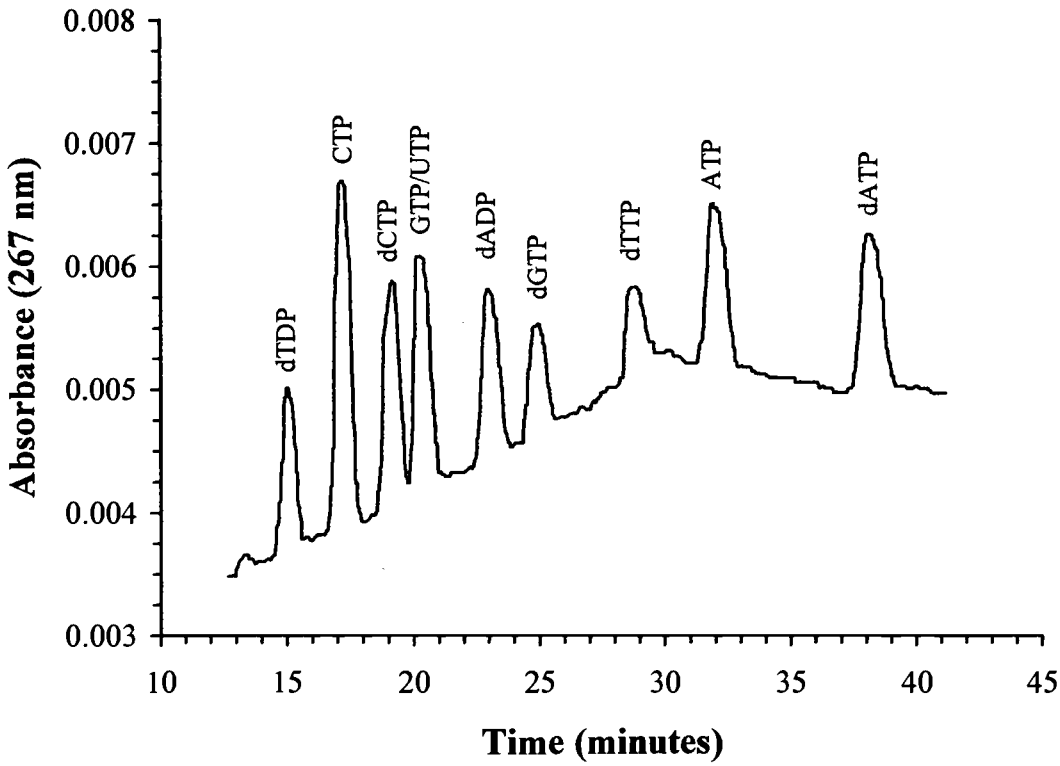


Figure 5.9 HPLC elution profile of a mixture of 250 pmol of each of the standard ribo- and deoxynucleoside triphosphates.
The nucleotides were detected by UV absorbance at a wavelength of 267 nm.

5.2.2. Electrochemical Detection of 8-oxo-dGTP

An electrochemical (EC) detector uses the electrochemical properties of compounds for their determination in a flowing stream. The EC detector used in this work was an amperometric detector, which applies a constant potential to the sample flowing over the surface of the working electrode. The applied potential represents the electrical energy that serves as the driving force for the redox reaction. In this case, 8-oxo-dGTP was the electrochemically active compound, and its oxidation required a positive applied potential. The loss of electrons from 8-oxo-dGTP during oxidation by the working electrode of the EC detector resulted in a current that is proportional to the

concentration of 8-oxo-dGTP in the flowing stream. In order to select the proper applied potential for the EC detection of 8-oxo-dGTP, a hydrodynamic voltammogram was constructed by making repeated injections of an 8-oxo-dGTP standard of known concentration, while recording the current generated at different applied potentials. The choice of the applied potential is dictated by the working and reference electrodes of the EC detector and the composition and pH of the mobile phase; therefore, it must be determined empirically when developing a new ECD method. The hydrodynamic voltammogram for 8-oxo-dGTP is shown in Figure 5.11. The strongest signal observed for 8-oxo-dGTP occurred at an applied potential of + 900 mV.

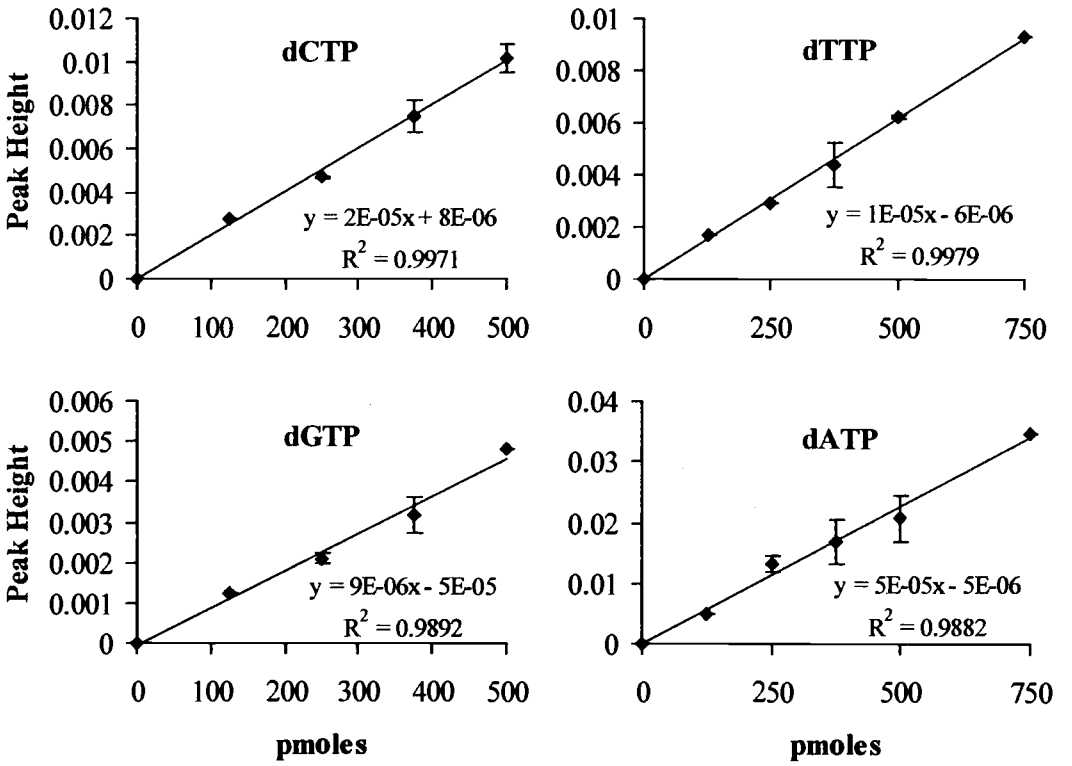


Figure 5.10 Calibration curves for each of the four normal dNTPs.

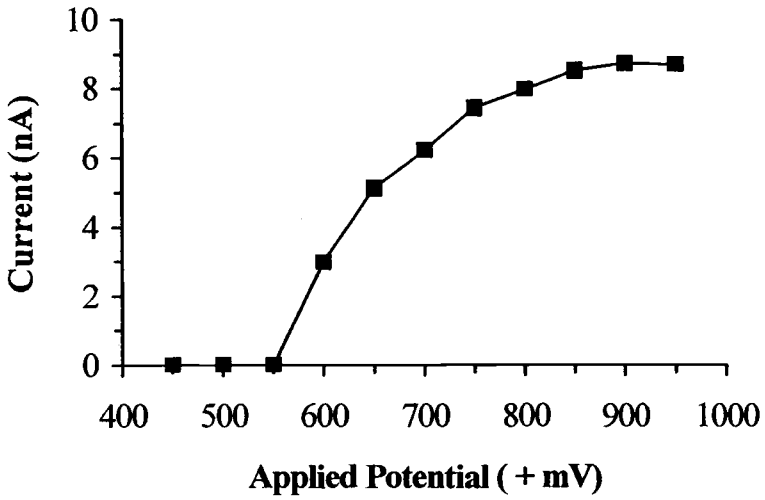


Figure 5.11 Hydrodynamic voltammogram of 8-oxo-dGTP at a glassy carbon electrode.
Maximal oxidation of 8-oxo-dGTP occurs at an applied potential of + 900 mV. 120 pmoles of the nucleotide was injected for each chromatographic run.

The high potential (+ 900 mV) required to maximally oxidize 8-oxo-dGTP resulted in a high background current, and hence a high noise level. This is a disadvantage when performing ECD because a high potential is more universal and could result in the detection of many electrochemically active compounds present in a complex nucleotide extract from cells. However, because the pool size of 8-oxo-dGTP was expected to be quite low, the assays were performed at the high potential required to maximally oxidize 8-oxo-dGTP in order to achieve the best signal for the nucleotide when measuring its levels in cell extracts. An offset current was applied to the EC detector to minimize the background current.

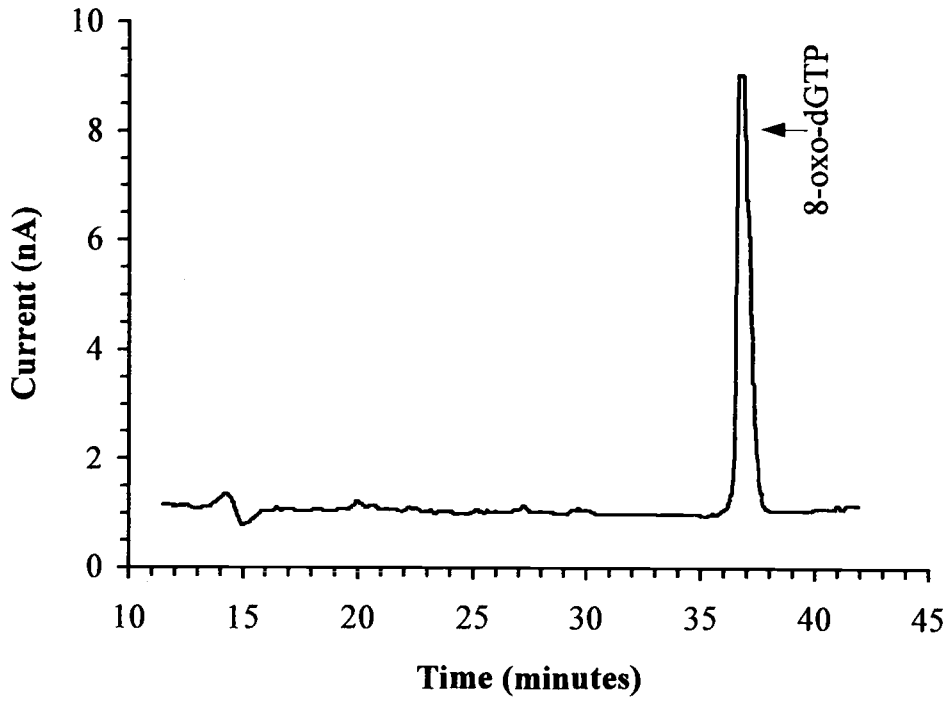


Figure 5.12 Electrochemical detection of 8-oxo-dGTP.
A 250 pmole sample of authentic 8-oxo-dGTP was detected electrochemically at an applied potential of + 900 mV.

The elution of 8-oxo-dGTP from the HPLC column, and its detection by ECD is shown in Figure 5.12. When using the Alltima column, 8-oxo-dGTP elutes at a retention time of approximately 37 minutes. When a mixture of 8-oxo-dGTP and all of the standard rNTPs and dNTPs was injected onto the column, 8-oxo-dGTP was the only nucleotide to show a discernible peak when the nucleotide mixture was analyzed by EC detection at + 900 mV. Therefore, this HPLC-ECD separation method made it possible to detect and quantitate 8-oxo-dGTP in the presence of an excess of each of the standard nucleoside triphosphates. The calibration curve for 8-oxo-dGTP measured by ECD is shown in Figure 5.13.

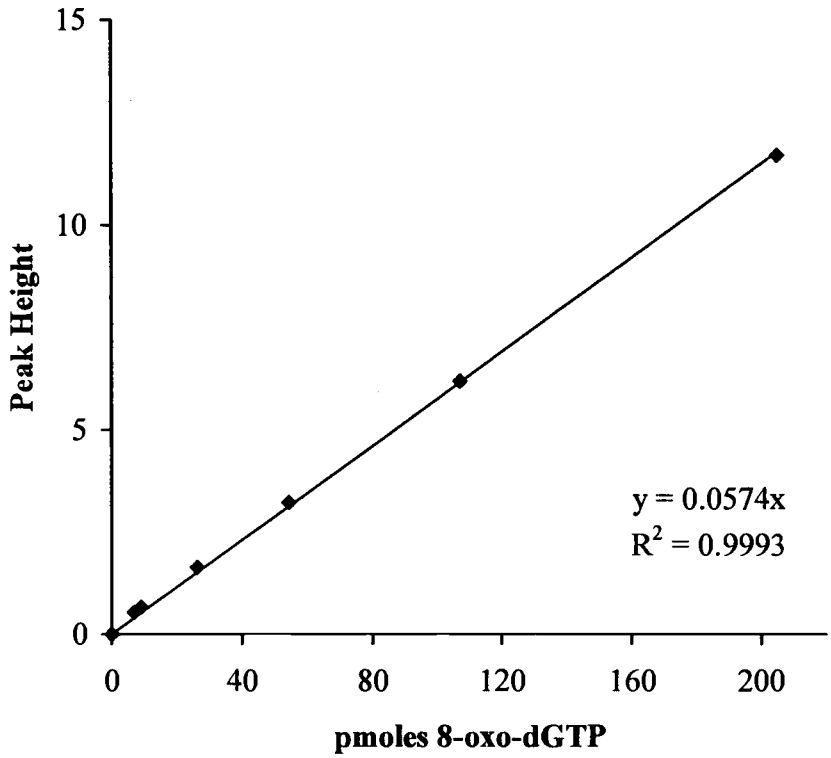


Figure 5.13 Calibration curve for 8-oxo-dGTP detected by ECD at + 900 mV.

5.2.3. HPLC-ECD Analysis of dNTP Pool Sizes from Bacterial Cultures – Initial Results

Initially, wild type *E. coli* (*E. coli* B) and strains lacking MutT activity (T-198, 58-278M, ES-1580) were grown under normal aerobic conditions, and their dNTPs were extracted for analysis by HPLC-ECD. In *mutT* mutant strains, the oxidized nucleotide, 8-oxo-dGTP was expected to accumulate; therefore, HPLC-ECD analysis of the dNTP pools in these strains provided a good starting point for these studies. The dNTP pool data from each of these strains are shown in Table 5.1. As expected, the dGTP pool was the smallest of the dNTP pools in each of the *E. coli* strains. However, the presence of 8-oxo-dGTP was not detected by ECD in any of the *E. coli* strains, including those that lacked MutT activity.

Table 5.1 dNTP pool sizes from *E. coli* grown under normal aerobic conditions. *E. coli* B is the wild type strain, and each of the other strains lacks a functional MutT enzyme. dNTP pool sizes are expressed as pmoles dNTP / 10^8 cells. The nucleotides extracted from approximately 10^9 cells were analyzed in each HPLC injection. dNTP pools were measured by UV absorbance at 267 nm, and 8-oxo-dTP was detected electrochemically at + 900 mV applied potential.

E. coli strain	dCTP	dGTP	dTTP	dATP	8-oxo-dGTP
E. coli B	7.8	4.6	18.0	13.7	-
T-198	13.9	5.5	18.5	13.4	-
58-278M	12.6	3.3	10.4	7.6	-
ES-1580	10.2	5.1	14.4	8.3	-

When the experiment was repeated, the size of the bacterial cultures was increased from 50 to 100 ml. Final dNTP extracts from these cultures were resuspended in the same volume (100 µl) that had been used in the preparation of the previous extracts, so that the dNTP concentrations in the second set of extracts would be greater. When the HPLC-ECD analysis of dNTP pools from these extracts was repeated, again no 8-oxo-dGTP was detected in any of the bacterial dNTP extracts. The results of dNTP pool measurements from the second set of bacterial dNTP extracts are shown in Table 5.2.

Table 5.2 dNTP pool sizes from *E. coli* grown under normal aerobic conditions. In this attempt to measure 8-oxo-dGTP pools in bacterial cells, the nucleotides extracted from approximately 10^{10} cells were analyzed in each HPLC injection. dNTP pool sizes are expressed as pmoles dNTP / 10^8 cells. dNTP pools were measured by UV absorbance at 267 nm, and 8-oxo-dGTP was detected electrochemically at + 900 mV applied potential.

E. coli strain	dCTP	dGTP	dTTP	dATP	8-oxo-dGTP
E. coli B	9.6	6.9	15.0	13.1	-
T-198	7.4	3.2	10.7	8.9	-
58-278M	15.4	2.4	14.9	11.9	-
ES-1580	13.1	6.8	19.8	9.4	-

The chromatograms generated by EC detection of nucleotides extracted from the *E. coli mutT* mutant strains, ES-1580, T-198, and 58-278M (Panel A, Figures 5.15-5.17), did show an electrochemically active compound eluting from the column at a retention time similar to that of 8-oxo-dGTP. This peak was not observed during EC detection of nucleotide extracts from *E. coli* B (see Figure 5.14, Panel A). This unknown compound eluted at a retention time of approximately 39 minutes, whereas an authentic standard of 8-oxo-dGTP eluted at a retention time of 37 minutes. To show that this peak did not represent 8-oxo-dGTP, an aliquot of each extract that had been spiked with 50 pmoles of authentic 8-oxo-dGTP was analyzed by HPLC-ECD. In the resulting chromatograms (see Figures 5.14-5.17, Panel B), two distinct peaks could be seen: the unknown electrochemically active compound that elutes at 39 minutes, and the added 8-oxo-dGTP that elutes at 37 minutes.

