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Comparative Effects of Various Metals on the Generation of Oxidative Damage Using 8-Hydroxy-2'-Deoxyguanosine as a Biomarker

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THESIS

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Presented in Partial fulfillment of the Requirements for

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By

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Texas Southern University

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In the recent years of scientific investigation, the role of DNA in disease pathology has become increasingly important. In attempts to understand the effects of foreign materials and energies on DNA structures and interactions, several types of genetic damage have been characterized. One type of genetic malfunction that is of interest to this laboratory is oxidative damage to DNA. Oxidative damage can result when cells are exposed to stimuli such as metallic ions, creating free radicals from metabolic processes that can attack and damage cellular components such as DNA that lead to elevated levels of oxidative stress. The objective of this investigation was to examine the effects of various metallic members of the periodic table in relation to their ability to induce oxidative damage of a particular DNA constituent. Furthermore, any possible effect of time and temperature was examined via changes made to a standard reaction setup in attempts to understand the impact of these variables on the generation of the analyte of interest. The investigation into the effects of various metals on oxidative damage was monitored and assessed by means of HPLC analysis of 8-Hydroxy-2-

deoxyguanosine, a specific biomarker of oxidative stress. The metals of interest include iron, manganese, cobalt, copper, lead, chromium, nickel, cadmium, mercury, and zinc. The results of this study found iron to be by far the most potent inducer of oxidative damage followed by manganese, cobalt, chromium, copper, nickel, lead, and cadmium respectively. In relation to the time and temperature study, two general trends were revealed that relate to an increase in temperature over time. Firstly, certain metals tend to exude an innate ability to increase or decrease the concentration of 8-OHdG detected over time. Secondly, the effects of increased temperature on the outcomes of the aforementioned trends seem to only push these reactions further in the directions in which they were already compelled, causing either a noticeable rise or reduction in concentration of 8-OHdG produced.

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Chairperson, Thesis Committee — Date 1/7/08

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LIST OF ABBREVIATIONS AND TERMS

2-dG	2'-Deoxyguanosine
8-OHdG	8-Hydroxy-2'-deoxyguanosine
Avg.	Average
°C	Degrees Celsius
DNA	Deoxyribonucleic Acid
ECD	Electrochemical Detector
EDTA	Ethylenediamine Tetraacetic Acid
Exp.	Experiment
HPLC	High Performance Liquid Chromatography
hr.	Hour
µl	Microliter
µM	Micromolar
mM	Millimolar
M	Molar
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
t	Time
UV	Ultraviolet

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CHAPTER 1

INTRODUCTION

The role of DNA in disease pathology has become increasingly important in recent scientific investigations. In attempts to understand the effects of foreign materials and energies on DNA structure and interactions, several types of genetic damage have been characterized. Typically, as cells metabolize life-sustaining reactants, byproducts are produced, which can have negative effects on their internally stored DNA. As these destructive byproducts are allowed to accumulate and cause damage to DNA, several diseases, typically emerging as some form of cancer, can result especially when the affected cells are allowed to proliferate uncontrollably. As a result, numerous diseases, disorders, and deficiencies have become associated with particular aspects of genetic failure and uncorrected mutation.

One type of genetic malfunction that has become commonly studied in contemporary scientific research is the measure of oxidative damage to DNA. Oxidative damage is characterized by free radical components, which are highly reactive molecules that cause damage to cellular mechanisms through the interaction of their unpaired electron. The most important reactions of free radicals in aerobic cells involve molecular oxygen and its radical derivatives, hence the oxidative damage caused by the byproducts of these reactions (De Zwart et al., 1998). There are several processes within a cell that can create reactive oxygen species (ROS) that cause oxidative damage to DNA.

Within aerobic organisms, basic cellular metabolism involves the production of oxygen free radicals as well as non-radical reactive species better known as reactive

oxygen species (Valavanidis et al, 2006). Furthermore, many natural processes that occur within cellular mechanisms use these metabolic by-products to stimulate other types of reactions. These metabolic processes include, but are not limited to, cellular respiration, enzymatic metabolism, and exogenous insults (Bolin et al., 2004). Also, oxidation of DNA components can be induced by a variety of internal and external factors including endogenous cell metabolism products, metals, chemicals, drugs, ionizing radiation, solar light, cigarette smoking, and air pollution (De Martinis and Bianchi, 2002). Typically, biologically advanced organisms are equipped with the appropriate cellular equipment in order to adequately regulate the production of free radicals as well as maintain "redox homeostasis" (Ames et al., 1993). Once an imbalance between the creation and destruction of reactive species occurs within an organism, a state known as oxidative stress ensues; therefore, the regulation and maintenance of such compounds become essential for the physiological health of such organisms (Davies, 1995; Ames et al., 1993). It is this very fact that leads researchers to ascertain the quantity of oxidative stress byproducts that are produced when DNA components are exposed to various stimuli. Furthermore, through testing of the aforementioned quandary, a common trend has emerged throughout the scientific community, making oxidative damage of DNA widely recognized to be at least partially involved in the process of cancer as well as playing some role in aging (De Martinis and Bianchi, 2002).

For some time now, researchers, in detecting when these types of reactions have begun to affect DNA and other cellular components, have used biomarkers of oxidative stress. Biomarkers are supposed to reflect changes in biological systems that are related to "exposure to," or "effects of" xenobiotics or other types of toxic materials/factors (De

Zwart et al., 1998). Currently, there are three recognized types of biomarkers used in scientific experimentation, which include biomarkers of exposure, effects, and susceptibility. While there are several biomarkers of oxidative stress, many prove to be inadequate due to their short lifetimes and extremely low concentrations. Fortunately, one particular biomarker for oxidative stress persists amongst scientists as an adequate determinant for cellular exposure to oxidative stress. Of the approximately 20 adducts of DNA that have been described by science, 8-Hydroxy-2-deoxyguanosine (8-OHdG) is the most studied oxidatively modified DNA base product due to its acute sensitivity and mutagenic potential (De Martins and Bianchi, 2002).