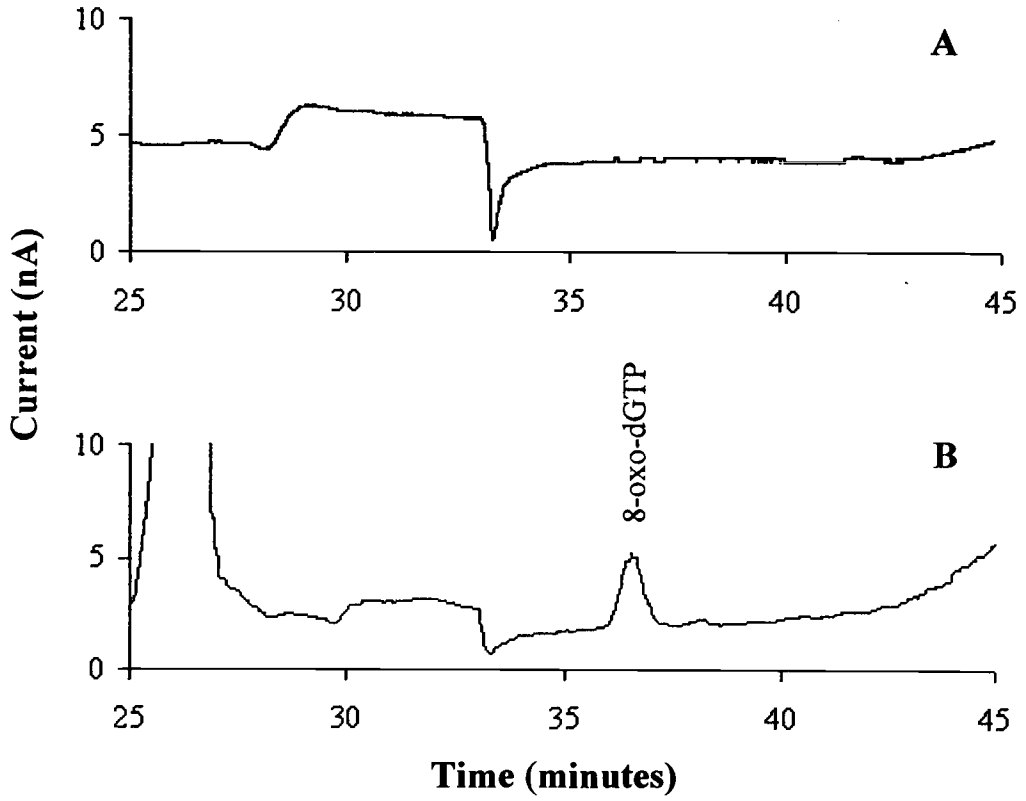


Figure 5.14 Analysis of nucleotide extracts from *E. coli* B by electrochemical detection.
Panel A shows the chromatogram generated by injecting 50 µL of the nucleotide extract and analyzing the nucleotides by electrochemical detection, and Panel B shows the chromatogram generated when an authentic 8-oxo-dGTP standard was added to the nucleotide extract. Each chromatogram represents the nucleotides extracted from 9.79 x 10^9 cells.

The results of these initial experiments to measure the 8-oxo-dGTP pool in *E. coli* were surprising because it is a widely accepted premise in the literature that the *E. coli* MutT enzyme functions to specifically degrade 8-oxo-dGTP to 8-oxo-dGMP, to eliminate the potentially mutagenic nucleotide from the nucleotide pool [11]. In the absence of MutT enzymatic activity, the pool size of 8-oxo-dGTP was expected to increase. The inability of the HPLC-ECD method to detect 8-oxo-dGTP from *E. coli* *mutT* mutant strains led to the question of whether or not pools of 8-oxo-dGTP were

being degraded during the extraction of nucleotides from cells, or if the 8-oxo-dGTP pool is lower than the lower limit of detection for this HPLC-ECD method.

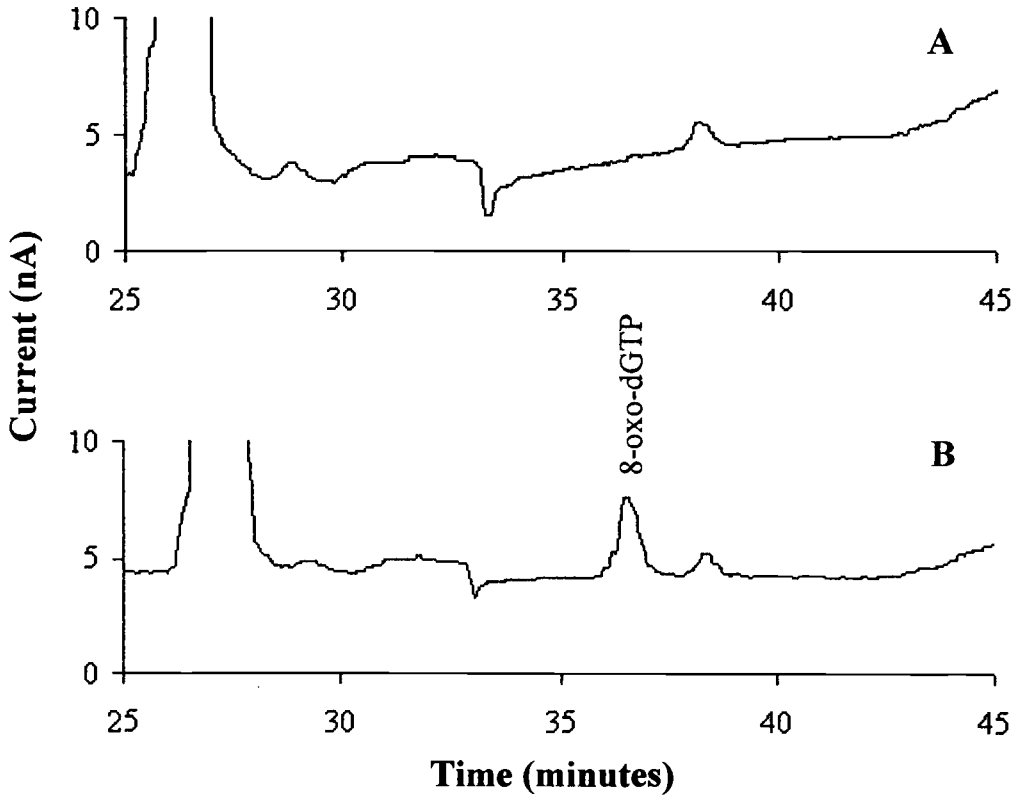


Figure 5.15 Analysis of nucleotide extracts from the *mutT E. coli* strain, T-198, by electrochemical detection.
Panel A shows the chromatogram generated by injecting 50 µl of the nucleotide extract and analyzing the nucleotides by electrochemical detection, and Panel B shows the chromatogram generated when an authentic 8-oxo-dGTP standard was added to the nucleotide extract. Each chromatogram represents the nucleotides extracted from 9.19 x 10^9 cells.

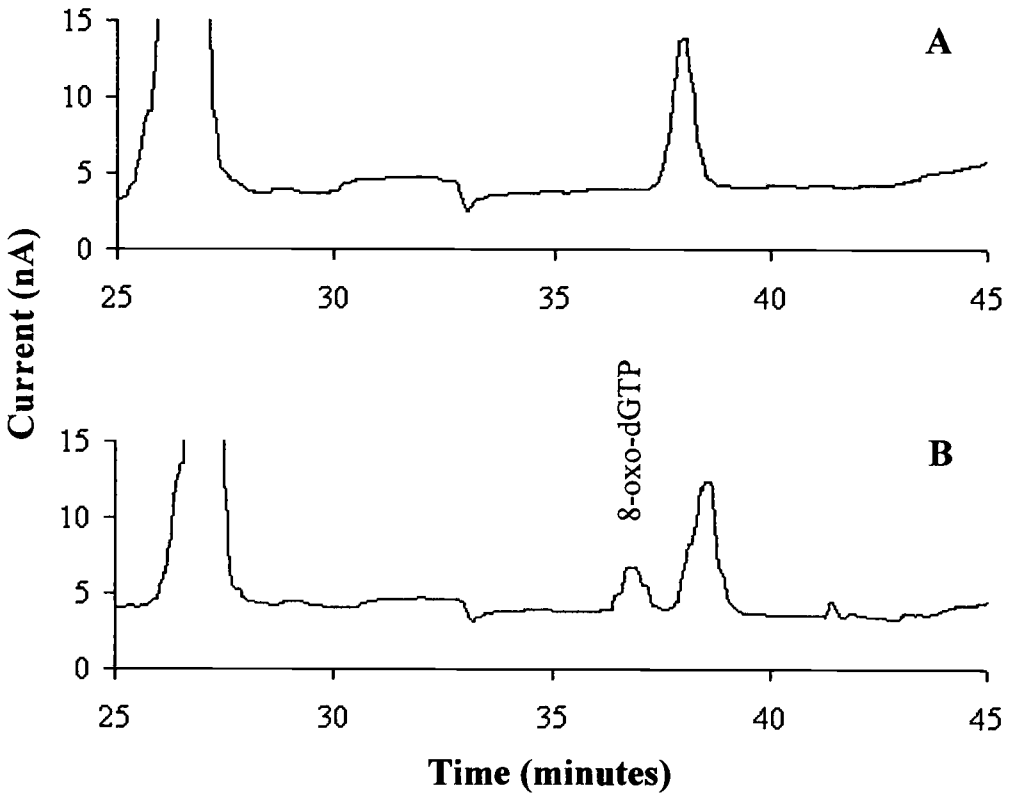


Figure 5.16 Analysis of nucleotide extracts from the *mutT E. coli* strain, 58-278M, by electrochemical detection.
Panel A shows the chromatogram generated by injecting 50 µl of the nucleotide extract and analyzing the nucleotides by electrochemical detection, and Panel B shows the chromatogram generated when an authentic 8-oxo-dGTP standard was added to the nucleotide extract. Each chromatogram represents the nucleotides extracted from 6.81 x 10^9 cells.

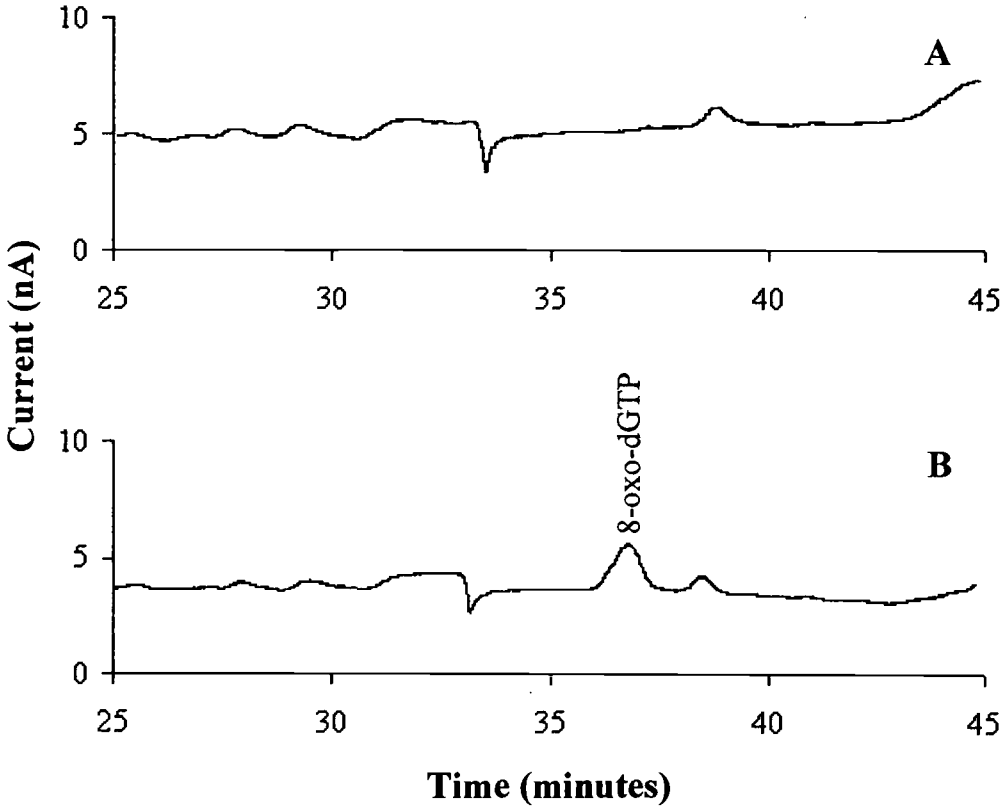


Figure 5.17 Analysis of nucleotide extracts from the *mutT E. coli* strain, ES-1580, by electrochemical detection.
Panel A shows the chromatogram generated by injecting 50 µl of the nucleotide extract and analyzing the nucleotides by electrochemical detection, and Panel B shows the chromatogram generated when an authentic 8-oxo-dGTP standard was added to the nucleotide extract. Each chromatogram represents the nucleotides extracted from 11.18 x 10^9 cells.

5.2.4. Calculation of the Lower Limit of Detection for 8-oxo-dGTP using HPLC-ECD

The lower limit of detection of 8-oxo-dGTP by UV absorbance at 267 nm and by EC detection at + 900 mV was determined by making serial dilutions of known concentration of 8-oxo-dGTP (λ_{max} = 246 nm, ε_{max} = 12.3 x 10^3) and injecting them onto the HPLC column. For UV detection, the lower limit of detection for 8-oxo-

dGTP was found to be approximately 50 pmoles. ECD is an order of magnitude more sensitive than UV for the detection of 8-oxo-dGTP, with the lower limit of detection by ECD being approximately 6 pmoles.

The lower limit of detection of 8-oxo-dGTP by the HPLC-ECD method (6 pmoles of 8-oxo-dGTP) was used to estimate the upper limit of the 8-oxo-dGTP pool in bacterial cells. Each *E. coli* dNTP extract was made from a culture of cells containing approximately 3×10^{10} cells, and one half of the entire extract was injected onto the HPLC column for analysis. Therefore, each injection represented the dNTPs extracted from approximately 1.5×10^{10} cells. Taking into account that 1 mole of 8-oxo-dGTP is 6.022137×10^{23} molecules, the HPLC-ECD assay could therefore detect as little as 240 molecules of 8-oxo-dGTP / cell. Since no 8-oxo-dGTP was detected by HPLC-ECD in the *E. coli* extracts, it was concluded that if 8-oxo-dGTP is stably present in the dNTP pools of *E. coli*, it is present at less than 240 molecules / cell.

To determine the intracellular concentration of 8-oxo-dGTP in *E. coli*, the dNTP pool measurements made by Mathews [134] were considered. In this publication, 7×10^4 molecules of dGTP / cell represented an intracellular concentration of 0.1 mM dGTP in *E. coli* B cells, based on a mean cell volume for *E. coli* B of about 0.9 μm^3. Accordingly, 240 molecules of 8-oxo-dGTP / cell is equivalent to an intracellular concentration of 0.34 μM. Therefore, based on the limit of detection of the HPLC-ECD method, the upper limit of the intracellular pool size of 8-oxo-dGTP in *E. coli* cells was calculated to be 0.34 μM.

5.2.5. Controls for the HPLC-ECD Assay of 8-oxo-dGTP Pools

The inability of the HPLC-ECD method to detect 8-oxo-dGTP from *E. coli* was surprising, since *mutT* mutant strains were expected to have high levels of the nucleotide. In order to determine if pools of 8-oxo-dGTP were being degraded during the extraction of nucleotides from cells, a number of control experiments was conducted. Desferal was added during the dNTP extractions to prevent oxidation of nucleotides [117], and its effects on the recovery of dNTPs was also evaluated. These

experiments were designed to test the recovery of dNTPs for a number of different dNTP extraction methods that have been published in the literature. The dNTP extraction methods that were tested are:

1. 500 µl of 0.4 M $HClO_4$, on ice for 20 minutes [135]
2. 2 ml of 10% TCA, on ice for 30 minutes [136]
3. 500 µl of 0.4 M $HClO_4$, on ice for 5 minutes [119]
4. 750 µl of 1.2 M $HClO_4$, on ice for 5 minutes [123]
5. 1 ml of 60% methanol + 1% toluene, -20°C for 1 hour, drying under vacuum, followed by the addition of 75 µl of ice-cold 5% TCA and immediate centrifugation for 30 minutes (this method is routinely used in the laboratory of C.K. Mathews for the extraction of dNTPs from *E. coli*).

A 45-µl sample containing a mix of 4,500 pmoles of the four normal dNTPs and 450 pmoles of 8-oxo-dGTP was added to each of the extraction conditions listed above. The extractions were set up in duplicate, and 0.1 mM desferal was added to one of the extractions. After carrying out the extractions, the samples were all neutralized by the addition of 1.5 volumes of TOA/Freon, dried under vacuum and resuspended in 45 µl of H_2O. Each of the "extracts" was then diluted 10-fold and analyzed by HPLC-ECD to determine the recovery of the dNTPs.

The results of these control experiments are shown in Table 5.3. Each dNTP extraction method tested gave incomplete recovery of the dNTPs. Method # 5 was the extraction method used previously in the initial attempts to measure pool sizes of 8-oxo-dGTP from bacterial cultures. Overall, this method (#5), which used methanol + toluene followed by a short extraction with 5% TCA, gave the highest percentage recovery for the dNTPs and 8-oxo-dGTP. The addition of 0.1 mM desferal to this extraction decreased the recovery of each of the nucleotides tested. Although there is some loss of 8-oxo-dGTP during the extraction procedure, the nucleotide is not completely degraded. Therefore, if the intracellular pool of 8-oxo-dGTP recovered

after cellular extraction is larger than the lower limit of detection for this HPLC-ECD method, it should be possible to quantitate levels of the nucleotide using this method.

Table 5.3 Percentage recovery of dNTPs for different extraction methods.
A known concentration of each of the four normal dNTPs and 8-oxo-dGTP was subjected to extraction by the methods listed in the text. The recovery of each dNTP was analyzed after extraction by the HPLC-ECD method. The effect of desferal on the recovery of nucleotides was evaluated by the addition of 0.1 mM desferal to each extraction.

Extraction Method	dCTP	dGTP	dTTP	dATP	8-oxo-dGTP
1	75	29	55	62	26
1 + desferal	64	23	25	56	34
2	63	23	39	44	0
2 + desferal	71	66	26	60	46
3	55	26	26	54	28
3 + desferal	55	24	51	53	25
4	63	19	22	37	26
4 + desferal	39	22	35	28	24
5	69	59	67	61	72
5 + desferal	60	58	60	51	64

5.2.6. EC Detection of Oxidized Nucleoside Triphosphates

The identity of the compound in the electrochemically active peak that eluted just after the retention time of 8-oxo-dGTP in *mutT* mutant *E. coli* strains was of interest, because this peak was absent when dNTP extracts of *E. coli* B were analyzed by HPLC-ECD. To determine if this peak represented an oxidized nucleoside triphosphate, standard nucleotides were treated with H_2O_2 and ascorbate as described in Chapter 4, and the reaction mixtures were analyzed by HPLC-ECD. The only

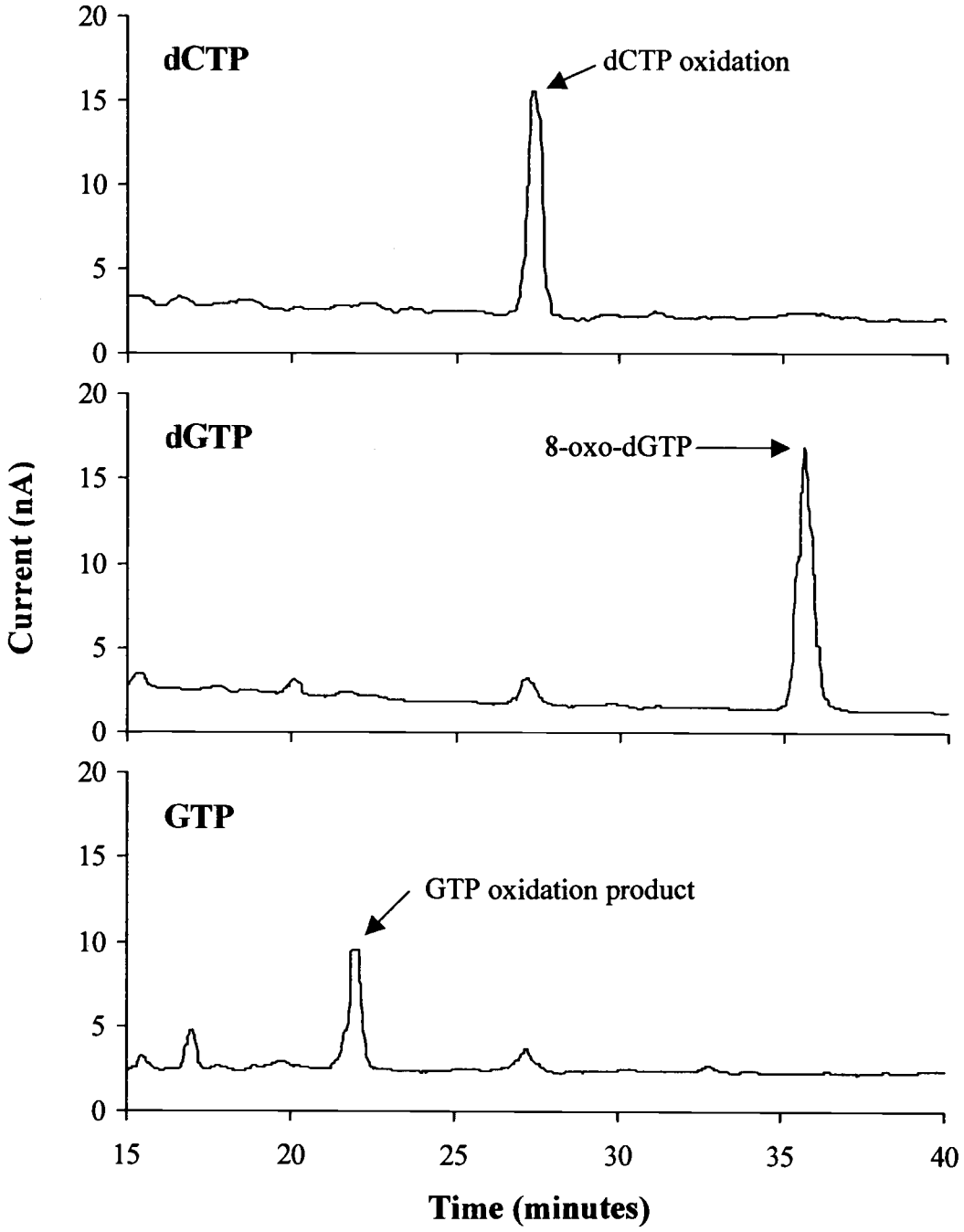


Figure 5.18 Electrochemical detection of oxidized nucleoside triphosphates. Each of the standard NTPs were oxidized by exposure to ascorbic acid and H_2O_2, as described in Chapter 4. The oxidation of dCTP, GTP and dGTP produced electrochemically active compounds that were detected at + 900 mV applied potential.

nucleotides that were measurable by EC detection at + 900 mV after this treatment were the oxidized forms of dCTP, GTP and dGTP. These chromatograms are shown in Figure 5.18. Oxidized dCTP was electrochemically active and eluted shortly after unoxidized dCTP at a retention time of 27 minutes. The GTP oxidation product eluted immediately before GTP at a retention time of approximately 22 minutes, and dGTP was oxidized to 8-oxo-dGTP under these conditions to yield a peak at the expected retention time of 37 minutes.

None of the NTPs oxidized by these conditions produced an electrochemically active peak at a retention time of 39 minutes. However, these results should not be interpreted to mean that the unidentified peak is not an oxidized derivative of one of the standard nucleoside triphosphates, since in this experiment only one oxidative condition was used. Reports in the literature have shown that dATP, dTTP, and dCTP can also be oxidized *in vitro* when exposed to iron and EDTA [126,127]. Oxidation of the nucleotides under these conditions (see Chapter 4 for details of the oxidation reactions) also failed to generate any electrochemically active compounds that could be detected at + 900 mV.

5.2.7. HPLC-ECD Analysis of dNTPs from Bacterial Cultures Exposed to Oxidative Stress

Growth of the *mutT* mutant strains of *E. coli* under normal aerobic conditions was expected to increase levels of 8-oxo-dGTP in the nucleotide pools. However, when dNTP pools from these cells were analyzed by HPLC-ECD, no 8-oxo-dGTP was detected. In an attempt to increase the pool of 8-oxo-dGTP so that it would be more easily detected by HPLC-ECD, wild type and *mutT E. coli* were grown in the presence of 2.5 mM H_2O_2 as described in Materials and Methods. The volume of the cultures used for this experiment was increased to 100 ml to increase the concentration of the nucleotides in the final dNTP extract. Desferal was omitted during the preparation of nucleotide extracts from these cultures, since previous experiments showed that the greatest recovery of 8-oxo-dGTP occurred in the absence of desferal (see Table 5.3).

The chromatograms generated from the electrochemical detection of nucleotides from bacterial cultures grown in the presence of H_2O_2 are shown in Figures 5.19 – 5.22. Many electrochemically active peaks are present in these chromatograms due to the omission of desferal during nucleotide extraction. No new electrochemically active compounds were detected in the extracts made from cells cultured in the presence of H_2O_2 (compare Panels A and B for each figure).

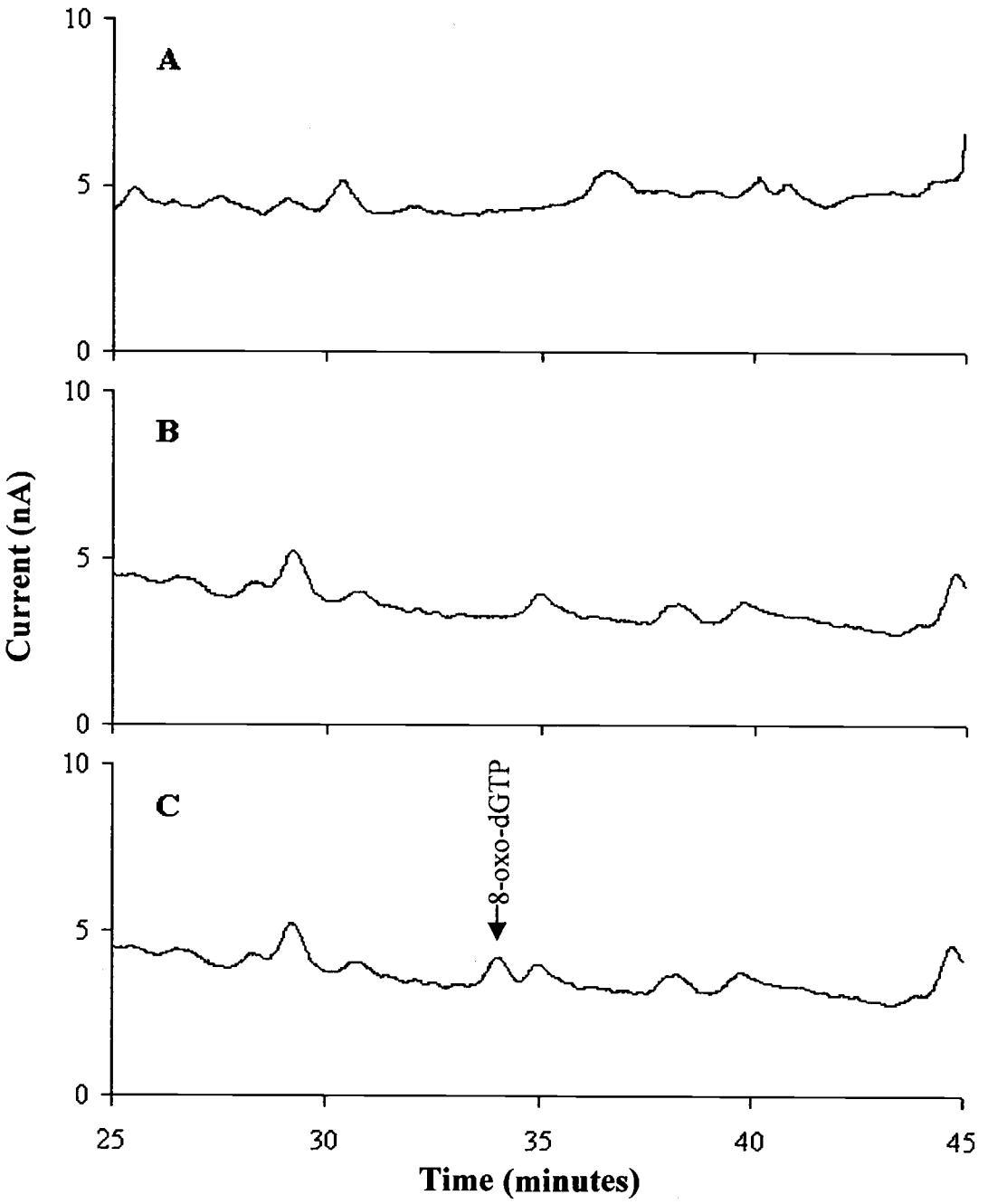


Figure 5.19 Analysis of nucleotides from *E. coli* B exposed to H_2O_2 by electrochemical detection at + 900 mV.
Bacteria were grown in the absence of H_2O_2 (Panel A), or in the presence (Panel B) of 2.5 mM H_2O_2. Panel C depicts an extract of nucleotides from *E. coli* B grown in H_2O_2 to which an authentic standard of 8-oxo-dGTP was added immediately prior to HPLC analysis.

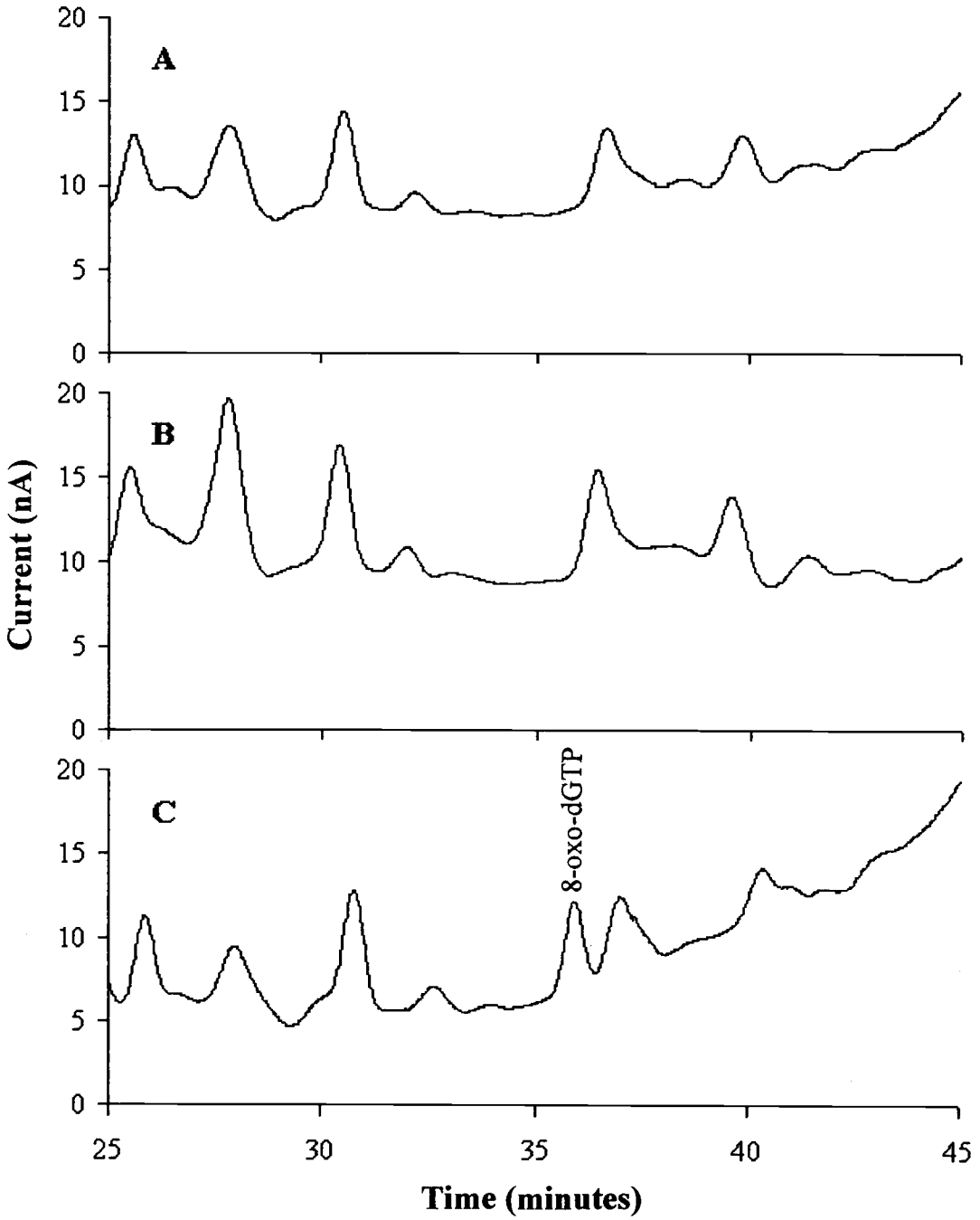


Figure 5.20 Analysis of nucleotides from the *mutT E. coli* strain, T-198, exposed to H_2O_2 by electrochemical detection at + 900 mV.
Bacteria were grown in the absence of H_2O_2 (Panel A), or in the presence (Panel B) of 2.5 mM H_2O_2. Panel C depicts an extract of nucleotides from T-198 grown in H_2O_2 to which an authentic standard of 8-oxo-dGTP was added immediately prior to HPLC analysis.

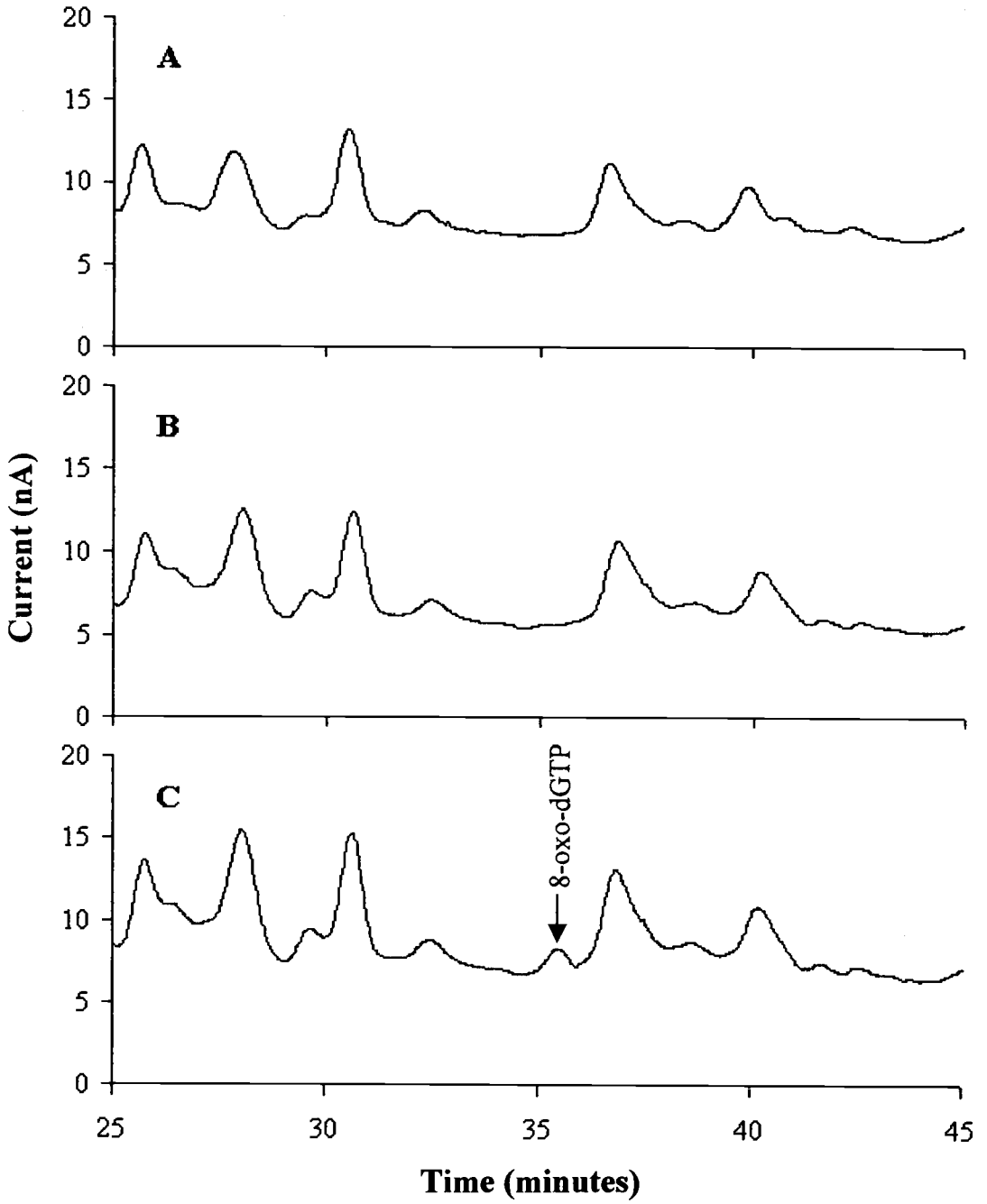


Figure 5.21 Analysis of nucleotides from the *mutT E. coli* strain, 58-278M, exposed to H_2O_2 by electrochemical detection at + 900 mV.
Bacteria were grown in the absence of H_2O_2 (Panel A), or in the presence (Panel B) of 2.5 mM H_2O_2. Panel C depicts an extract of nucleotides from 58-278M grown in H_2O_2 to which an authentic standard of 8-oxo-dGTP was added immediately prior to HPLC analysis.