8-Hydroxy-2-deoxyguanosine, a product of oxidative damage to DNA, can be generated by various endogenous and exogenous means. One of the more common methods in which this compound is produced appears to involve metal dependant reactions yielding potent hydroxyl radicals that in return cause damage to DNA. Metals play numerous roles in many biological processes, making them both critically important as well as necessary constituents of cellular mechanisms. Furthermore, humans are constantly exposed to metals in their various oxidative states on a daily basis. From consumption in food and liquids to their presence in the air we breathe, our exposure to metals is ultimately unavoidable. While many biological processes use metallic ions in productive ways to yield products that will be beneficial to the organism, sometimes the presence of metals in reactions that do not require them can lead to harmful effects. Moreover, many times when metals react with a substrate it is not an actual product that is directly generated to cause the negative effects, but it is their presence with the combined substrate that will lead to problems in later reactions. Relative to oxidative

damage of DNA, it is not necessarily the interaction of cationic metals with DNA's anionic components, as much as it is the binding and later reaction of those bound cationic metals with sources of ROS, typically in the form of hydroxyl radicals, that yields the damage at the particular points of binding (Colwell and Morris, 2003). It is believed that this very principle is what influences both the generation of hydroxyl radicals and their attack on DNA at certain sites such as phosphate groups and the nucleoside 2-deoxyguonosine. Also, through the efforts of others it is realized that multiple types of metals can be involved in this process. Unfortunately, a general understanding for the various types of metals has not been generated in relation to their affinity to induce oxidative damage of DNA and the quantity to which the adduct 8-OHdG is produced. While several studies have determined the effects of one particular metal or a specific group of metals, as of now, no one has done a comprehensive study comparing representatives of the different types of metals: particularly transition metals, heavy metals, and cationic metalloids. Hopefully, with this study some understanding can be attributed to various metallic members of the periodic table in relation to their activity in causing oxidative damage to DNA.

This study will examine the effects of various metals on oxidative damage to a specific DNA component using 8-hydroxy-2-deoxyguanosine as the accepted biomarker for analysis. The hypothesis of this study is that certain metals may have an affinity to induce oxidative damage more readily and produce the analyte of interest in greater amounts than others. Moreover, an examination of changes to time and temperature variables in a standard reaction setup should prove to have direct impacts on the generation of 8-hydroxy-2-deoxyguanosine. The metals of interest include iron,

manganese, cobalt, copper, lead, chromium, nickel, cadmium, mercury, and zinc. These issues may be interpreted by assessing the levels of specific biomarkers of oxidative stress via analytical means.

The specific aims to be investigated within the experimental confines of this research are as follows:

1. To examine the effects of several metals on 2-Deoxyguanosine, a nucleoside component of DNA, using *in vitro* means.
2. To observe any differences amongst the various metals in relation to oxidative damage and the generation of 8-Hydroxy-2-deoxyguanosine.
3. To determine the effects of metals on 2-deoxyguanosine by employing methodologies that measure levels of 8-Hydroxy-2-deoxyguanosine using High Performance Liquid Chromatography (HPLC) coupled with an ElectroChemical Detector (ECD) and/or Ultra-Violet Detector (UV).

CHAPTER 2

LITERATURE REVIEW

Generation of Hydrogen Peroxide from Superoxide Radicals

Hydrogen peroxide is a very common molecule that is often a product of everyday biological processes. Unfortunately, this product can also prove to be a highly unstable compound when placed under certain conditions and can become the source of a very undesirable byproduct. Often being a direct or indirect result of several enzymatic processes, hydrogen peroxide is typically produced from superoxide anion radicals, $O_2^{-\bullet}$, that are generated by redox cycling of toxicants or by NADPH oxidase in activated macrophages and granulocytes during moments of rapid cellular respiration (Klaassen, 1996). Moreover, the introduction of various xenobiotics into biological systems often leads to their conversion into radical species by their acceptance of an electron from different reductases in enzymatic mechanisms. Unfortunately, the radicals generated often transfer the additional electron that they possess to molecular oxygen, thus forming the superoxide anion radical and allowing them to regenerate back into their original state to persist further (Klaassen, 1996; Kappus, 1986). These superoxide anion radicals can be converted to hydrogen peroxide via spontaneous conversion or catalysis by the superoxide dismutase (SOD) enzyme. Once hydrogen peroxide has been generated, another toxicologically important radical molecule can persist in the form of the hydroxyl radical. Both the superoxide radical and the hydroxyl radical are notorious forms of Reactive Oxygen Species.

Reactive Oxygen Species

A term used to describe reactive intermediates of oxygen and oxygen containing compounds, reactive oxygen species, are highly energetic molecules that are typically the products of respiration in aerobic organisms and tend to cause damage during their interactions with neighboring biologically relevant compounds. The primary damage to cells resulting from ROS induced alteration typically manifests itself in macromolecules such as proteins, fatty acids in various cellular membrane lipids, and DNA (Hofer and Moller, 1998). While there are numerous means by which oxidative damage may occur in regards to reactive oxygen species, two compounds persist as the most studied forms of ROS. The superoxide radical and hydroxyl radical are well established ROS molecules, and are known to cause damage to critical cellular materials (Valavanidis et al, 2006). Typically, the former is inadvertently generated during mitochondrial transfer of electrons to oxygen in the electron transfer portion of aerobic respiration. Furthermore, the superoxide radical, $O_2^{\cdot}$ -, has a propensity to oxidize several critical cellular molecules including antioxidant vitamins, catecholamines, and thiols. It also has the ability to inactivate various enzymes including the highly important catalase and peroxidases (Kono and Fridovich, 1982; Blum and Fridovich, 1985). Similarly, the hydroxyl radical ($^{\cdot}OH$) is one of the most important and active forms of ROS, exuding a powerful oxidative potential as well as a lack of discrimination when reacting with other cellular components (Jackson and Loeb, 2001). In addition, the formation of this compound typically results from a metal-dependant decomposition of hydrogen peroxide, H_2O_2, as well as other hydroxyl containing molecules (Colwell and Morris, 2003). Ultimately, it is the polyanionic nature of several important biomolecules, including DNA and cell

membranes, that provide a substrate for the adherence of metals and other cations. This adherence then has the capability of initiating Fenton or Fenton-like reactions that generate hydroxyl radicals and result in oxidative damage at very specific locations (Ercal et al., 2001; Colwell and Morris, 2003).

Fenton Generation of Hydroxyl Radicals

Hydroxyl radicals are often the direct result of the homolytic cleavage of hydrogen peroxide (Klaassen, 1996). While there are several means by which this process may occur, historically, transition metals have been known to accomplish this task with great efficiency. In addition, the Fenton reaction, a term given to the degradation of hydrogen peroxide via metal catalysis, is a major toxification mechanism for hydrogen peroxide, its precursor the superoxide anion, and various transition metals (Klaassen, 1996). Typically, the metal of choice for this reaction is iron; however, copper, manganese, chromium, and nickel all have the potential to be just as effective and are considered to be the most common transition metals associated with Fenton-like reactions. The following equation exemplifies the process by which hydroxyl radicals are produced via Fenton or Fenton-like reactions:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + \cdot OH + OH^-$$

In the above reaction, M^{n+} represents a metal ion prior to reaction with hydrogen peroxide, and $M^{(n+1)+}$ represents the same metal ion with an increased oxidative state after its electron(s) have been stripped during the cleavage of hydrogen peroxide into a hydroxyl radical and a hydroxyl ion (Colwell and Morris, 2003). The image below (Figure 1) represents the process by which hydroxyl radicals are created, beginning with

the production of hydrogen peroxide from the superoxide anion radical (a mechanism described previously).