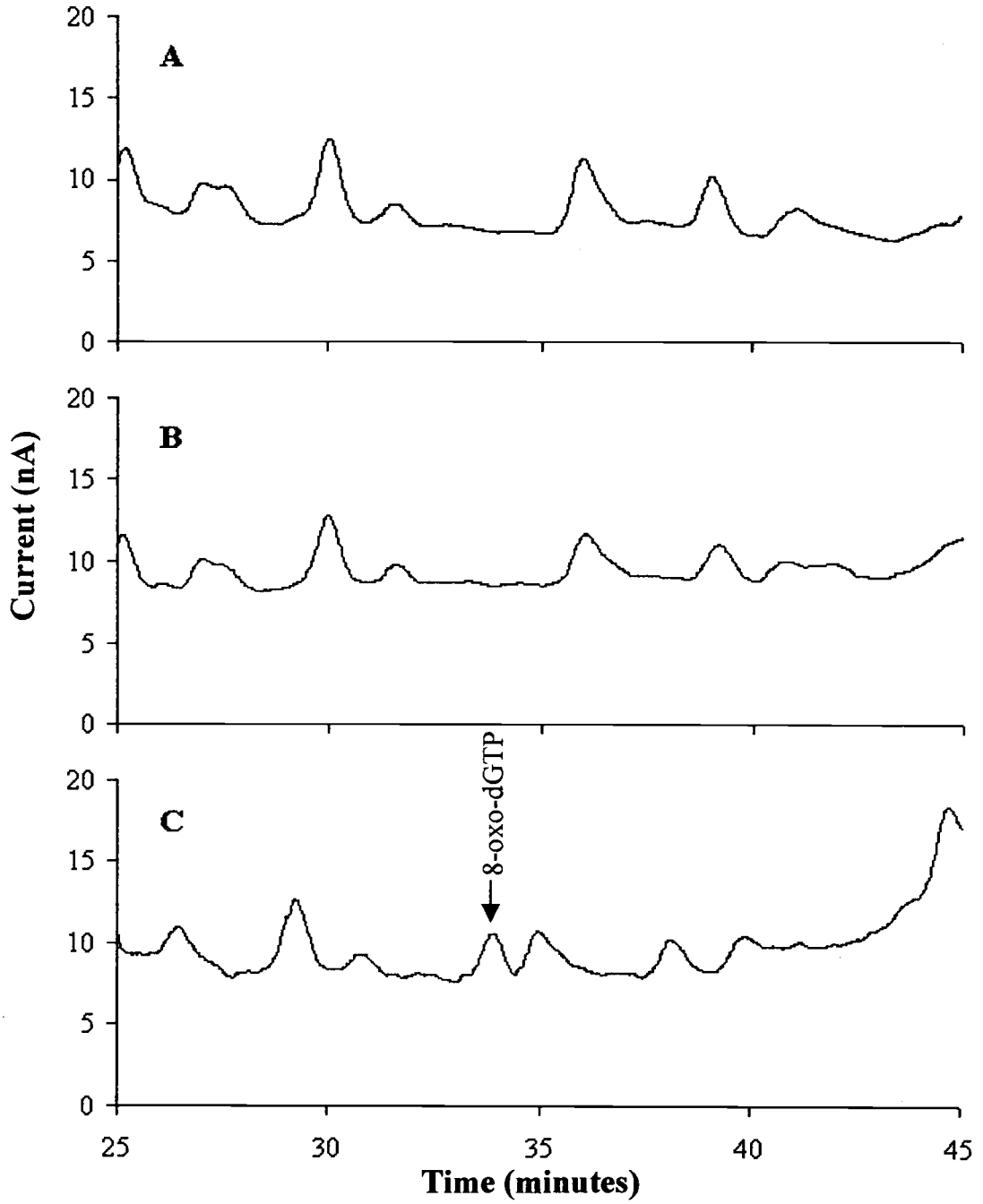


Figure 5.22 Analysis of nucleotides from the *mutT E. coli* strain, ES-1580, exposed to H_2O_2 by electrochemical detection at + 900 mV.
Bacteria were grown in the absence of H_2O_2 (Panel A), or in the presence (Panel B) of 2.5 mM H_2O_2. Panel C depicts an extract of nucleotides from ES-1580 grown in H_2O_2 to which an authentic standard of 8-oxo-dGTP was added immediately prior to HPLC analysis.

When comparing the chromatograms in Figures 5.19 – 5.22, it is clear that the retention time for 8-oxo-dGTP varies for each chromatographic run. During the course of these experiments, it was observed that the age of the column greatly affected the retention time of compounds. This made it necessary to make duplicate injections of the dNTP extracts, adding an internal standard of the 4 normal dNTPs and 8-oxo-dGTP to the second injection to verify the retention time for each nucleotide. Panel C of each figure shows the chromatogram generated when the extract from H_2O_2-exposed cells was mixed with an authentic standard of 8-oxo-dGTP and analyzed by HPLC-ECD. A comparison of Panels B and C for each figure shows that 8-oxo-dGTP was not detected in any of the bacterial extracts.

5.2.8. Bacterial Mutagenesis

Prior to extracting the nucleotides from the bacterial cells in the experiment described above, the H_2O_2 level in the culture medium was measured by the horseradish peroxidase method described in Chapter 3. For each strain, very low levels of H_2O_2 were measured in the cultures after a 15-minute exposure to 2.5 mM H_2O_2, indicating that cells are able to rapidly decompose H_2O_2. Hydrogen peroxide is not very reactive when present in culture medium, and it is known to be short-lived. However, its breakdown generates the very reactive hydroxyl radical ($OH\cdot$), which can damage all classes of cellular macromolecules. In order to show that the conditions used in these experiments were sufficient to generate oxidative stress, the level of oxidative mutagenesis in these cultures was assessed.

By plating the cultures on rifampicin-containing media and measuring the number of rifampicin-resistant colonies, the ability of this H_2O_2 exposure to generate rifampicin-resistant mutants was monitored. The target of the antibiotic rifampicin is the β subunit of RNA polymerase, and resistant mutants possess an altered β subunit due to mutations in the *rpoB* gene that are usually base substitutions [137]. Thus, monitoring the level of rifampicin-resistant mutagenesis is a relevant assay for the class of mutational events that are induced by the incorporation of 8-oxo-dGTP into

DNA. The results of this experiment (see Table 5.4) show that exposure to H_2O_2 under these experimental conditions does increase the number of rifampicin-resistant mutants, and that the mutant fraction is higher in *mutT* strains as compared to wild type *E. coli*, regardless of H_2O_2 exposure.

Table 5.4 Evaluation of the mutagenic effect of H_2O_2 on bacterial cultures. *E. coli* strains grown in the presence (+) or absence (-) of 2.5 mM H_2O_2 were plated in the presence of rifampicin, and the number of rifampicin-resistant colonies was counted.

***E. coli* strain**	**relevant genotype**	**H_2O_2 exposure**	**H_2O_2 (μM)**	**cells plated (x 10^8)**	**Rif^R colonies**	**Rif^R colonies per 10^8 cells**
E. coli B	wild type	-	0	0.253	3	11.9
E. coli B	wild type	+	112	0.297	13	43.8
T-198	*mutT*	-	0	0.315	36	114.3
T-198	*mutT*	+	88	0.315	80	254.0
58-278M	*mutT*	-	0	0.750	60	80.0
58-278M	*mutT*	+	27	0.700	500	714.3
ES-1580	*mutT*	-	0	0.815	316	387.7
ES-1580	*mutT*	+	38	0.670	613	914.9

5.3. 8-oxo-dGTPase Assay

The 8-oxo-dGTPase activity assay, first described by Bialkowski and Kasprzak [114], was used to test the hypothesis that cells growing under conditions of oxidative stress are expected to have higher levels of 8-oxo-dGTP and a higher rate of turnover of 8-oxo-dGTP by the activity of the MutT enzyme. Due to the very small dNTP pool sizes in mammalian cells and the limit of detection for the HPLC-ECD method developed to measure 8-oxo-dGTP pools, this assay could not be used to measure the 8-oxo-dGTP pool in mammalian or bacterial cells. Instead, the 8-oxo-dGTPase

activity assay was employed to look at the rate of turnover of 8-oxo-dGTP in mammalian cells.

5.3.1. Purification of 8-oxo-dGMP

Although a chromatographic standard of pure 8-oxo-dGTP was commercially available, it was necessary to synthesize a chromatographic standard of 8-oxo-dGMP for use in the assay of 8-oxo-dGTPase activity. The oxidation of dGMP with ascorbic acid and H_2O_2 yielded two main UV-absorbing peaks, as shown in Figure 5.23. dGMP that had not undergone oxidation eluted in the gradient at approximately 87 mM TEAB. As observed previously by Bialkowski and Kasprzak [114], 8-oxo-dGMP eluted as the last UV-absorbing peak in the chromatographed mixture. The elution of

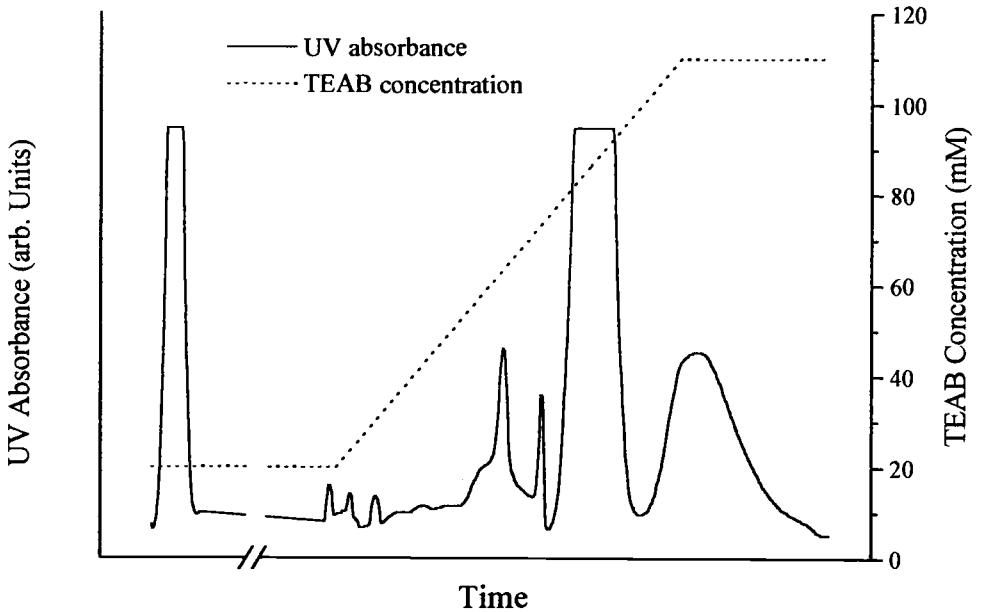


Figure 5.23 Purification of 8-oxo-dGMP.
A chromatographic standard of 8-oxo-dGMP was prepared by oxidation of dGMP with H_2O_2 and ascorbic acid, and purified by anion exchange chromatography on a DEAE-sephadex A-25 column with a TEAB gradient. The reaction mixture was loaded on the column which was equilibrated with 20 mM TEAB (pH 8.5) and eluted with a gradient of 20-110 mM TEAB (pH 8.5).

8-oxo-dGMP began at a point near the end of the gradient that corresponded to an approximate concentration of 103 mM TEAB. The remainder of the 8-oxo-dGMP was eluted from the column isocratically at a concentration of 110 mM TEAB, resulting in a broad peak in the chromatographic profile. Fractions of 8-oxo-dGMP were collected and pooled, and after further purification the final yield of 8-oxo-dGMP synthesized was calculated to be 1.6%.

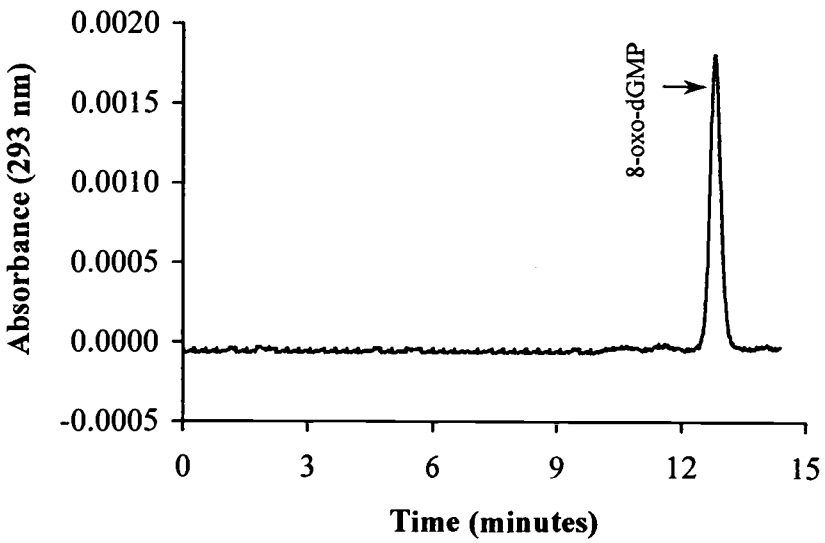


Figure 5.24 Purity check of the synthesized 8-oxo-dGMP.
The final preparation of 8-oxo-dGMP (175 pmoles) was separated by HPLC using a reverse phase column as described. The nucleotide was detected by UV absorbance at 293 nm and had an approximate retention time of 12.8 minutes.

5.3.2. Separation of 8-oxo-dGTPase Reaction Products by HPLC

The purity of the synthesized 8-oxo-dGMP was shown by HPLC analysis (see Figure 5.24). Synthesized 8-oxo-dGMP eluted from the reverse-phase HPLC column as a single peak at a retention time of approximately 12.8 minutes. Figure 5.25 shows that the elution order of the compounds on the reverse phase column depends on the number of phosphate residues in the nucleotide, with 8-oxo-dGTP eluting first

(retention time = 6.2 minutes) and the monophosphate, 8-oxo-dGMP, eluting last. The lower limit of detection of 8-oxo-dGMP was 0.58 μM, which corresponds to a concentration of 29 pmoles of 8-oxo-dGMP formed in a 50 μl reaction mixture. As shown in Figure 5.26, there was a linear relationship between 8-oxo-dGMP or 8-oxo-dGTP concentration and absorbance at 293 nm, making the assay of 8-oxo-dGTPase activity quantitative.

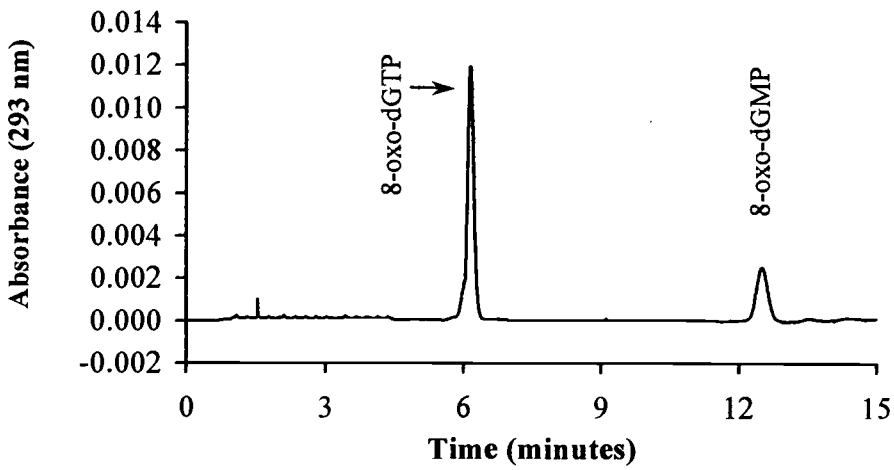


Figure 5.25 Elution of nucleotide standards from the reverse phase HPLC column used for the 8-oxo-dGTPase activity assay.
8-oxo-dGTP elutes at a retention time of approximately 6.2 minutes, and 8-oxo-dGMP elutes at a retention time of approximately 12.8 minutes.

5.3.3. 8-oxo-dGTPase Activity Assay for Mammalian Cells

Bialkowski and Kasprzak first developed the 8-oxo-dGTPase activity assay for cultured Chinese hamster ovary (CHO) cells [114],[138], and they also used the method to measure 8-oxo-dGTPase activity in rat testis [139], and in fetal and maternal tissue from Swiss mice [140]. In their experiments, Bialkowski and Kasprzak typically used 1-5 x 10^7 cultured mammalian CHO cells per extract, and their reaction mixtures contained a volume of 10 μl of ultrafiltrate obtained from a cell extract containing 0.5-3 mg protein/ml, for a final protein concentration in the reaction

mixture of 132 μg/ml. Using these reaction conditions, they were able to measure the baseline activity of the 8-oxo-dGTPase in CHO cells to be approximately 1200 U/mg protein.

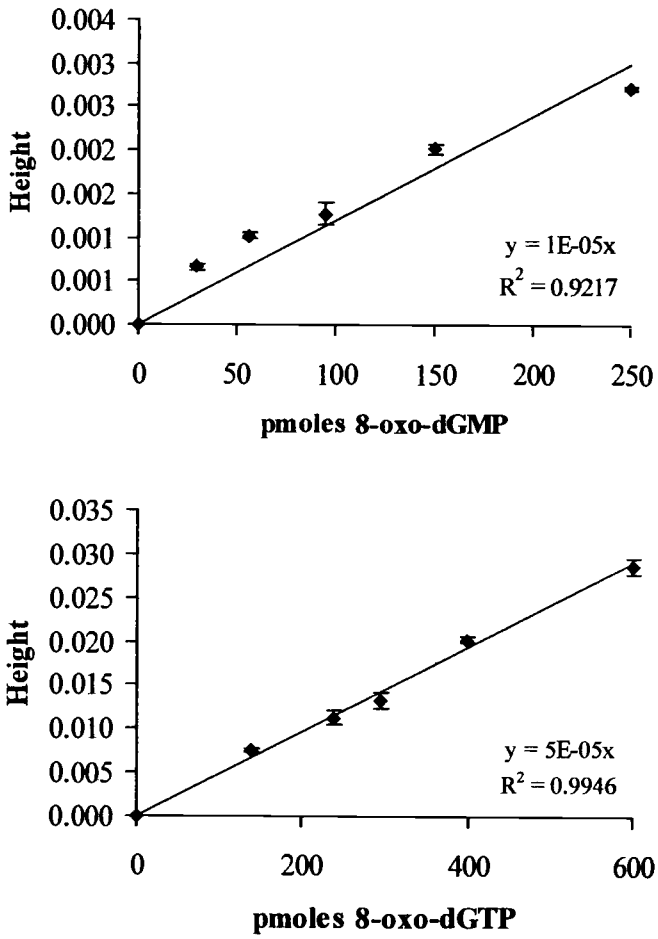


Figure 5.26 Calibration curves for 8-oxo-dGMP and 8-oxo-dGTP.
There is a linear relationship between concentration and peak height or peak area for the nucleotides being measured in the 8-oxo-dGTPase activity assay.

In order to measure the 8-oxo-dGTPase activity of V79 cells (Chinese hamster lung cell line), cells were grown at 20% oxygen and in a 2% oxygen atmosphere, and cell ultrafiltrates were prepared exactly as described by Bialkowski and Kasprzak [114]. On separate occasions, three sets (2% O_2 and 20% O_2) of cell ultrafiltrates from V79 cells were prepared from cell extracts with protein concentrations ranging from 0.3 – 3.7 mg/ml. In each case, no 8-oxo-dGTPase activity was measurable in any of the ultrafiltrates prepared from V79 cell extracts. Mammalian homologs of the MutT enzyme have a molecular weight of 18 kDa [38,39,141], and the enzyme would therefore be isolated in the cell ultrafiltrate by its ability to pass through the 30 kDa ultrafiltration membrane. There was measurable phosphatase activity in the whole cell extracts from V79 cells (see Figure 5.27). With increasing assay time, the 8-oxo-dGTP peak (retention time 6.2 minutes) decreased in size, and two new peaks were seen in the chromatograms. One peak corresponded to the nucleotide 8-oxo-dGMP, and was seen at a retention time of 12.8 minutes. The other peak was present at an intermediate time of 8.4 minutes, and was presumed to be the nucleoside diphosphate, 8-oxo-dGDP, based on observations of the retention time for 8-oxo-dGDP made by Bialkowski and Kasprzak [114]. The enzymatic activity measured in the cell extracts is most likely due to the consumption of 8-oxo-dGTP by unspecific phosphatase activity in the V79 extracts, which is indicated by the dephosphorylation of 8-oxo-dGTP to 8-oxo-dGDP and finally to 8-oxo-dGMP, rather than the activity of an 8-oxo-dGTP-specific pyrophosphohydrolase. Similar interfering phosphatase activity was seen by Bialkowski and Kasprzak when the assay was conducted using extract prepared from CHO cells, whereas an ultrafiltrate obtained from the CHO cell extract hydrolyzed 8-oxo-dGTP exclusively to 8-oxo-dGMP [114].

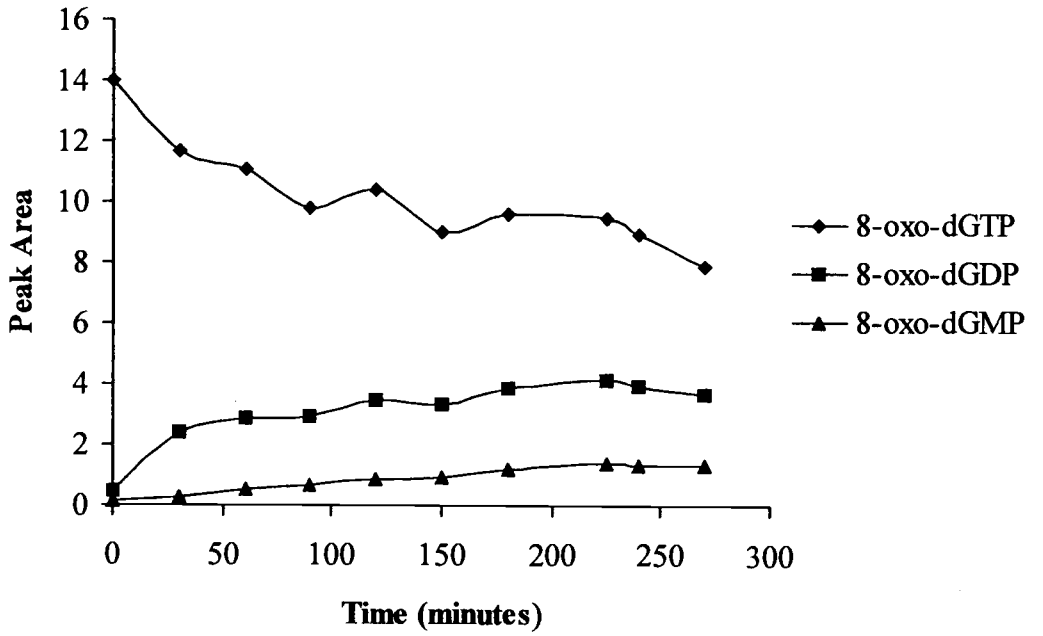


Figure 5.27 Time course of dephosphorylation of 8-oxo-dGTP by V79 cell extract. Extracts from V79 cells grown at 20% oxygen (50 µl) were mixed with 5 mM $MgCl_2$, 100 mM tris-Cl (pH 8.5), and 40 µM 8-oxo-dGTP in a total volume of 600 µl. Aliquots of 50 µl were chromatographed every 30 minutes. No 8-oxo-dGDP chromatographic standard was available for calibration, so the data are represented by peak area.

Unlike CHO cells, V79 cells had no measurable 8-oxo-dGTPase activity that could be contributed to the enzymatic activity of a MutT-like enzyme. Under conditions of oxidative stress, when V79 cells were grown in the presence of 5 mM H_2O_2 or in the presence of 5 mM H_2O_2 and 1.2 mM $FeSO_4$, there was still no measurable 8-oxo-dGTPase activity in ultrafiltrates prepared from cell extracts. If dGTP is being oxidized in the presence of reactive oxygen species to form 8-oxo-dGTP, and if this oxidized nucleotide is a substrate for the mammalian MutT homolog, then increased levels of 8-oxo-dGTPase activity might be expected under conditions of oxidative stress. Protein concentrations of the extracts used to prepare the ultrafiltrates used in these experiments were in the range used by Bialkowski and

Kasprzak for CHO cells, so the results of these experiments could be indicative of a very low concentration of the protein in this cell line.

5.3.4. 8-oxo-dGTPase Activity Assay for E. coli Cells

As shown in Figure 5.28, the 8-oxo-dGTPase activity assay confirmed the phenotype of the *E. coli* strains lacking a functional MutT enzyme. Ultrafiltrates prepared from each of the *mutT* mutant strains were unable to convert 8-oxo-dGTP to 8-oxo-dGMP, as measured by the 8-oxo-dGTPase activity assay, regardless of the oxygen conditions that they were grown in. In wild type *E. coli*, the 8-oxo-dGTPase activity was measured to be 56.4 ± 5.2 U/mg protein. When the cells were grown in the presence of 5 mM H_2O_2, the 8-oxo-dGTPase activity increased slightly to 69.0 ± 10.1 U/mg protein. The 8-oxo-dGTPase activity of wild type *E. coli* that were grown anaerobically was significantly lower than that measured in aerobic cultures, indicating that the transcription of the *mutT* gene may be regulated by oxygen conditions, so that under low oxygen conditions when there is little or no oxidation of dGTP to 8-oxo-dGTP, levels of the enzyme would decrease.

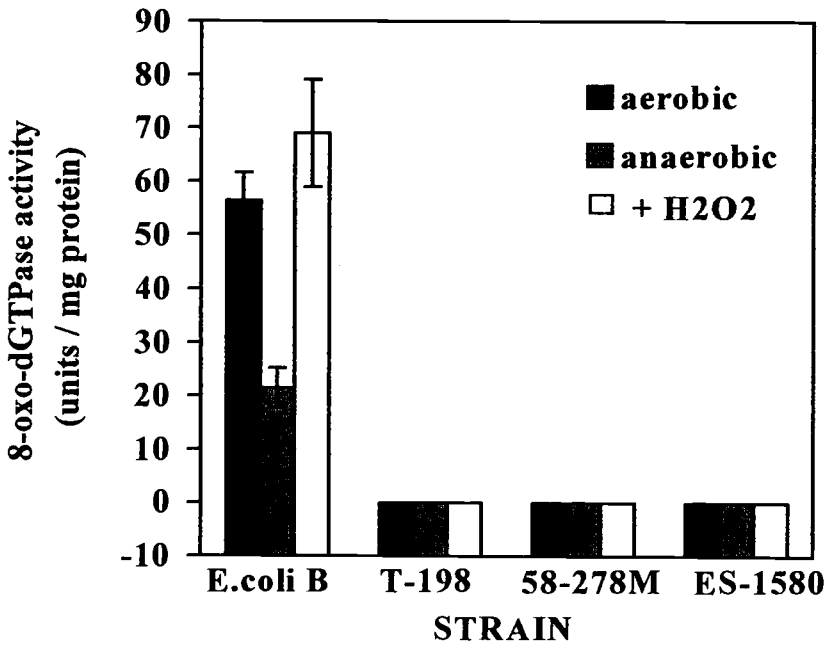


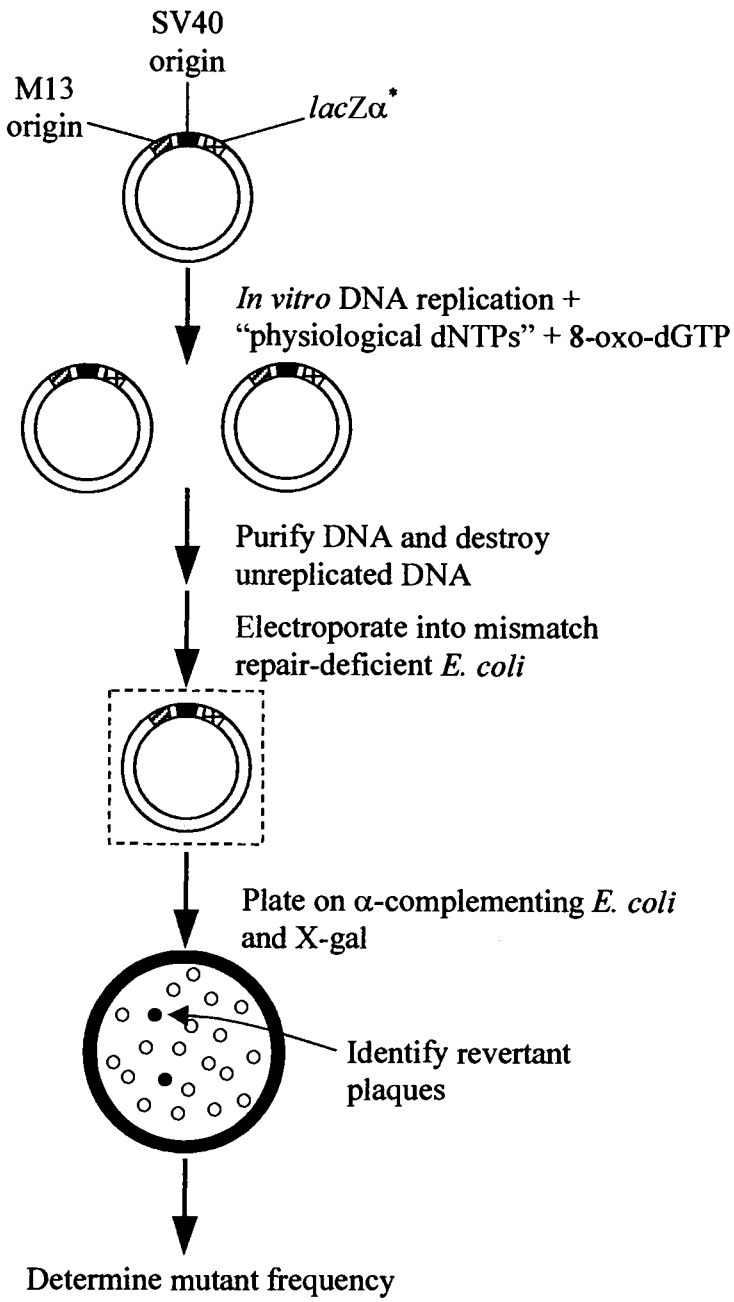
Figure 5.28 8-oxo-dGTPase activity in wild type and *mutT* mutant *E. coli* strains. One activity unit is defined as the amount of enzyme converting 1 pmol of 8-oxo-dGTP to 8-oxo-dGMP per minute. Error bars represent the standard error for three independent cultures treated identically.

5.4. *In vitro* DNA Replication with 8-oxo-dGTP

The experimental approach used to measure the fidelity of DNA replication in the presence of 8-oxo-dGTP is shown in Figure 5.29. The template DNA, M13mp2SV/opal-7, contains an opal (TGA) codon at position 7 of the *lacZα* peptide [5], which generates colorless plaques in the replication fidelity assay if no reversion in the sequence of the opal codon has occurred. This template DNA does not produce a functional α-peptide, and as a result no Xgal is utilized when the DNA is plated with the α -complementation host cells.

Figure 5.29 Experimental outline for the DNA replication fidelity assays.
The M13mp2SV/opal-7 DNA molecule is replicated *in vitro* in the presence of HeLa S3 cytoplasmic extract and SV40 T antigen or purified Klenow (*exo*$^-$) polymerase, physiological concentrations of dNTPs and varying concentrations of 8-oxo-dGTP. Replicated DNA molecules are purified and treated with *Dpn*I to destroy any unreplicated template molecules. The replicated DNA is transfected into electrocompetent mismatch repair-deficient *E. coli*, and the electroporated cells are plated on a lawn of the α-complementation *E. coli* strain. In the reversion assay, the original DNA generates colorless plaques, and the revertants are seen as blue plaques when the cells are plated in the presence of the chromogenic substrate, Xgal.

* containing TGA (opal) codon


SV40
origin
M13
origin
lacZα*
In vitro DNA replication +
"physiological dNTPs" + 8-oxo-dGTP
Purify DNA and destroy
unreplicated DNA
Electroporate into mismatch
repair-deficient E. coli
Plate on α-complementing E. coli
and X-gal
Identify revertant
plaques
Determine mutant frequency

However, when the M13mp2SV/opal-7 template DNA is replicated in the presence of 8-oxo-dGTP, single base substitutions can occur at the TGA codon sequence. If the sequence of the M13mp2SV/opal-7 DNA reverts back to wild type or psuedo-wild type sequence, a functional α -peptide is produced, which then complements the β-galactosidase activity of the α -complementation host cells, Xgal is hydrolyzed, and dark blue M13 plaques are formed. This reversion assay is highly sensitive due to the low background reversion frequency (~$1x10^{-5}$) [115].

The opal codon reversion assay was used to determine the mutagenicity of 8-oxo-dGTP at the concentration determined to be the upper limit present in *E. coli* (0.34 μM). In addition, each of the four dNTPs that are normally present during DNA replication were present at their physiological concentrations found in HeLa cell nuclei (60 μM dATP, 60 μM dTTP, 30 μM dCTP, and 10 μM dGTP) [20]. *In vitro* DNA replication reactions were conducted with purified Klenow (*exo*$^-$) polymerase and with HeLa (S3) cell cytoplasmic extract. The results of the reversion assays are shown in Tables 5.5 and 5.6.

Table 5.5 Results of *in vitro* DNA Replication Assays using purified Klenow (*exo*$^-$) polymerase.

8-oxo-dGTP (μM)	**total plaques counted**	**revertants**	**reversion frequency ($x10^{-4}$)**
0	179,200	48	2.7
0.34	170,400	60	3.5
1	160,800	63	4.0
3.4	540	-	-
10	175,840	196	11.1

Table 5.6 Results of *in vitro* DNA Replication Assays with HeLa (S3) cell cytoplasmic extract.
"Background" refers to the results seen when the replication reaction was completed in the absence of T antigen.

8-oxo-dGTP (μM)	**total plaques counted**	**revertants**	**reversion frequency ($x10^{-5}$)**
0	160,000	6	3.8
0.34	190,200	7	3.7
1	239,400	10	4.2
3.4	313,000	15	4.8
10	198,600	9	4.5
background	726,200	23	3.2

When replication reactions were performed using Klenow (*exo*$^-$) polymerase, the background reversion frequency was 10-fold higher than replication reactions completed with cytoplasmic extract from HeLa S3 cells. This elevated reversion frequency has been observed previously [15] in similar experiments using the purified polymerase, which lacks exonucleolytic proofreading activity. As shown in Table 5.5, when 8-oxo-dGTP was added to the replication reaction at the "physiological" concentration of 0.34 μM, there was a slight increase in the reversion frequency over background, and the reversion frequency increased further when the concentration of 8-oxo-dGTP in the replication reaction reached 1 μM. The DNA collected from the replication reaction containing 3.4 μM 8-oxo-dGTP was not successfully transfected into the NR9162 competent cells, so no reversion data were obtained for this concentration of 8-oxo-dGTP. However, at a concentration of 10 μM 8-oxo-dGTP, the reversion frequency was 4-fold higher than the background reversion frequency. These data show that the presence of 8-oxo-dGTP during replication of the M13mp2SV/opal-7 DNA by the Klenow fragment of *E. coli* DNA polymerase I

caused an increase in the reversion frequency that is proportional to the concentration of 8-oxo-dGTP in the replication reaction.

Table 5.6 shows reversion data for a similar experiment using replication competent cytoplasmic HeLa S3 extract rather than purified polymerase. The goal of these *in vitro* DNA replication experiments was to determine the mutagenic potential of 8-oxo-dGTP under physiological conditions, making the experiment conducted using HeLa cell extract a much more physiologically relevant experiment than that conducted using purified exonuclease-deficient polymerase. When background reversion frequencies for the two reactions are compared in Tables 5.5 and 5.6, it is clear that replication by the HeLa S3 cell cytoplasmic extract is much more accurate, with a 10-fold lower background reversion frequency. When replication of the M13mp2SV/opal-7 DNA was completed in the presence of 0.34 μM 8-oxo-dGTP, the upper limit of the intracellular concentration that is expected to be present under physiological conditions, the mutant fraction was indistinguishable from that seen with an otherwise identical replication mixture containing no 8-oxo-dGTP. There was a slight increase in the reversion frequency in the presence of 8-oxo-dGTP at higher concentrations (1-10 μM); however, because the numbers of mutant plaques counted were quite small, these increases in the mutant fraction are likely not statistically significant.

These results suggest that 8-oxo-dGTP present during replication is not significantly mutagenic at its calculated highest possible intracellular concentration in *E. coli*, 0.34 μM, when replication is completed in a cell extract that contains the proteins necessary for breakdown of the oxidized nucleotide or removal of the oxidized base from DNA. These experiments were conducted in the presence of dGTP at its estimated concentration in HeLa cell nuclei, 10 μM. However, in *E. coli* the estimated concentration of dGTP is 100 μM, 10-fold higher [134]. Therefore, if 0.34 μM 8-oxo-dGTP is not detectably mutagenic in the presence of 10 μM dGTP, it is unlikely to cause mutagenesis in the presence of dGTP at a concentration of 100 μM, as found in *E. coli*.

Chapter 6. Conclusions

6.1. Oxygen Effects on Mammalian dNTP Pools

In the dNTP pools of mammalian cells, dGTP is consistently underrepresented. It often accounts for less than 5% of the total dNTPs in the cell (see Table 1.1), which is of interest considering that the dNTP pool sizes might be expected to reflect the relative abundance of each of the four deoxyribonucleotides in DNA. The hypothesis that the underrepresentation of dGTP is due to its oxidation to 8-oxo-dGTP and subsequent turnover was tested by culturing mammalian cells in varying oxygen conditions and measuring the dNTP pools to correlate the intracellular pool sizes with oxygen tension. If the dGTP pool were a target for the damaging effects of reactive oxygen species, then one would expect the pool to expand under low oxygen conditions.

The results presented in this dissertation do not support the above-mentioned hypothesis. HeLa cells grown in 2% and 20% oxygen showed no difference in the levels of any of the four dNTPs utilized for DNA synthesis (see Figure 5.1). HeLa cells, being a tumor-derived cell line, are thought to rely heavily on anaerobic metabolism. So, the experiments were repeated in a non-tumor-derived cell line: V79, derived from Chinese Hamster lung tissue. dGTP pool measurements from an asynchronous culture of these cells did show changes with varying oxygen, as the pool of dGTP doubled when the cells were grown in 2% oxygen as compared to the pool measured from cells grown at 20% oxygen. This same result was observed in a synchronized S-phase population of V79 cells; however, the oxygen-dependent pool changes were not limited to the dGTP pool (see Figure 5.7). The dGTP, dATP and dTTP pools all increased when cells were grown in low oxygen, while the dCTP pool decreased under low oxygen conditions. These results indicate that growth in low oxygen does not specifically cause the dGTP pool to expand. Although these results suggest that there may be increased turnover of the dGTP pool under high oxygen

conditions, the reason for this oxygen-dependent change in the dGTP pool was not addressed by these experiments.

The observation that the dCTP pool decreased when cells were grown in low oxygen was of interest because similar changes in the dNTP pools had previously been reported in the literature in Ehrlich ascites cells cultured in a 0.02% oxygen atmosphere [142]. This oxygen-dependent change in the dCTP pool was suggested to be the cause of specific inhibition of DNA replication in hypoxic cells, and the authors hypothesized that under hypoxic conditions the activity of ribonucleotide reductase is affected so that the dCTP pool is specifically lowered. The hypothesis that oxygen limitation affects the substrate specificity of ribonucleotide reductase was tested to determine whether this was the basis for the observed decline in the dCTP pool [143]. The results of these experiments showed that CDP reduction is not specifically sensitive to oxygen depletion, but GDP reduction is specifically sensitive. Cells growing *in situ* are exposed to low oxygen tensions, comparable to those used in the above-mentioned experiments with ribonucleotide reductase. Therefore, intracellular oxygen limitation may cause the observed underrepresentation of dGTP in mammalian cells by inhibiting the reduction of GDP. However, this explanation for the underrepresentation of dGTP is difficult to resolve when one considers that the same underrepresentation of dGTP with respect to the other dNTP pools is seen in mammalian cell cultures grown at ambient (approximately 20%) oxygen.