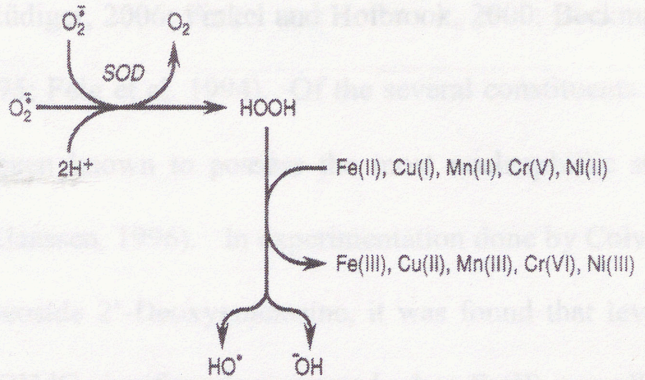


Figure 1. Represents the production of hydrogen peroxide from the superoxide anion radical

(Klaasen (ed.), 1996, Chapter 3: Mechanisms of Toxicity, p.41, McGraw Hill Health Professions Division, USA)

To further exacerbate the effects of this reaction, several metals are believed to bind to components of DNA at specific nucleophillc sites that provide locations for DNA insults to occur (Cowell and Morris, 2003; Noblitt et al., 2007).

Metals, Oxidative Damage, and DNA

In attempts to understand interactions of xenobiotics with DNA, scientists have explored numerous factors, both endogenous and exogenous, influencing the integrity of this delicate and extremely critical macromolecule. In the process of genetic exploration many have come to realize that oxidative damage to DNA is an important factor in the pathophysiological processes involved in the development of cancer and acceleration of aging (Pilger and Rüdiger, 2006). Furthermore, reactive oxygen species in the form of oxygen-based free radicals have been observed to cause various types of damage that include, but are not limited to, DNA single and double strand breaks and oxidation of bases that can lead to mutations.(Pilger and Rüdiger, 2006; Hofer and Moller, 1998;

Breen and Murphy, 1995). These same malformations are believed to be involved in the mechanisms involved in carcinogenesis, tumor formation, liver damage, and accelerated aging (Pilger and Rüdiger, 2006; Finkel and Holbrook, 2000; Beckman and Ames, 1997; Wiseman et al., 1995; Feig et al. 1994). Of the several constituents of DNA, the purine base guanine has been known to possess the most nucleophillic site, N7 position, of genetic structure (Klaassen, 1996). In experimentation done by Colwell and Morris with Fe(II) and the nucleoside 2'-Deoxyguanosine, it was found that levels of the oxidative damage product 8-OHdG significantly increased when Fe(II) was allowed to bind to the nucleoside prior to the addition of hydrogen peroxide (2003). Over time, other experiments have been performed examining the relationship between oxidative damage, DNA, and several metals including copper, chromium, cobalt, and zinc (Cowell and Morris, 2003; Noblitt et al., 2007; Ivancsits et al., 2002).

8-Hydroxy,2-Deoxyguanosine

The formation of 8-Hydroxy-2-deoxyguanosine (8-OHdG) through the use of oxygen radicals was first reported by Kasai and Nishimura in 1984 while making observations about the effects of mutagens on heated glucose (Kasai, 1997; Kasai et al., 1984; Kasai and Nishimura, 1984). Further exploration has revealed that during conditions of severe oxidative stress the c-8 position of 2′-deoxyguanosine, which is a constituent of DNA, is hydroxylated, and the result produces an altered form of the constituent known as 8-hydroxy-2-deoxyguanosine (Hayashi et al., 1999). Below is a depiction, Figure 2, of this reaction obtained from the Japanese Institute for the Control of Aging Web site (2007).

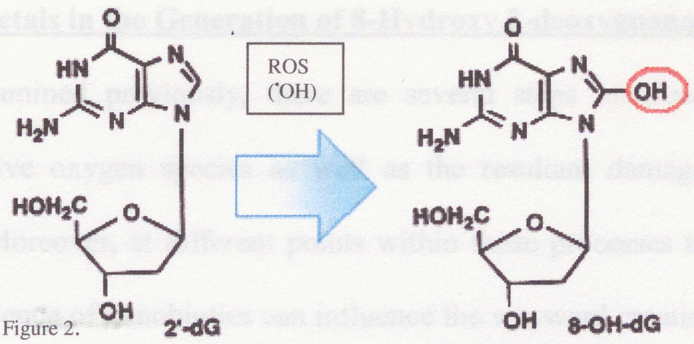


Figure 2.

Formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) by oxigen radicals.
H Kasai: Environmental Mutagen Research, Vol. 10, p73-78 (1988)

Through subsequent experiments with this particular compound, it has been observed that various agents are effective in the hydroxylation of the deoxyguanosine residue in DNA (Piger and Rüdiger, 2006; Kasai, 1997). Also, this particular compound provides dual roles by not only serving as a biomarker of oxidative stress, but also having biological importance through its promutagenic properties. 8-OHdG is considered to be promutagenic due to its tendency to preferentially pair with adenine over cytosine during DNA replication, thus inducing G:C to T:A transversions (Bolin et al., 2004). While the ultimate results of 8-OHdG accumulation are unclear, it is known to have deleterious effects in both proliferating and differentiating cells leading to either the initiation step of carcinogenesis in proliferating cells or apoptosis in differentiating cells (Bolin et al., 2004). Furthermore, the transversions attributed to the formation of 8-OHdG are frequent recurrences in human cancers and tend to be widespread in the mutational spectrum of the p53 tumor suppressor gene (Pilger and Rüdiger, 2006; Hollstein et al., 1996). The tendency of this compound to cause mutagenic effects on DNA is a highly significant aspect of this particular biomarker since genetic alteration has directly been connected to the presence of this molecule, linking it to carcinogenesis (Bolin et al., 2004).