6.2. HPLC-ECD Method for Detection of 8-oxo-dGTP

Attempts to directly measure the turnover of the dGTP pool in mammalian cells were unsuccessful (see Appendix). Also, it was difficult to correlate the underrepresentation of dGTP with increased dGTP turnover under high oxygen conditions using dNTP pool measurements, since these measurements do not give any information regarding the dynamic nature of dNTP turnover. In order to determine whether the dGTP pool is low because of its oxidation by reactive oxygen species to

8-oxo-dGTP, followed by its turnover (see Figure 1.3), it was of interest to directly measure the intracellular pool of 8-oxo-dGTP.

8-oxo-dGTP is thought to be highly mutagenic, because the nucleotide is able to be misincorporated across from template adenine during DNA replication *in vitro*, which leads to A·T→C·G transversions (see Figure 2.5). The occurrence in evolutionarily distant organisms of an enzyme that degrades 8-oxo-dGTP to 8-oxo-dGMP to eliminate the potentially mutagenic substrate from the nucleotide pool available for DNA synthesis, and the 10^2- to 10^4-fold increased spontaneous mutation frequency in *E. coli* strains lacking this enzymatic activity (described in Chapter 2), emphasizes the importance of preventing this mutagenic pathway. The presence of 8-oxo-dGTP in the nucleotide pool of cells is an accepted premise in the scientific literature; however, prior to this study no measurements of the intracellular levels of 8-oxo-dGTP had been published.

This dissertation (see Chapter 4) describes the development of an HPLC-ECD assay for the detection of each of the four normal dNTPs and 8-oxo-dGTP from cell extracts. The assay has both advantages and disadvantages. While it is possible to simultaneously detect the nucleotides of interest in a short period of time and to sensitively quantitate 8-oxo-dGTP levels by electrochemical detection by using this assay, the HPLC method itself is problematic. The solvent system used in the assay is highly viscous because it consists of a mixture of buffer and methanol. This caused the HPLC system and column to be exposed to extremely high pressures throughout the course of each chromatographic run, which shortens the lifetime of both the column and the seals of the HPLC system. Therefore, constant maintenance was necessary to keep the HPLC system in working order while these assays were being conducted.

In addition, this separation used a C18 column, and exposure of this type of column to mobile phases that contain a high percentage of aqueous solvent tends to have an adverse affect on the stationary phase. Constant exposure to the mobile phase used in this assay caused the very hydrophobic C18 chains of the column to lie flat on the silica surface in order to minimize the surface area exposed to the highly polar

mobile phase. This reduced capacity factors of the compounds being resolved and also caused non-reproducibility of injections. This problem could be resolved by making duplicate injections of the dNTP extracts, adding an internal standard of the 4 normal dNTPs and 8-oxo-dGTP to the second injection to verify the retention time for each nucleotide (as described in Chapter 4). However, as the lifetime of the column increased, it was observed that the retention times for each nucleotide would decrease (see Chapter 5). Therefore, it was frequently necessary to wash the column extensively to remove the salts, and then regenerate the column by extensive washing with 50% methanol. Although these procedures were very time-consuming, they were essential for maintaining both the efficiency and resolution of the assay, and for maximizing the lifetime of the HPLC column.

The electrochemical detection of 8-oxo-dGTP also required that the working electrode of the EC detector be cleaned regularly. It was common to experience electrode passivation problems due to the composition of the mobile phase, which resulted in a decrease in the signal generated by the oxidation of 8-oxo-dGTP with time of exposure to the mobile phase. The high applied potential (+ 900 mV) required for the oxidation of 8-oxo-dGTP also contributed to this problem by causing oxidation of the mobile phase itself. Therefore, it was necessary to polish the surface of the glassy carbon-working electrode on a regular basis using standard metallographic procedures, and to sonicate the electrode to remove any debris left from the polishing procedure.

6.3. Measurements of the Pool Size of 8-oxo-dGTP

Once an analytical method for the measurement of 8-oxo-dGTP pools was established, levels of the nucleotide were measured in both wild type *E. coli*, and in strains lacking a functional MutT enzyme. Maki and Sekiguchi showed that the *E. coli* MutT enzyme has a major role in preventing the A·T→C·G transversions caused by the incorporation of 8-oxo-dGTP into DNA [11], due to its ability to degrade the nucleotide and "sanitize" the nucleotide pool. Therefore, the presence of the MutT

enzyme should correlate with levels of the oxidized nucleotide, 8-oxo-dGTP. The results described in this dissertation show that this is not the case. When the nucleotides extracted from both wild type and *mutT* mutant *E. coli* strains were analyzed by HPLC-ECD, no 8-oxo-dGTP was detected in any of the bacterial nucleotide extracts (Figures 5.14-5.17). Even when the cells were cultured in the presence of H_2O_2, a condition that elevates the levels of reactive oxygen species and should therefore promote the formation of 8-oxo-dGTP, no measurable level of the nucleotide could be detected (Figures 5.19-5.22), even though oxidative mutagenesis was occurring in these cultures (see Table 5.4). Bacteria cultured in the presence of H_2O_2 have only a slightly increased level of 8-oxo-dGTPase activity, although this increase was not statistically significant (see Figure 5.28).

6.4. *In vitro* DNA Replication with "Physiological" Levels of 8-oxo-dGTP

The lower limit of detection of 8-oxo-dGTP using the HPLC-ECD method was used to calculate the upper limit of the intracellular concentration of 8-oxo-dGTP in *E. coli* cells. This was determined to be approximately 240 molecules of 8-oxo-dGTP per cell, or 0.34 μM. If 8-oxo-dGTP is as highly mutagenic *in vivo* as the *in vitro* data suggest, then levels of the oxidized nucleotide should correlate with mutation rate. Is the "physiological" concentration of 8-oxo-dGTP as determined by HPLC-ECD sufficient to cause mutagenesis *in vivo*?

Much of the current evidence suggesting that 8-oxo-dGTP causes mutagenesis *in vivo* by its incorporation into actively replicating DNA comes from studies that used 8-oxo-dGTP in an *in vitro* DNA replication system in which replication errors could be scored as mutations. In these studies [14,15,111], the addition of 8-oxo-dGTP stimulated replication errors that were shown by sequence analysis to be transversions, mutations that are characteristically seen when 8-oxo-dGTP is incorporated into DNA. In the earliest study of this kind, Cheng *et al.* [14] found that during DNA replication in *E. coli*, when 8-oxo-dGTP was substituted for dGTP, it had the mutagenic ability to pair with A to generate transversions. This effect was seen only when 8-oxo-dGTP

was present in a 10- to 100-fold greater concentration than dATP, dCTP and dTTP (5 μM each) in the replication reaction. Minnick *et al.* [111] found that the presence of 8-oxo-dGTP at equimolar concentration (100 μM) with each of the four normal dNTPs resulted in a >46-fold increase in error rate for A·T→C·G transversions over that observed in the absence of 8-oxo-dGTP. Similarly, DNA replication in the presence of 8-oxo-dGTP at equimolar concentration (50 or 100 μM) with the normal dNTPs by purified exonuclease-deficient Klenow, T4, and *Thermus thermophilus* DNA polymerases decreased the fidelity of replication [15]. These investigators also found that, when present during SV40 origin-dependent replication of double-stranded DNA in HeLa cell extracts at a concentration equal to those of the four normal dNTPs (100 μM), 8-oxo-dGTP was 13-fold more mutagenic for A·T→C·G transversions than was a 100-fold excess of dGTP.

These data have led researchers to suggest that the presence of 8-oxo-dGTP could be highly mutagenic during both mitochondrial and nuclear genomic replication *in vivo* in eukaryotes. However, it is important to note that in each of these studies, each of the four normal dNTPs usually present during DNA replication was present in equimolar concentrations. Also, in each case, 8-oxo-dGTP was present in the reactions at equimolar concentrations to the normal dNTPs, or was present at a 10- to 100-fold excess. Detailed studies in both prokaryotic [134] and eukaryotic cells (see Table 1.1) of the intracellular concentrations of each of the four normal dNTPs utilized during DNA replication have shown that there is a natural asymmetry in the dNTP pool sizes. Thus, when considering the replication of DNA in eukaryotic cells and the asymmetry of the dNTP pools, it becomes clear that the conditions used in these *in vitro* DNA replication studies with 8-oxo-dGTP are not physiologically relevant. Given the experimental conditions that were used, the results of these *in vitro* studies should not be interpreted to suggest that the nucleotide, 8-oxo-dGTP, is involved in oxidative mutagenesis *in vivo*.

A more recent publication evaluated the mutagenicity of 8-oxo-dGTP *in vivo* by introducing the oxidatively damaged nucleotide into competent *E. coli* and selecting

mutants of the chromosomal *lacI* gene [144]. The results of this study showed that the addition of 8-oxo-dGTP induced 12-fold more substitution mutations than the spontaneous event, and from these results the authors concluded that the nucleotide is mutagenic *in vivo*. However, an evaluation of the experimental conditions used in this study shows that when 8-oxo-dGTP was introduced into the cells, the intracellular concentration of the nucleotide was at least 250 μM. Based on the upper level of the intracellular concentration of 8-oxo-dGTP determined by HPLC-ECD in this dissertation (0.34 μM), the experimental conditions used in the "*in vivo*" experiments performed by Inoue *et al.* are also not physiologically relevant.

The goal of the *in vitro* DNA replication reactions and reversion assay experiments described in this dissertation was to determine the mutagenicity of 8-oxo-dGTP when it and each of the four normal dNTPs were present during DNA replication at physiologically relevant concentrations. Based on the observation that the concentration of dNTPs in bacterial cells is typically greater than 10-fold higher than the dNTP pools in mammalian cells, it was hypothesized that any mutagenic effect of 8-oxo-dGTP present during DNA replication in HeLa cell extracts should be detectable when the nucleotide is present at the estimated maximal concentration in *E. coli* of 0.34 μM. Previously, Leeds *et al.* [20] estimated the dNTP concentrations in S-phase HeLa cell nuclei to be 60 μM dATP, 60 μM dTTP, 30 μM dCTP, and 10 μM dGTP. Therefore, in order to determine the mutagenic potential of 8-oxo-dGTP under physiologically relevant experimental conditions, these replication reactions contained the four normal dNTPs at concentrations found in HeLa cell nuclei, and 8-oxo-dGTP was added to the reactions in concentrations ranging from 0 to 10 μM.

The opal codon in the M13mp2SV/opal-7 DNA used in these *in vitro* DNA replication reactions is susceptible to mutagenesis in the presence of the nucleotide, 8-oxo-dGTP. Although it is unlikely that this mutational target would revert back to the wild type sequence (TCA) in the presence of 8-oxo-dGTP, this region of the *lacZα* gene is flexible and most mutations within this region retain wild type function. Therefore, single base substitutions in the opal codon will generate a wild type

phenotype and be scored as mutations in the reversion assay. Because 8-oxo-dGTP can base pair with both template cytosine [74] and template adenine [75], 8-oxo-dGTP present during the DNA replication reaction could potentially be incorporated opposite the template adenine at position 3 in the opal (TGA) codon ((+) strand). Incorporation of 8-oxo-dGTP at this site and transcription of the (-) strand would result in a transcript encoding UGC at this site, which codes for the amino acid, cysteine. Reversion to the wild-type sequence would produce the codon UCA, which encodes serine. The amino acids cysteine and serine have similar molecular structures, with the only difference being that cysteine has a sulfhydryl group where serine has a hydroxyl group in the side chain. Kunkel et al. [115] have shown that a serine to cysteine substitution in this region of the *lacZα* mutational target does generate a blue plaque phenotype. Therefore, it is possible that a change in the DNA sequence due to the incorporation of 8-oxo-dGTP that results in an amino acid change from serine in the wild type sequence to cysteine when the M13mp2SV/opal-7 DNA undergoes mutagenesis could produce a functional α-peptide, which would generate a blue plaque phenotype.

It is also important to note that these replication reactions were carried out in a HeLa S3 cell cytoplasmic extract. If the mammalian MutT homolog is present in the cell extract, it could presumably break down the 8-oxo-dGTP added to the replication reaction in order to prevent its incorporation into DNA. Similarly, even if 8-oxo-dGTP becomes incorporated into the actively replicating DNA, the presence of the bacterial MutM protein would result in the removal of 8-oxo-dG in the template strand, and if this occurred prior to transcription, the sequence of the original M13mp2SV/opal-7 DNA would be preserved. As shown in Table 5.6, only under experimental conditions where the concentration of 8-oxo-dGTP was greater than 1 μM was a slight increase in the mutation frequency observed. Even when the concentration of 8-oxo-dGTP in the replication reactions was equimolar to the dGTP concentration (10 μM), no significant increase in the mutation frequency was seen. And, as noted earlier, in *E. coli* the estimated concentration of dGTP is 100 μM [134], which is 10-fold higher than the

concentration of dGTP used in these experiments. Therefore, it is unlikely that a concentration of 0.34 μM 8-oxo-dGTP would cause mutagenesis in the presence of dGTP at a concentration of 100 μM if this concentration of 8-oxo-dGTP is not detectably mutagenic in the presence of 10 μM dGTP, as seen in these results. When considering that these *in vitro* DNA replication reactions were carried out under experimental conditions that closely resemble the physiological conditions expected during intracellular DNA replication, it was not unexpected that the results of these experiments would show that the addition of physiologically relevant concentrations of 8-oxo-dGTP during DNA replication does not significantly increase the mutation frequency.

The ability of different DNA polymerases to use 8-oxo-dGTP as a substrate for *in vitro* DNA synthesis has been studied. Maki and Sekiguchi [11] were the first to show that 8-oxo-dGTP can be inserted opposite the A residue as well as C in a synthetic DNA template by the α subunit of *E. coli* DNA polymerase III which lacks 3′→5′ exonuclease activity. Pavlov *et al.* [15] also showed that 8-oxo-dGTP could be incorporated opposite both C and A by Klenow (exo$^-$), T4 polymerase (exo$^-$) and *Thermus thermophilus* DNA polymerase. The kinetics of incorporation of 8-oxo-dGTP opposite template C in both a DNA and RNA template by HIV-1 and murine leukemia virus reverse transcriptases and DNA polymerases α and β were reported by Kamath-Loeb *et al.* [109]. More recently, the kinetics of 8-oxo-dGTP incorporation into DNA by *E. coli* polymerase I (exo$^-$), *E. coli* polymerase II (exo$^-$), HIV-1 reverse transcriptase and bacteriophage T7 polymerase (exo$^-$) was examined [44]. For each of these four prokaryotic polymerases, 8-oxo-dGTP was found to be a poor substrate, with insertion efficiencies $>10^4$-fold lower than for dGTP incorporation opposite C. The mammalian enzyme, polymerase δ, was also shown by Einolf and Guengerich [41] to incorporate 8-oxo-dGTP with low efficiency, only approximately 10^{-3} that of dGTP, in the presence of proliferating cell nuclear antigen. This result is of particular interest because polymerase δ is thought to be the main polymerase involved in leading strand DNA replication in mammalian cells. It may be possible that another

mammalian polymerase inserts 8-oxo-dGTP into actively replicating DNA with high efficiency. However, the results of the *in vitro* DNA replication experiments described in this dissertation are consistent with the observation that for most polymerases studied to date, the tendency to insert 8-oxo-dGTP appears to be low.

6.5. General Discussion

Sekiguchi and his colleagues were the first investigators to describe the 8-oxo-dGTPase activities of both the *E. coli* MutT enzyme [11] and its mammalian homolog [40]. Their proposal that the role of these enzymes in the elimination of the oxidized form of dGTP from the nucleotide pool is important for the high fidelity of DNA synthesis has been widely accepted in the scientific literature. However, the research described in this dissertation and other recent studies have presented evidence that suggests that this proposal needs to be evaluated. Einolf and Guengerich [41] have questioned the significance of the MutT enzyme and its mammalian homolog in preventing oxidative mutagenesis, based on their kinetic studies of the insertion of 8-oxo-dGTP into DNA by various bacterial polymerases and mammalian polymerase δ. The inability of the HPLC-ECD method described in this dissertation to detect a measurable pool of 8-oxo-dGTP in *E. coli*, and the results of *in vitro* DNA replication assays also show that physiologically relevant levels of the oxidized nucleotide are insufficient to cause mutagenesis.

Based on these results, it is possible that the critical substrate for the MutT enzyme may not be 8-oxo-dGTP, but instead may be another yet uncharacterized nucleotide. The observation that the mammalian MutT homolog, hMTH1, has variable substrate specificity and can hydrolyze oxidized forms of dATP and dGTP [48] gives precedent to this suggestion. MutT belongs to a family of pyrophosphohydrolase enzymes, the NuDiX hydrolases [106], that have a versatile nucleotide binding and catalytic site and are able to hydrolyze a nucleoside diphosphate linked to some other moiety, X. Members of this family of enzymes have disparate substrate preferences, which include nucleoside triphosphates, coenzymes, nucleotide sugars, and

dinucleoside polyphosphates. The MutT enzyme is not the only enzyme that has the ability to hydrolyze 8-oxo-dGTP. GTP cyclohydrolase II is a protein that efficiently hydrolyzes both 8-oxo-dGTP and 8-oxo-GTP to the respective monophosphates, and is able to reduce the increased level of mutation frequency of the *mutT* strain to almost the normal level, provided that the gene product is overproduced [145]. The very low intracellular level of 8-oxo-dGTP that was determined by HPLC-ECD is likely insufficient to account for the levels of *mutT* mutator activity in cells, and it is therefore probable that the *E. coli* MutT enzyme has another preferred substrate *in vivo*.

The results presented in this dissertation (see Figures 5.14-5.17) did show the presence of an electrochemically active compound that eluted during HPLC separation at a similar time as the other dNTPs and that was present only in nucleotide extracts from *E. coli* strains lacking a functional MutT enzyme. This compound is not an oxidized derivative of any of the standard nucleoside triphosphates, and the identity of this compound is currently unknown. Identification of this compound may provide a clue to additional biochemical functions of the *E. coli* MutT enzyme and a better understanding of the mutator phenotype of *mutT* mutants.

Other unexpected results regarding the function of the MutT protein are reported in the literature. The first was a report that showed that starvation of *mutT* mutants of *E. coli* causes accumulation of mutations even though measurable DNA replication does not occur during starvation [146]. The *mutT* mutagenesis pathway as it is currently understood is believed to require DNA synthesis for incorporation of the oxidized nucleotide. Bridges interpreted the results of this study to indicate that a considerable amount of DNA synthesis is occurring under these experimental conditions that uses precursors derived from DNA turnover rather than exogenously provided precursors. However, these results can also call into question the premise that 8-oxo-dGTP is the preferred substrate of the MutT enzyme because there is no reason to expect that the generation of reactive oxygen species or the relative levels of

8-oxo-dGTP should be greater in stationary-phase bacteria when compared to growing bacteria.