The Use of Metals in the Generation of 8-Hydroxy,2-deoxyguanosine

As examined previously, there are several steps involved in the process of creating reactive oxygen species as well as the resultant damage produced by their production. Moreover, at different points within those processes there are instances in which the presence of xenobiotics can influence the wayward creation of rather insidious forms of genetic damage. 8-Hydroxy-2-deoxyguanosine exemplifies such a compound whose production is dependant upon the direct fabrication of ROS. As stated earlier, metals have the capacity to intervene in the generation of reactive oxygen species and the ultimate creation of any resultant 8-OHdG. For several years now, many scientists have delved into trying to understand how individual species of metal ions affect the outcome of oxidative damage to DNA from various aspects including Fenton based chemistry. As a result, a variety of conclusions have been made in regards to multiple facets such as exposing DNA to ROS, proper excision of 8-OHdG from genetic material, proper DNA extraction procedure for oxidatively damaged products, proper selection of the appropriate means of analysis, and binding of metal ions to DNA constituents.

Several metals are sequestered by biological systems on a regular basis to later become integral components as either direct constituents of macromolecules or as catalysts of critical reactions. For humans, these metals comprise a group loosely defined as essential metals, whose members include cobalt, copper, iron, magnesium, manganese, molybdenum, selenium, and zinc (Klaassen, 1996). Iron, one of the more commonly used of the essential metals, is a very important biological trace metal whose presence can be both beneficial and harmful to the organisms that employ it to thrive. With regard to 8-Hydroxy-2-deoxyguanosine, iron tends to also be one of the more commonly used

metals involved in experiments because of its characteristic properties in the Fenton based chemistry that are used to generate hydroxyl radicals.

One of the more notable studies on iron that has been conducted fairly recently is that of Colwell and Morris. In the study, attempts to gain some insight into the generation of 8-OHdG from the nucleoside 2-deoxyguanosine using Fe(II) mediated oxidative damage were examined (2003). Moreover, this group of scientists was able to present evidence of iron binding to 2-dG in the course of their study. As reported by Colwell and Morris, the analyte of interest, 8-OHdG, was produced in significantly higher amounts when the iron ions in their reaction were allowed time to "bind" to the nucleoside prior to the addition of hydrogen peroxide, the source of ROS for their experimental reactions (2003). The idea of metal binding to DNA substrates is a fairly new approach to the investigation of oxidative damage to DNA. The thought surrounding this issue emanates from the realization of investigators that the resultant damage to the guanine base of DNA is highly specific; however, the ability of the hydroxyl radical to persist in reaction conditions is very limited (Colwell and Morris, 2003; Klaassen, 1996; Roots and Okada, 1975). This suggests that DNA has the capability to bind metal ions at specific locations, and the reaction of hydrogen peroxide at or near the site of binding results in the apparently selective manner in which any hydroxyl radicals formed cause damage (Colwell and Morris, 2003). Moreover, this offers an explanation as to why the short lifetime of the hydroxyl radical, $\sim 4 \times 10^{-9}$s in experimental conditions, is still able to affect components of DNA in proximity to its existence (Klaasen, 1996; Colwell and Morris, 2003).

Similar studies by Noblitt and colleagues further substantiate this point in a study that not only used iron, but copper and chromium as well (2007). In their study, the metals of interest were allowed to react with the nucleoside, 2-deoxyguanosine, and the nucleotide, 2-deoxyguanosine-5'-monophosphate in an attempt to examine the influence of the additional phosphate group in relation to the guanine base with respect to site specific oxidative damage (Noblit et al., 2007). The results of this study report the formation of 8-OHdG in reactions involving all three elements. However, iron and copper exuded trends of metallic binding of the DNA constituents in a manner that more or less promoted the generation of 8-OHdG while chromium displayed a behavior that was more favorable for the integrity of the two DNA components (Noblit et al., 2007).

The effects of other metals, such as cobalt, nickel, cadmium, lead, vanadium, and zinc, on the generation of 8-OHdG have been explored by numerous scientists over the years. Experiments such as those performed by Mikhailova et al., Lloyd and Phillips, Kawanishi et al., Prasad et al., and Ivancsits et al. explore the effects of cadmium; copper, iron, and nickel; various nickel compounds; zinc; and cobalt, respectively, on DNA and its components via direct interaction or cellular exposure studies (1997, 1999, 2001, 2004, 2002). While these studies are very broad in investigating different properties in the generation of 8-OHdG, common terminology such as Fenton chemistry, reactive oxygen species, and oxidative DNA damage all persist during the examination of their respective hypotheses, which tend to result in the conclusion that 8-OHdG is created in various amounts depending upon the metal under investigation and the experimental conditions that were implemented. Unfortunately, there are not many studies that have compared a large group of metals, especially greater than three, and those that may have

looked at a small group of metals often explore the same transition metals or small nuances among the different oxidative states of a particular element. Ultimately, a broader comparison is needed in an attempt to gain an understanding of which element is generally better at mediating the generation of reactive oxygen species and their ultimate damage to DNA.

CHAPTER 3

DESIGN OF THE STUDY

Materials and Reagents

The following materials used in this study were purchased from Sigma Aldrich Inc., USA.

Metallic Salts

- Manganese(II) Sulfate Monohydrate ($MnSO_4 \cdot H_2O$)
- Cobalt Sulfate Hydrate ($CoSO_4 \cdot xH_2O$)
- Nickel(II) Sulfate Heptahydrate ($NiSO_4 \cdot 7H_2O$)
- Zinc Sulfate Heptahydrate($ZnSO_4 \cdot 7H_2O$)
- Cadmium Sulfate 8/3-Hydrate ($CdSO_4 \cdot {}^{8}/_{3}H_2O$)
- Iron(II) Sulfate Heptahydrate ($FeSO_4 \cdot 7H_2O$)
- Copper(II) Sulfate Pentahydrate ($CuSO_4 \cdot 5H_2O$)
- Chromium(III) Sulfate Hydrate ($Cr_2(SO_4)_3 \cdot xH_2O$)
- Mercury(II) Nitrate Monohydrate ($Hg(NO_3)_2 \cdot H_2O$)
- Lead(II) Nitrate ($Pb(NO_3)_2$)

Standards

- 8-Hydroxy-2'-deoxyguanosine
- 2'-Deoxyguanosine Monohydrate

The following materials used in this study were purchased from Fisher Scientific Inc., USA:

Salts

- Citric Acid
- Sodium Acetate

Reagents

- Deionized Ultra Filtered Water (D.I.U.F.)
- 3% Hydrogen Peroxide, Certified
- Acetic Acid, Glacial, HPLC Grade
- Methanol, Optima Grade
- Acetonitrile, Optima Grade

Equipment

- Stovall Shaking Water Bath
- Laboratory Thermometer

The following reagent used in this study was purchased from Amresco Inc., USA:

- 0.5M Ethylenediamine Tetraacetic Acid (EDTA)

The following materials used in this study were purchased from Phenomenex, USA:

Analytical Column

- Synergi 4u Hydro-RP 80A, 75 x 4.6mm
- SecurityGuard Cartridges AQ C-18, 4 x 3mm

Generation of Reactive Oxygen Species

In vitro studies involving the DNA adduct 2-deoxyguanosine (2-dG) were used in an attempt to generate the analyte of interest, 8-hydroxy,2-deoxyguanosine, from the individual interaction of the various test metals with hydrogen peroxide. A method similar to that generated by Colwell and Morris (2003) was employed; however, the metals used were expounded upon in an attempt to observe differences among the various metals used. Sulfated hydrates of iron, nickel, cadmium, copper, zinc, chromium, cobalt, aluminum, and manganese were used in this study. Also, hydrogen peroxide was introduced into all the reactions to act as the source of hydroxyl radicals, the driving force for oxidation of the nucleoside base, 2-dG. The use of 2-dG was employed because it is a well established precursor to 8-OHdG. Furthermore, purified 2-dG was chosen to be used directly in a reaction mixture with the other constituents because results can be observed without the impedance of cellular mechanisms designed to either eliminate or reduce reactive oxygen species and the products of oxidative damage. Part I of this methodology was used to strictly observe the effectiveness among the various metals at generating oxidative damage to 2-Deoxyguanosine. Part II of this methodology was used with the more successful metallic species from Part I with the intention of monitoring the generation of 8-Hydroxy-2-deoxyguanosine over time as well as observing the effects of temperature on the experimental reactions. The analysis of Part I reactions involved a High Performance Liquid Chromatography (HPLC) system with an Ultraviolet (UV) detector which exuded an excellent ability at observing the various reaction samples and had the ability to quantitate moderate levels of 8-OHdG. For further analysis, Part II reactions were analyzed via an HPLC system with an Electrochemical Detector (ECD).

This system allowed for lower detection of the analyte of interest, resulting in an added dimension of accuracy.

Part I

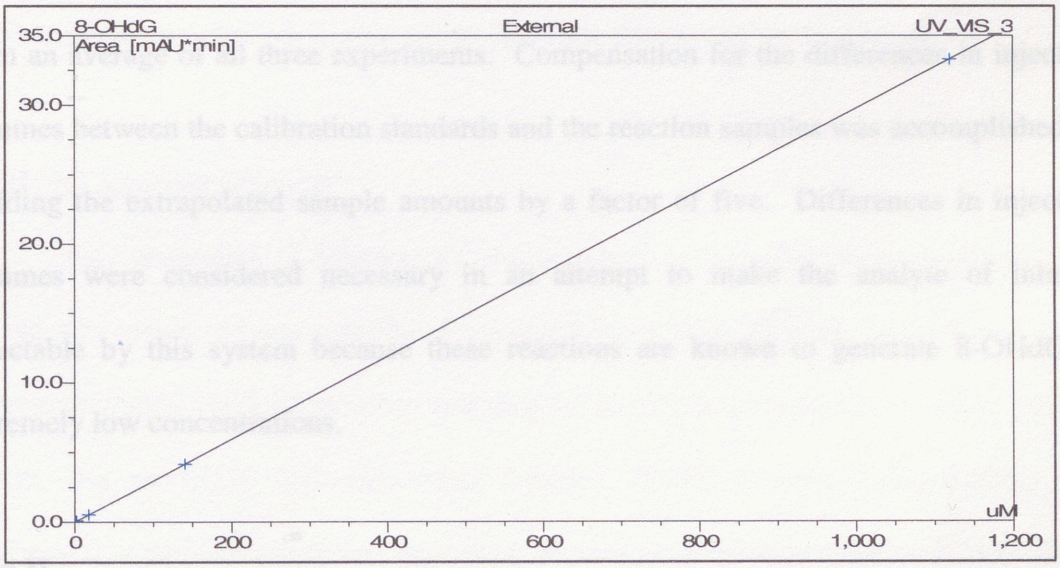
As outlined by Colwell and Morris (2003), a stock solution of 2.5mM 2-Deoxyguanosine obtained from Sigma Aldrich was prepared in Deionized Ultra Filtered (D.I.U.F.) water obtained from Fisher Scientific. Next, fresh 100mM stock solutions of the various metallic salts were made by reconstituting them in degassed D.I.U.F. water. The water was degassed with helium just prior to hydrating the metallic salts in an attempt to reduce the oxidation of the metal ions by any oxygen species present. The following metallic salts used in these reactions were purchased at the highest purity available from Sigma Aldrich Incorporated: Manganese(II) Sulfate Monohydrate ($MnSO_4 \cdot H_2O$), Cobalt Sulfate Hydrate ($CoSO_4 \cdot xH_2O$), Nickel(II) Sulfate Heptahydrate ($NiSO_4 \cdot 7H_2O$), Zinc Sulfate Heptahydrate($ZnSO_4 \cdot 7H_2O$), Cadmium Sulfate 8/3-Hydrate ($CdSO_4 \cdot {}^{8}/_{3}H_2O$), Aluminum Sulfate Octadecahydrate ($Al_2(SO_4)_3 \cdot 18H_2O$), Iron(II) Sulfate Heptahydrate ($FeSO_4 \cdot 7H_2O$), Copper(II) Sulfate Pentahydrate ($CuSO_4 \cdot 5H_2O$), and Chromium(III) Sulfate Hydrate ($Cr_2(SO_4)_3 \cdot xH_2O$). Once all solutions were prepared, reaction mixtures totaling 0.5ml were combined in 1.5ml microcentrifuge tubes. The make up of each reaction mixture was as follows: 1) 380µl of the 2.5mM 2-dG solution was combined with each test metal at a concentration of 25mM obtained from diluting the previously made 0.1M stock solution; 2) the mixture was briefly vortexed and allowed to incubate at room temperature for two minutes; 3) following the incubation, 14µl of water was added so that a volume of 470µl could be achieved prior to the

addition of the final two components; 4) 5µl of 3% hydrogen peroxide (Fisher Scientific) was added, the reaction mixture was lightly vortexed and allowed to incubate for ten seconds; 5) finally, 25µl of 0.1M Ethylenediamine Tetraacetic Acid (EDTA) (Amresco Inc.) was added to complete the reaction mixture. Prior to the addition of the EDTA and H_2O_2 to the reaction mixture, the brief incubation time of two minutes was implemented to allow any possible binding of the metallic cations to the 2-dG structure. Ten seconds were allowed to pass between the addition of the hydrogen peroxide and the chelation of the reaction by EDTA in an attempt to give enough time for the creation of any hydroxyl radicals. A one-hour incubation of each reaction mixture occurred at ambient temperature (~24°C ± 2 degrees), once all components had been added to the microcentrifuge tube. For incubations at ambient conditions, the final reaction mixtures were put in a closed laboratory drawer which provided the proper temperature conditions, and shielded the reactions from light sources that could possibly contribute to variability in results. After the one-hour incubation period, all reaction mixtures were immediately analyzed using a Dionex HPLC with a UV detector. It is important to note that all reaction components were created or diluted using high purity D.I.U.F. or HPLC grade water.