The second puzzling observation in the literature regarding MutT is that under anaerobic growth conditions, the mutation rate of *mutT* bacteria is influenced by the richness of the growth medium. Fowler *et al.* [43] showed that a *mutT* strain of *E. coli* grown in rich medium showed nearly identical mutation frequencies under either aerobic or anaerobic culture conditions. In contrast, *mutT E. coli* cultured in minimal medium showed low mutation frequencies when cultured anaerobically, as expected if mutagenesis involves the oxidation of dGTP to 8-oxo-dGTP, followed by its incorporation into DNA. The mechanism of mutagenesis in *mutT* bacteria cultured in rich medium under anaerobic conditions is not well understood, although it should be noted that rich medium is poorly defined, and the production of reactive oxygen species and oxidative mutagenesis have been reported in *Salmonella typhimurium* that had been grown anaerobically [147]. Levels of reactive oxygen species may be influenced by the nutrient conditions as well as oxygen conditions, or it may be that the MutT enzyme acts on a substrate other than 8-oxo-dGTP during anaerobic growth. It is also possible that *mutT*-induced mismatches are more efficiently repaired when the cells are grown anaerobically in minimal medium because cells grown under these conditions would be expected to grow very slowly, allowing more time for DNA repair.

Taken together, the results presented in this dissertation and the observations discussed above suggest that the mechanism by which the MutT protein counteracts mutagenesis *in vivo* should be reevaluated. *In vitro* experiments have clearly demonstrated that 8-oxo-dGTP is a highly mutagenic compound that gives rise to A·T→C·G transversions, and that the MutT enzyme efficiently hydrolyzes the nucleotide to prevent *in vitro* mutagenesis. However, intracellular levels of the nucleotide are insufficient to account for the levels of *mutT* mutator activity. Therefore, the possibility that the MutT enzyme has an alternative mode of action and may prefer an alternative substrate *in vivo* should be considered.

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APPENDIX

Appendix – dGTP Pool Turnover in V79 Cells

A.1. Labeling of V79 Cells with [^{3}H]-Hypoxanthine

Initially, [^{3}H]-hypoxanthine was chosen as a radiolabeled precursor for use in the dGTP pool turnover experiments. Hypoxanthine is utilized in purine salvage pathways in mammalian cells, and is converted into the nucleotide, inosine 5′-monophosphate (IMP), by the addition of 5-phosphoribosyl-α-pyrophosphate (PRPP). This reaction is catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HGPRT), as shown below:

$$\textbf{hypoxanthine} + \textbf{PRPP} \leftrightarrow \textbf{IMP} + \textbf{PP}_i$$

Hypoxanthine had been previously used in similar experiments conducted by Bianchi *et al.* to label the dATP pool [128]. These experiments showed that the dATP pool turnover rate is equal to the rate of incorporation of dAMP into DNA. IMP is converted into both AMP and GMP during de novo synthesis of nucleotides. Therefore, labeling cells with [^{3}H]-hypoxanthine results in labeling of both the dATP and dGTP pools. The use of this precursor provided an important internal control for the dGTP turnover experiments, because dATP is thought to be a far less significant target for oxidation than dGTP [127]. Labeled dATP should have only one metabolic fate, incorporation into DNA, whereas dGTP could presumably be incorporated into DNA or enzymatically degraded if it had undergone prior oxidation.

Before attempting to measure dGTP pool turnover, it was necessary to determine the time course of equilibration of the label into the acid-soluble nucleotide pools. This was achieved by labeling the synchronized cells immediately after release from aphidicolin block by the addition of 0.3 μM [^{3}H]-hypoxanthine (specific activity 14.1 Ci/mmol) directly to the culture medium, and the acid-soluble pools were extracted at 30-minute intervals for the following 4 hours. The cells were grown in 35-mm culture dishes, and duplicate cultures were prepared for each time point.

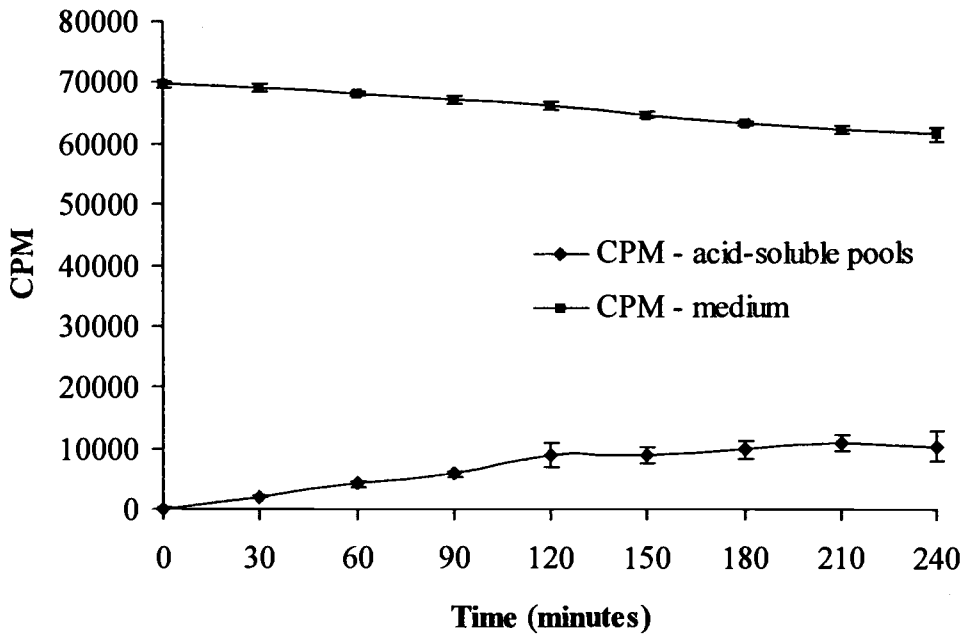

Figure A. 1. Time course of equilibration of acid-soluble pools in synchronized V79 cells labeled with 0.3 μM [3H]-hypoxanthine.
Time zero is the time at which the cells were released from aphidicolin block.

At each time point, extracts of the acid-soluble nucleotides were prepared by aspirating the labeled medium and washing the cells twice with ice-cold PBS, followed by the addition of 0.5 μl of 0.5 N TCA to each culture dish. The dishes were rocked gently for 5 minutes, and the TCA collected into microcentrifuge tubes. The acid-precipitable material was collected by centrifugation of the TCA extract for 30 minutes at 14,000 rpm, 4°C, and the supernatant was neutralized by the addition of 1.5 volumes of 0.5 M TOA/Freon. Four 20 μl samples of the aqueous phase of the neutralized supernatant were added to scintillation fluid and counted for 5 minutes to determine the amount of tritium incorporated into the acid-soluble pools. Four 5-μl samples of the labeled medium were also counted.

The results of this preliminary experiment are shown in Figure A.1. The amount of label incorporated into the acid-soluble fraction equilibrated at approximately 210

minutes after the addition of labeled hypoxanthine to the medium. At this time point, flow cytometry had previously shown that the aphidicolin-synchronized cells are in late S-phase (see Figure 5.3). Beginning the cold chase-period at this late point in S-phase, and attempting to measure dGTP pool turnover after this point was not ideal. The lengthy time required for complete labeling of the acid-soluble pools was possibly due to the fact that the [^{3}H]-hypoxanthine was supplied to the cells at a very low concentration, yet the ribonucleotide and deoxyribonucleotide pools both were labeled during this time. The deoxyribonucleotide pools should equilibrate more rapidly than the total time required because it is a much smaller pool, so it became necessary to determine the amount of time required for the deoxyribonucleotide pools to equilibrate.

Thin layer chromatography was used to determine when pools of dAMP and dGMP equilibrated with the label. 20 µl each of 100 mM AMP, dAMP, GMP, dGMP, dCMP, CMP, and dTMP standards were run on PEI-cellulose thin layer sheets in 200 ml of a solvent system containing 0.5 M LiCl, 0.45 M acetic acid, and 354 mM $Na_2B_4O_7$, pH 7.0, and the nucleotides were visualized with UV light. As shown in Figure A.2, this solvent system showed a good separation of the standard nucleotides. It was then used to separate nucleotides contained in samples that had been collected at 15-minute intervals for two hours after the cells were released from aphidicolin. Prior to loading the time-course samples onto the PEI-cellulose, each sample was heated to 37°C for 30 minutes to hydrolyze phosphorylated nucleotides to monophosphates. 20 µl of each sample was separated by thin layer chromatography. After developing the plates, the sections containing the nucleotides of interest were cut from the thin layer plate and placed in 0.5 M HCl for 30 minutes in a scintillation vial to elute the nucleotides, scintillation fluid was added, and the samples were counted.

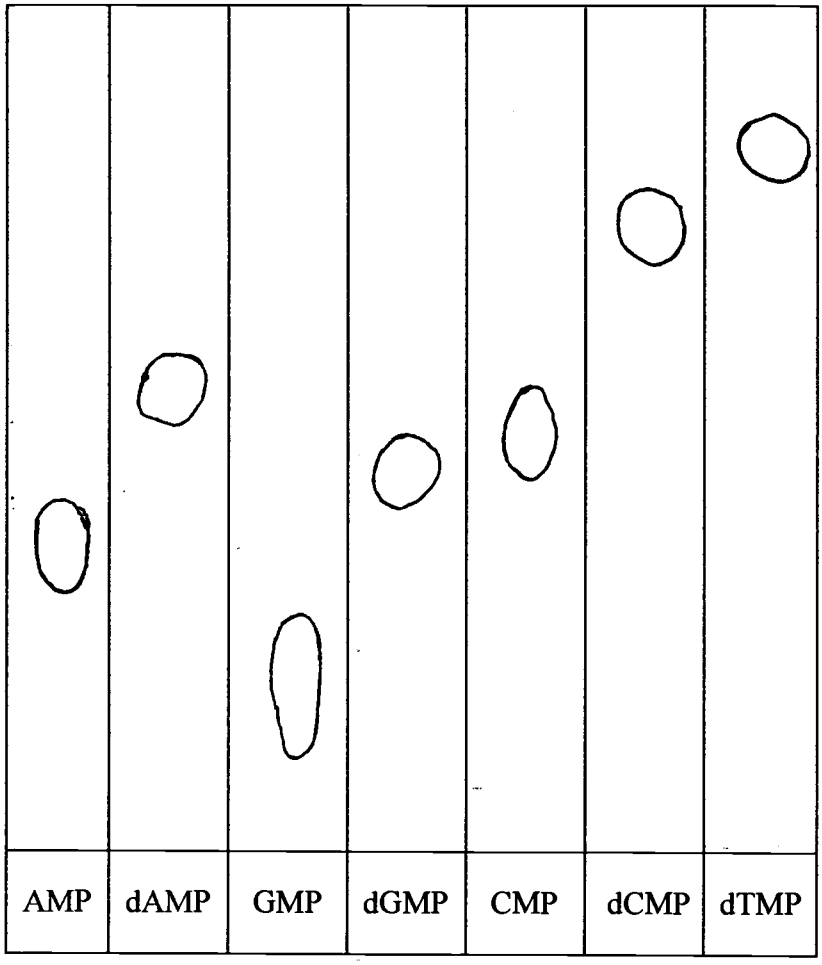

Figure A. 2 Separation of nucleoside monophosphate standards by thin-layer chromatography.
The solvent system contained 0.5 M LiCl, 0.45 M acetic acid, and 354 mM $Na_2B_4O_7$, pH 7.0. Circles indicate the locations of the nucleoside monophosphates visualized by UV light.

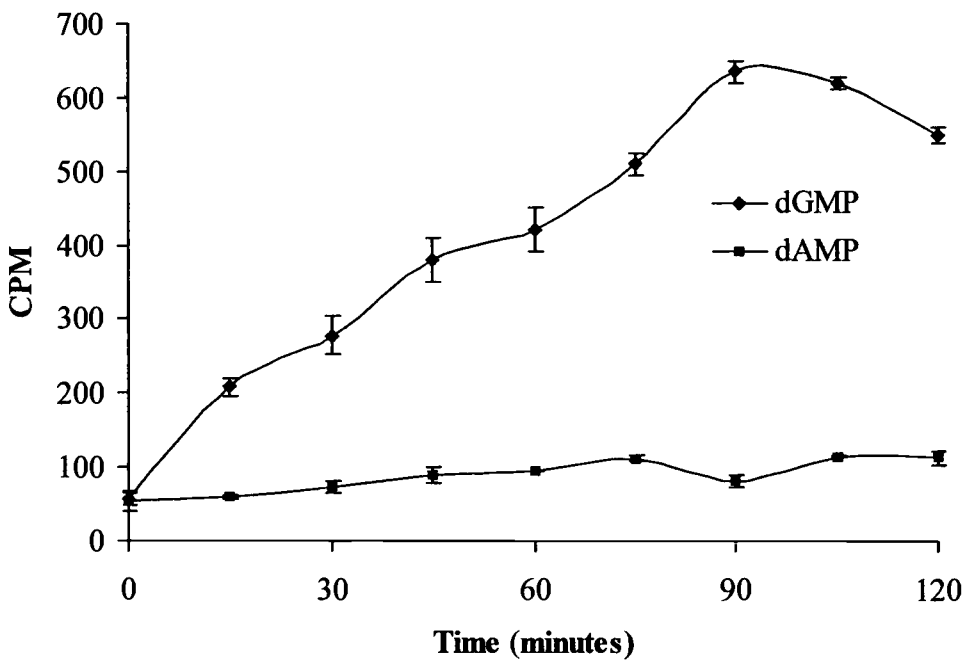

Figure A. 3 Time course of equilibration of dGMP and dAMP pools in synchronized V79 cells labeled with 0.3 μM [3H]-hypoxanthine. Time zero is the time at which the cells were released from aphidicolin block.

The results of this experiment are shown in Figure A.3. The dGMP pool incorporated label slowly and equilibrated after approximately 100 minutes, but there was no obvious plateau for the dAMP pool. Note that the amount of label incorporated into the pools was very small. At the 90-minute time point, only approximately 600 CPM was present in 20 μl of the sample, indicating that only a very small fraction of the tritium supplied by the [^{3}H]-hypoxanthine was actually incorporated into the nucleotide pools. In the dNTP enzymatic assay used to measure dGTP turnover, only 5 μl of the cell extract is added to a reaction mix. Taking into account the background counts, these data made it seem unlikely that the dGTP pool would be sufficiently labeled to a high enough specific activity by the [^{3}H]-hypoxanthine to be able to measure the turnover of the pool.

Nevertheless, synchronized V79 cells that had been grown in both 2% and 20% oxygen were used to prepare dNTP extracts. Immediately after release of the cells from the aphidicolin block, the cells were incubated with 0.3 μM [^{3}H]-hypoxanthine and allowed to incorporate the label for 100 minutes. After this time, the labeled medium was replaced with unlabeled conditioned medium and dNTPs were extracted at 0, 3, 6, 9, 12, 30, and 60- minute time points. DNA was also extracted from the cells at each time point by dissolving the cell layer that remained after methanol + toluene extraction of the dNTPs with 2 ml of 0.3 M NaOH at 37°C overnight. The radioactivity incorporated into the DNA was measured by the method of Nicander and Reichard [148]. The dGTP pools were measured using the DNA polymerase-based dNTP enzymatic assay, as described in Materials and Methods, with a α^{32}P-labeled complementary nucleotide. The ^{3}H and ^{32}P in each dNTP pool assay reaction was counted, and the specific activity (CPM/pmol dNTP) of the pools at each time point was calculated.

The results of this initial attempt to measure dGTP pool turnover are shown in Figure 5.6. The pool of dGTP present in V79 cells that had been grown in 20% oxygen was extremely low (<2 pmoles dGTP / 10^6 cells). However, the pool size of dGTP in cells grown at 2% oxygen was 3-fold higher. This result supported the hypothesis that cells grown at higher oxygen tensions would have higher levels of oxidative stress and the resulting damage to the dGTP pool would cause the pool to be smaller at higher oxygen tensions.

However, the mechanism responsible for this change in the pool sizes was not made clear by these experiments because the turnover of the dGTP pool could not be measured. The counts attributed to ^{3}H in the dGTP pool for each sample were indistinguishable from background counts. This indicated that the dGTP pool was not adequately labeled by incubation of the cells with 0.3 μM [^{3}H]-hypoxanthine. When 5 μl samples of the cell extracts used in the dNTP enzymatic assay were counted, all samples from each time point and oxygen condition contained 10,000-20,000 CPM. These cell extracts are composed of all ribonucleotides and deoxyribonucleotides in

the cells, only a small fraction of which are dNTPs. However, as suggested by preliminary experiments, the results of which are shown in Figure A.3, the amount of [^{3}H]-label incorporated into the deoxyguanosine nucleotide pools is very small and treatment with the hypoxanthine precursor at 0.3 μM fails to label the dGTP pool to a high enough specific activity for its turnover to be measured by this approach.

A.2. Labeling of V79 Cells with [^{3}H]-Guanosine

Previous attempts to label the dGTP pool with the precursor, hypoxanthine, were unsuccessful. This may have been due to the fact that hypoxanthine is used in the synthesis of both adenine and guanine nucleotides, and much of the label incorporated into cells was probably in the ribonucleotide pool, specifically in the ATP pool. In a further attempt to radioactively label the dGTP pool, the precursor [^{3}H]-guanosine was used.

During nucleotide metabolism, the nucleoside, guanosine, is cleaved by the action of purine nucleoside phosphorylase (PNP) to yield the corresponding base, guanine. Guanine, in turn, is phosphorylated to GMP by the addition of PRPP, which is catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). GMP can then be phosphorylated to GDP by the action of guanylate kinase. After reduction to dGDP and further phosphorylation, dGTP derived from the guanosine precursor is available for DNA synthesis. This pathway is shown below:

Guo → Gua → GMP → GDP → dGDP → dGTP

Alternatively, guanosine can be phosphorylated by nucleoside kinase to form GMP, which is further metabolized to dGTP as shown below:

Guo → GMP → GDP → dGDP → dGTP

Two attempts were made to label the dGTP pool of V79 cells with 0.3 μM [^{3}H]-guanosine (specific activity = 9.2 Ci/mmol), as previously described. Initially, the length of time required to fully label the dGTP pool was determined by preparing dNTP extracts at 30, 60, 90, and 120 minutes after application of the label and measuring the specific activity of the dGTP pool at each time point using the dNTP enzymatic assay.

As described above for cells that had been labeled with [^{3}H]-hypoxanthine, counts attributed to the incorporation of [^{3}H]-guanosine into dGTP in both sets of these samples were only slightly above background when the dNTP extracts were used in the dNTP enzymatic assay and counted. This could have been due to the fact that the precursor, [^{3}H]-guanine, was incorporated into the pool of guanine ribonucleotides instead of the deoxyribonucleotide pool. The very low counts incorporated into the dGTP pool, coupled with the fact that the dGTP pool is so low during S-phase in the V79 cells, made calculation of the dGTP pool specific activity (CPM/pmol dGTP) impossible. Hence, the labeling of the dGTP pool with [^{3}H]-guanosine at 0.3 μM was also insufficient to label the pool to a high enough specific activity for its turnover to be measured.