8-OHdG Analysis (Part I)

Any generation of 8-hydroxy-2-deoxyguanosine from the experimental reactions above was monitored and quantitated using a Dionex Summit High Performance Liquid Chromatography (HPLC) system consisting of an ASI-100 automated sample injector, a P680 gradient pump, and a UVD170U UV-Vis Detector. Separations were performed at

ambient conditions according to methods similar to those created by Cordis et al. and implemented by Colwell and Morris. (1998; 2003). A Synergi Hydro-RP (4µ, 80A, 75 x 4.6mm) polar embedded, reverse-phase C18 column with guard was purchased from Phenomenex Incorporated, and employed in conjunction with an isocratic mobile phase consisting of 0.1% aqueous acetic acid in 3% acetonitrile at a flow rate of 2ml/min. The 8-OHdG analyte was monitored at a wavelength of 250nm, and the typical retention time for this compound was approximately 3.7 minutes in this system. Injection volumes of 25µl were used for analysis of the experimental reactions. Prior to analysis of experimental reactions, a calibration curve was constructed using 1.12mM to 1.094µM concentrations of 8-OHdG (Sigma Aldrich) in water. The typical R^2 value for these curves at the concentrations listed above would be 0.999 or better, exuding excellent linearity for the constraints of this system. An example of a reaction curve resultant of such parameters can be observed below (Figures 3-4).



No.	Ret.Time min	Peak Name	Cal.Type	Points	Coeff.Det. %	Offset	Slope	Curve
1	3.80	8-OHdG	Lin	5	100.0000	0.0000	0.0297	0.0000

Figure 3 Displays a typical calibration curve resultant from the various 8-OHdG concentrations used to calibrate the HPLC-UV system.

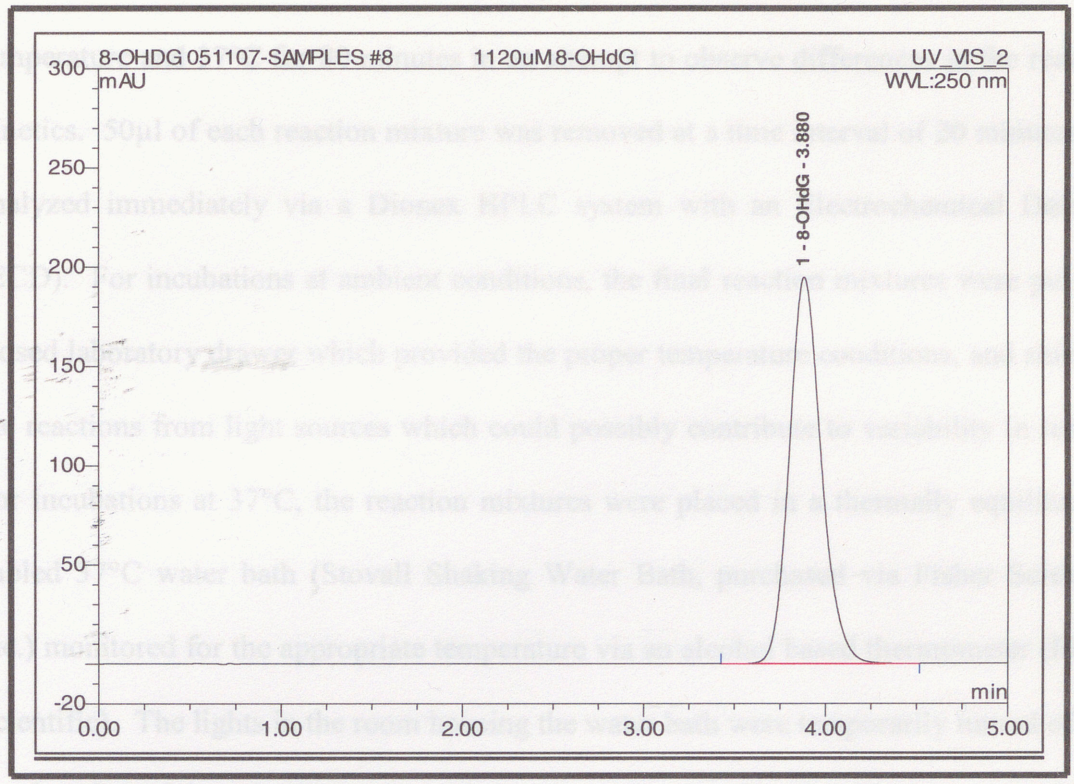


Figure 4. Displays a typical chromatograph of the 8-OHdG standard used to calibrate the HPLC-UV system.

All reactions were independently performed in triplicate with results being correlated from an average of all three experiments. Compensation for the differences in injection volumes between the calibration standards and the reaction samples was accomplished by dividing the extrapolated sample amounts by a factor of five. Differences in injection volumes were considered necessary in an attempt to make the analyte of interest detectable by this system because these reactions are known to generate 8-OHdG in extremely low concentrations.

Part II

The setup for these reactions involved the same procedure outlined in Part I; however, the incubation of these reactions in the final step occurred at both room

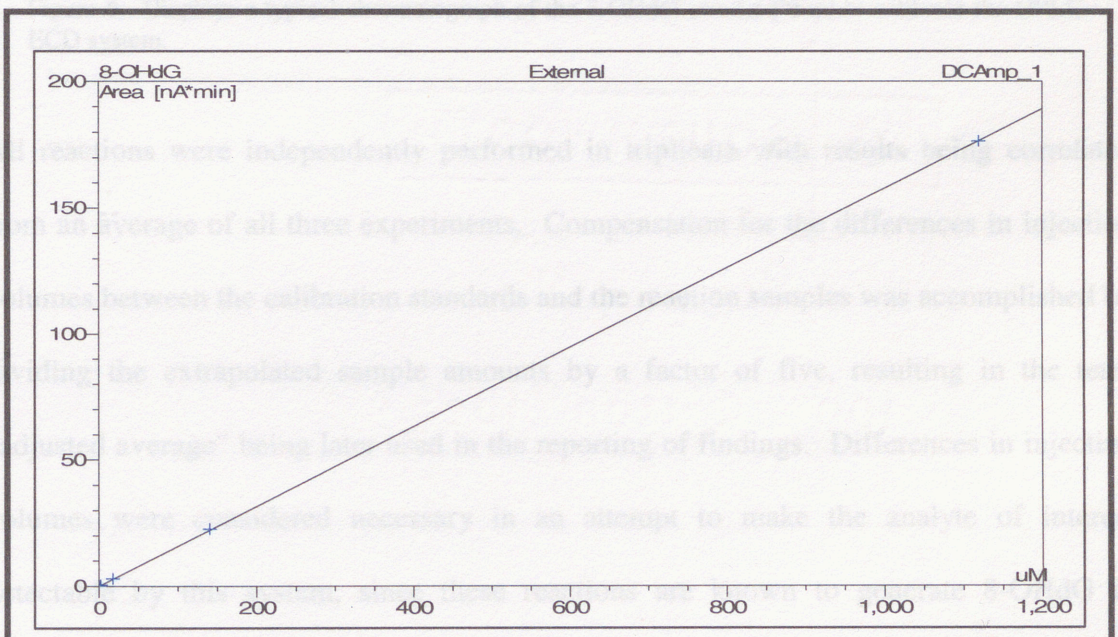
temperature and 37°C for 80 minutes in an attempt to observe differences in the reaction kinetics. 50µl of each reaction mixture was removed at a time interval of 20 minutes and analyzed immediately via a Dionex HPLC system with an Electrochemical Detector (ECD). For incubations at ambient conditions, the final reaction mixtures were put in a closed laboratory drawer which provided the proper temperature conditions, and shielded the reactions from light sources which could possibly contribute to variability in results. For incubations at 37°C, the reaction mixtures were placed in a thermally equilibrated, gabled 37°C water bath (Stovall Shaking Water Bath, purchased via Fisher Scientific Inc.) monitored for the appropriate temperature via an alcohol based thermometer (Fisher Scientific). The lights in the room housing the water bath were temporarily turned off for the duration of the experiment for added measures in ensuring that light was not a factor contributing to variability of results. The temperature parameter allowed for an added dimension in this study by providing insight into how 8-OHdG is generated over time as well as observing changes in this pattern amongst the various metal species.

8-OHdG Analysis (Part II)

Any generation of 8-hydroxy-2-deoxyguanosine from the experimental reactions above was monitored and quantitated using a Dionex DX-120 High Performance Liquid Chromatography (HPLC) system consisting of an AS50 autosampler, a GS50 gradient pump, and an ED50 Electrochemical Detector (ECD) Detector. Separations were performed at ambient conditions according to methods similar to those created by Zhang et al., 2004. A Synergi Hydro-RP (4µ, 80A, 75 x 4.6mm) polar embedded, reverse-phase C18 column with guard was purchased from Phenomenex Incorporated, and employed in

conjunction with an isocratic mobile phase consisting of 125mM citric acid, 250mM sodium acetate, 100mM acetic acid, and 10% methanol at a flow rate of 1ml/min. The amperometry based ECD cell was set to 0.85V, and the typical retention time for this compound was approximately 3.0 minutes in this system. Injection volumes of 25µl were used for investigation of the experimental reactions.

Prior to analysis of experimental reactions, a calibration curve was constructed using 1.12mM to 0.0547µM concentrations of 8-OHdG (Sigma Aldrich) in water using an injection volume of 5µl. The typical R^2 value for these curves at the concentrations listed above would be 0.999 or better exuding excellent linearity for the constraints of this system. An example of a reaction curve and peak resultant of such parameters can be observed below (Figure 5-6).



No.	Ret.Time Min	Peak Name	Cal.Type	Points	Coeff.Det. %	Offset	Slope	Curve
1	2.98	8-OHdG	Lin	5	99.9982	0.0000	0.1576	0.0000

Figure 5. Displays a typical calibration curve resultant from the various 8-OHdG concentrations used to calibrate the HPLC-ECD system.

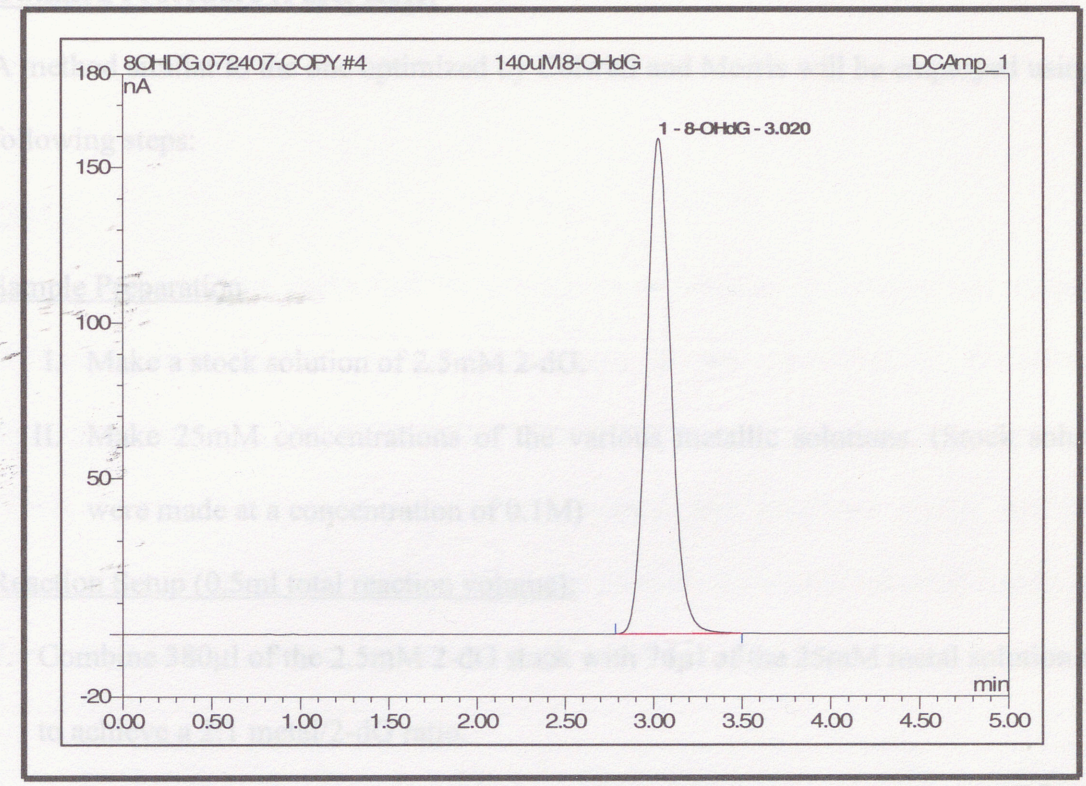


Figure 6. Displays a typical chromatograph of the 8-OHdG standard used to calibrate the HPLC-ECD system.