Previously, Arecco *et al.* [10] had succeeded in elevating the dGTP pool slightly in V79 cells by the addition of 0.1 mM guanine. Their results showed an elevation of the dGTP pool from 9.9 pmoles dGTP / 10^6 cells to 17.1 pmoles dGTP / 10^6 cells when the cells were incubated with guanine for 30 minutes, and this treatment did not significantly alter the other dNTP pools. While this result was seen with an asynchronous population of V79 cells, it was worthwhile to attempt to elevate the dGTP pools in synchronized V79 cells by using the same approach.

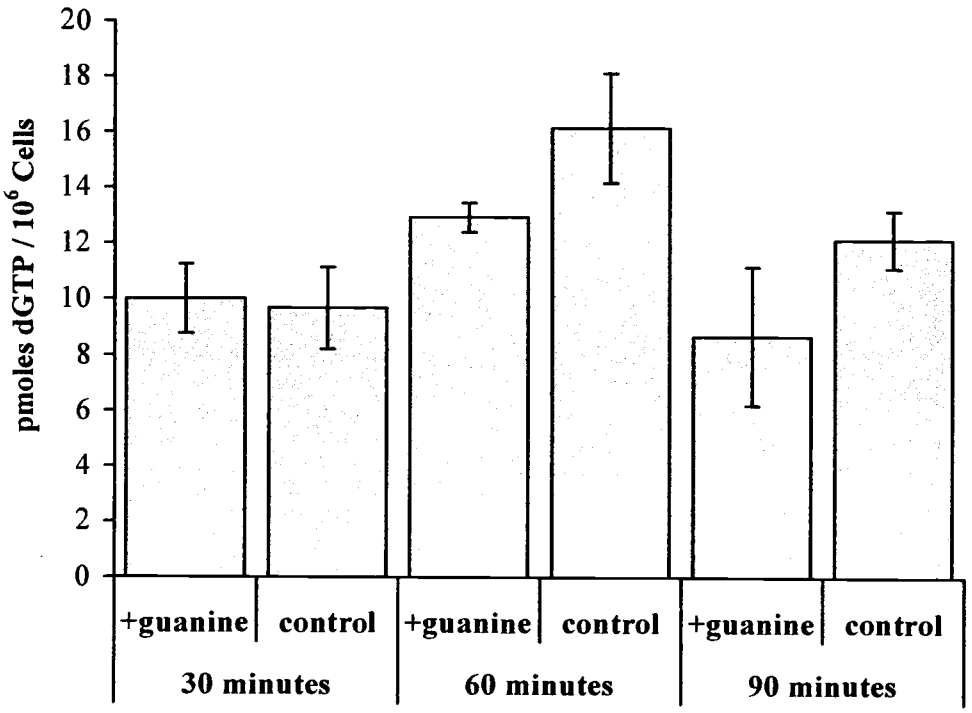

Figure A. 4 Effect of 0.1 mM guanine on the dGTP pool of aphidicolin-synchronized V79 cells.
Cells were incubated in the presence of guanine for the indicated times following release from the aphidicolin block. Control cells were grown in the absence of guanine. dNTPs were extracted from the cells and their pools were analyzed by the dNTP enzymatic method as described in Materials and Methods.

Consequently, V79 cells were synchronized by aphidicolin treatment and immediately following release from the aphidicolin block, they were incubated for 30, 60, and 90 minutes with 0.1 mM guanine. At each time point, dNTP extracts were prepared, and the dGTP pool was measured and compared to the dGTP pool from control extracts that had not been incubated with guanine.

As shown in Figure A.4, exposure of aphidicolin-synchronized V79 cells to 0.1 mM guanine in the culture medium immediately following the release of the cells from aphidicolin treatment did not significantly elevate the dGTP pools. This result

was in contrast to the result seen by Arecco *et al.* [10] for an asynchronous population of V79 cells treated with 0.1 mM guanine. In this publication, treatment of cells with deoxyguanosine (dGuo) and the purine nucleoside phosphorylase inhibitor, 8-aminoguanosine (AGR), also caused dGTP to accumulate, although this treatment did perturb the other dNTP pools. Because this attempt to elevate the S-phase dGTP pool using guanine was unsuccessful, cells were treated with deoxyguanosine and 8-aminoguanosine in the following attempt.

A.3. Labeling of V79 Cells with [^{3}H]-Deoxyguanosine

The metabolism of deoxyguanosine in salvage pathways used to produce dGTP is similar to the metabolism of guanosine, as shown in Figure A.5. The catabolism of dGuo to Gua is prevented by the addition of the purine nucleoside phosphorylase inhibitor, 8-aminoguanosine (AGR). Therefore, the use of [^{3}H]-dGuo in conjunction with AGR should prevent the radiolabeled precursor from being incorporated into guanine ribonucleotide pools. Arecco *et al.* [10] showed that the addition of 0.1 mM dGuo and 0.1 mM AGR to V79 cells for 30 minutes caused a 3-fold increase in the dGTP pool. Consequently, synchronized V79 cells were labeled as previously described by using 0.3 μM [^{3}H]-dGuo (specific activity, 8.1 Ci/mmol) and 0.1 mM AGR. Once again, the dGTP pool from cells labeled with the [^{3}H]-dGuo precursor was not sufficiently labeled to measure dGTP turnover. The addition of the PNP inhibitor, AGR, to these cells during labeling should have sufficiently inhibited the conversion of dGuo to Gua, so a series of experiments were designed and performed to determine the fate of the [^{3}H]-dGuo precursor that had been used.

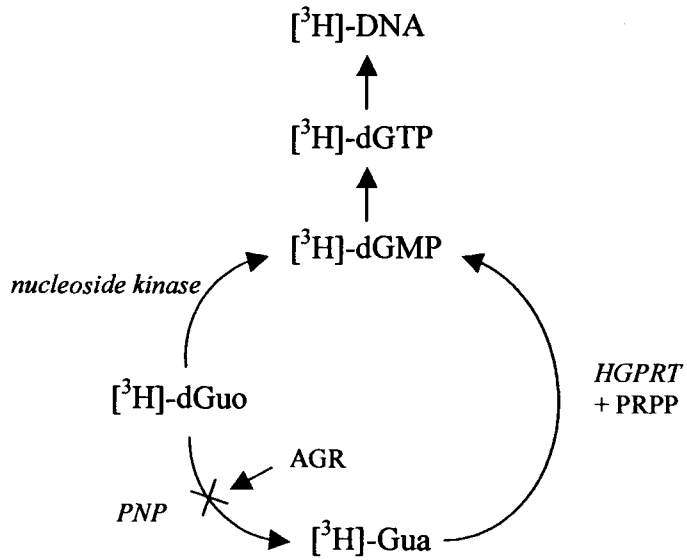

Figure A. 5. Labeling of the dGTP pool by the precursor, [^{3}H]-dGuo. The catabolism of dGuo to Gua is prevented by the addition of the purine nucleoside phosphorylase inhibitor, 8-aminoguanosine (AGR). Enzymes involved in the salvage pathway are shown in italics.

When the total radioactivity present in the medium, DNA and dNTP extracts was analyzed, it was determined that virtually 100% of the label remained in the medium, even though the cells were incubated in the presence of [^{3}H]-dGuo for 135 minutes. In order to determine whether the concentration of dGuo was limiting in this experiment and if the dGuo was incorporated into cells in the absence of AGR, cells were incubated in the absence and presence of AGR with varying concentrations of dGuo (0.3-30 μM) with the [^{3}H]-dGuo being diluted with unlabeled dGuo. Then, the radioactivity of the medium was measured at 30-minute intervals for 6 hours.

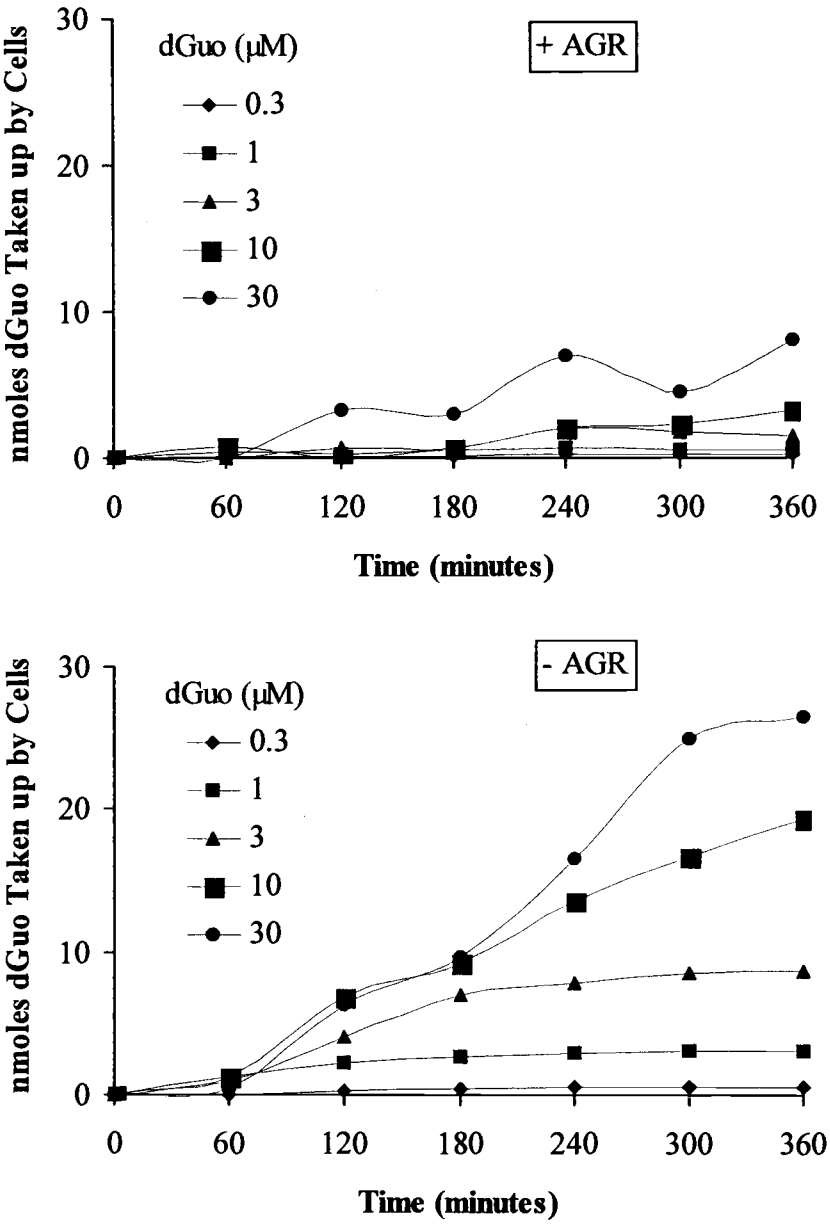

Figure A. 6 Uptake of deoxyguanosine by V79 cells in the presence (upper panel) and absence (lower panel) of 100 μM 8-aminoguanosine.
The concentrations of dGuo added to the cell medium are indicated in the legend on the right. Time is the number of minutes after addition of the nucleosides.

The results of this experiment are shown in Figure A.6. The upper panel shows the uptake of dGuo from the medium when 8-aminoguanosine is present at a concentration of 100 μM. The lower panel shows the uptake of dGuo into cells when 8-aminoguanosine was absent from the medium. These results show that cells took up dGuo much more readily in the absence of AGR, which suggests that the addition of 100 μM AGR inhibits the uptake of dGuo by the cells. It is possible that both dGuo and AGR use the same nucleoside transporter to enter the cells, and competition between the two nucleosides may have prevented the uptake of [^{3}H]-dGuo in this and the previous experiment. AGR was present at a greater than 300-fold excess over dGuo, so this could explain why no labeling of the dGTP pool occurred after incubation of the cells with 0.3 μM [^{3}H]-dGuo and 100 μM AGR.

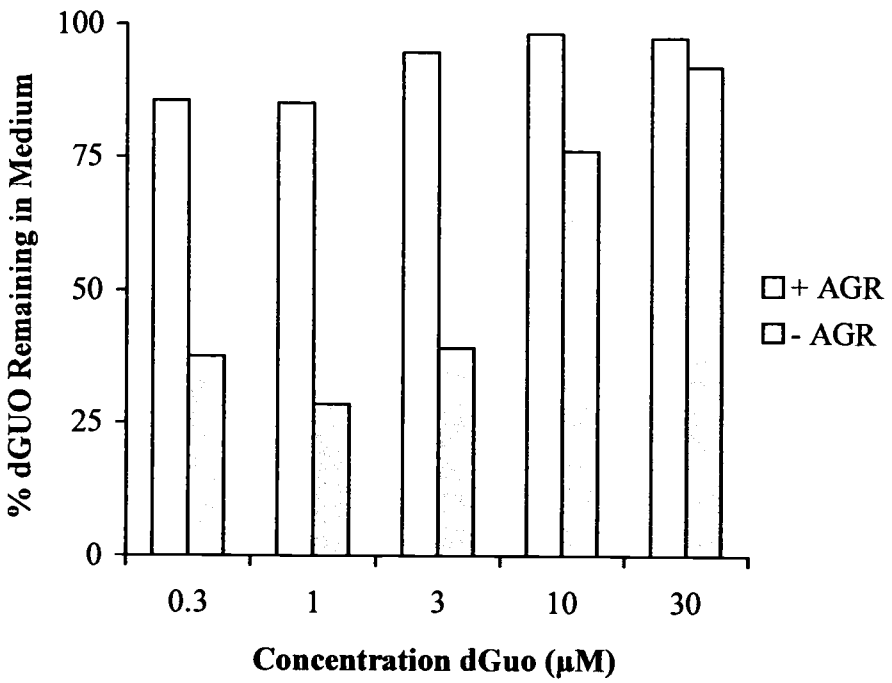

Figure A. 7 Percentage of deoxyguanosine remaining in the medium 180 minutes after addition in the presence and absence of 8-aminoguanosine. The concentration of dGuo added is indicated on the lower axis.

For the above-mentioned experiment, an interesting observation was made when the percentage of dGuo remaining in the medium was calculated for each time point. Figure A.7 shows the percentage of dGuo remaining in the medium as a function of dGuo concentration for the 180-minute time point. The same trend in the data was seen at each time point. These data show that as the concentration of dGuo increased above 1 μM, the percentage of dGuo taken up by cells decreased, but only in the absence of AGR. This could be due to saturation of the nucleoside transporter system at higher concentrations of total nucleoside (dGuo + AGR). Together, these data indicate that the concentration of dGuo remaining in the medium is lowest when AGR is absent and dGuo is present at a concentration of 1 μM.

The following experiment was designed to test the uptake of dGuo in the presence of varying concentrations of AGR. In this experiment, the concentration of dGuo used was 1 μM, since cells incubated with this concentration showed the greatest uptake of dGuo previously. The concentration of AGR added varied from 0-100 μM. The results of this experiment showed that the concentration of dGuo remaining in the medium decreased with time as a function of decreasing AGR concentration (see Figure A.8). Therefore, in order for the dGTP turnover experiments to be successful using [^{3}H]-dGuo as a precursor, it was necessary to somehow balance the inhibition of dGuo uptake by AGR and the necessary inhibition of purine nucleoside phosphorylase by AGR.

The previous experiment showed that cells were taking up approximately 20% of the dGuo (supplied at a concentration of 1 μM) from the medium at 120 minutes after addition in the presence of 1 μM AGR. Under these conditions, the two nucleosides that are competing for uptake by the cells are present at equimolar concentrations in the medium. These conditions were chosen for the following experiment, since a significant amount of the [^{3}H]-dGuo was still incorporated into cells, but AGR was present at a concentration that should be sufficient to inhibit PNP activity. An incubation time of 120 minutes was used because the cells are cycling into S-phase

during this time, and they should be actively synthesizing dNTPs and DNA, causing incorporation of the [^{3}H]-label into both acid-soluble and acid-precipitable pools.

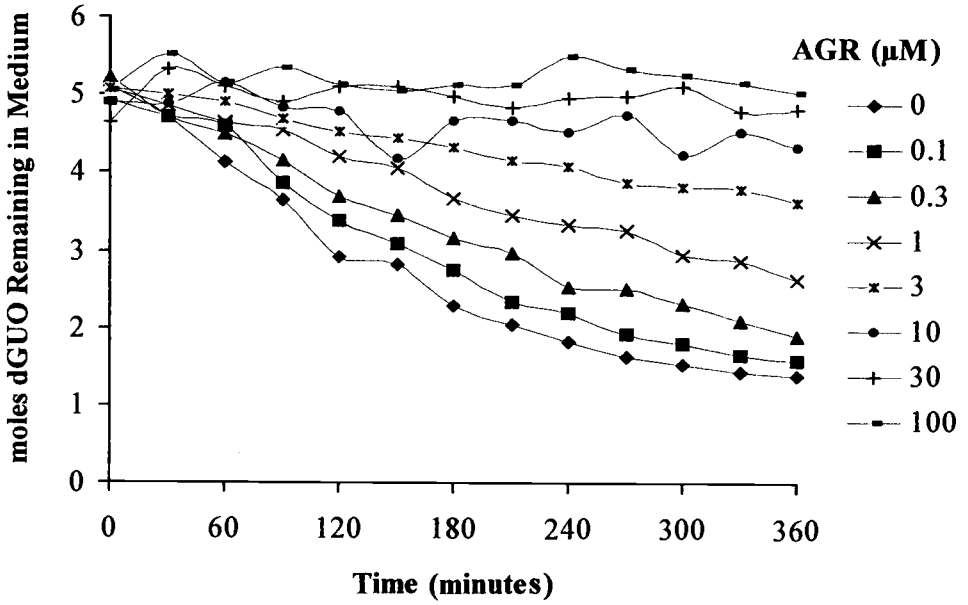

Figure A. 8 Concentration of deoxyguanosine remaining in the medium with varying concentrations of 8-aminoguanosine.
The concentrations of AGR added to the cell medium are indicated in the legend on the right. Time is the number of minutes after addition of the nucleosides.

In order to determine if a sufficient amount of the [^{3}H]-label to measure pool turnover was incorporated into the dGTP pool under these conditions, the number of counts in the acid-soluble and acid-precipitable pools was measured. The results of this final experiment showed that under these conditions (1 μM dGuo, 1 μM AGR, 120 minutes incubation time) less than half of the [^{3}H]-counts became incorporated into the acid-soluble pool. Assuming that the addition of 1 μM AGR is sufficient to completely inhibit the purine nucleoside phosphorylase activity, this [^{3}H]-labeled acid-soluble pool would consist only of the [^{3}H]-guanine deoxynucleosides and deoxynucleotides derived from the [^{3}H]-dGuo precursor. If the unrealistic assumption

was made that 100% of the counts in the acid-soluble pools were in the dGTP pool, and the counting efficiency, yield of recovery of dGTP after extraction, dilution of the extract in the dNTP enzymatic assay and sensitivity of the assay were all taken into account, it was determined that still not enough counts would be present in the [^{3}H]-dGTP pool to detect in the dNTP enzymatic assay for the measurement of dGTP pool turnover. Therefore, no further attempts were made to measure dGTP turnover. Instead, a method to directly detect and quantitate pools of 8-oxo-dGTP from cell extracts was developed.