All reactions were independently performed in triplicate with results being correlated from an average of all three experiments. Compensation for the differences in injection volumes between the calibration standards and the reaction samples was accomplished by dividing the extrapolated sample amounts by a factor of five, resulting in the term "adjusted average" being later used in the reporting of findings. Differences in injection volumes were considered necessary in an attempt to make the analyte of interest detectable by this system, since these reactions are known to generate 8-OHdG in extremely low concentrations.

Outlined Procedure (Parts I&II)

A method similar to the one optimized by Colwell and Morris will be employed using the following steps:

Sample Preparation

I. Make a stock solution of 2.5mM 2-dG.

II. Make 25mM concentrations of the various metallic solutions. (Stock solutions were made at a concentration of 0.1M)

Reaction Setup (0.5ml total reaction volume):

1. Combine 380µl of the 2.5mM 2-dG stock with 76µl of the 25mM metal solution so as to achieve a 2:1 metal/2-dG ratio.
2. Briefly vortex, and allow mixture to sit for 2 minutes. (This allows for any possible binging of the metallic cations to the 2-dG structure.)
3. Add 14µl of H_2O. (compensates for differences in total final volume)
4. Add 25µl of 0.1M EDTA. (a chelating agent that helps prevent free metallic ions from interacting with any 8-OHdG formed (Colwell and Morris, 2003))
5. Add 5µl of 3% hydrogen peroxide to the reaction mixture.
6. Allow reaction to incubate at room temp. and/or 37°C for 1 hr.
7. (If Applicable) Collect samples from the reaction mixtures at intervals of 20 minutes, starting with the initial reaction at t = 0.
8. Analyze using HPLC-ECD and/or HPLC-UV. (Injection volume=25 µl)
9. Quantitate data via calibration curve previously acquired using an 8-OHdG standard.

**All solutions and reagents are to be prepared using HPLC grade water or better.

CHAPTER 4

RESULTS AND DISCUSSION

Part 1

The reactions performed were done in an attempt to observe any noticeable differences between the various metals used in the generation of 8-Hydroxy-2-deoxyguanosine from the nucleoside, 2-Deoxyguanosine. The following supplements (Table 1 and Figures 7-9) depict the numerical and graphical results for Part 1 experiments. Starting with Table 1, this table presents the overall adjusted results of the Part 1 experiments in terms of 8-OHdG produced in micromolar (μM) concentrations.

	Cr	Ni	Co	Cd	Pb	Cu	Mn	Fe
Exp. 1	0.1111	0.0288	0.1166	0	0	0.0918	0.8681	89.7411
Exp. 2	0.1139	0	0.1045	0	0	0.0878	0.7159	87.5126
Exp. 3	0.1031	0	0.1094	0	0	0.0864	0.6807	96.1500
Average	0.1094	0.0096	0.1101	0	0	0.0887	0.7549	91.1346
Std. Dev.	0.0056	0.0166	0.0061	0	0	0.0028	0.0996	4.4842
Rel. Std. Dev.	5.1462	173.2051	5.5057	0	0	3.1445	13.1870	4.9204

Table 1. Displays the overall results from experiments testing the individual introduction of the metals Chromium, Nickel, Cobalt, Cadmium, Lead, Copper, Manganese, and Iron in the standard reaction setup previously described. The results in this table are the amounts (uM) of 8-OHdG produced from each experiment as well as the average, standard deviation, and relative standard deviation of each set of reactions.

Figures 7-9 graphically represent the data of Table 1. Beginning with Figure 7, this image plots the averages of 8-OHdG produced from each of the nine metals used in the experimentation of Part 1. Figure 8 excludes the results from Iron testing which adjusts the scale of the data plotted in the previous figure, allowing for a more substantial examination of the metals producing 8-OHdG in lesser amounts. Finally, Figure 9

compares the results of the three most effective, non-ferrous metals used in the experimentation of Part 1.

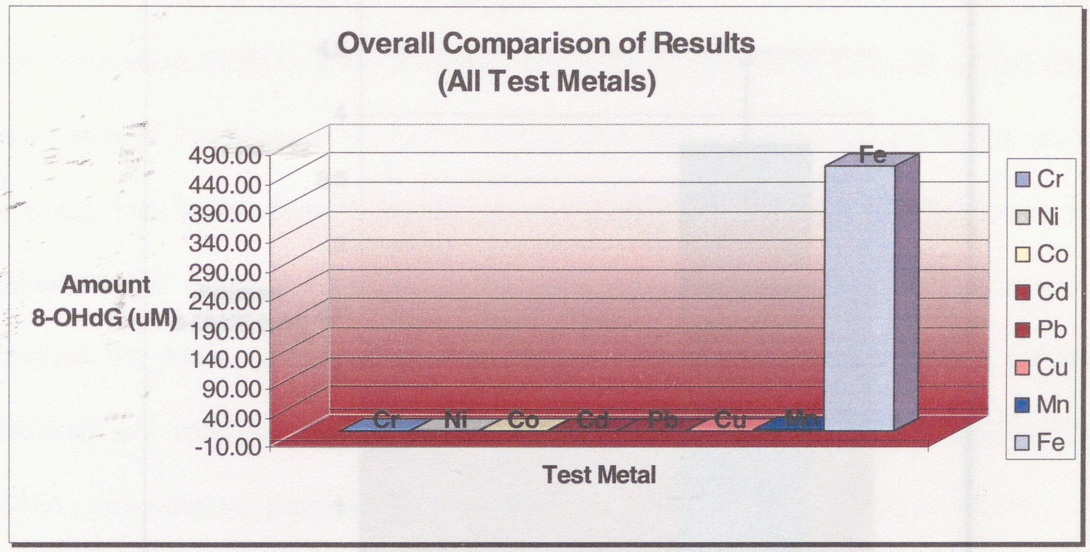


Figure 7. Graphically depicts the overall results from experiments testing the effectiveness of individual introduction of the metals Chromium, Nickel, Cobalt, Cadmium, Lead, Copper, Manganese, and Iron in terms of 8-OHdG produced via the standard reaction setup previously described.

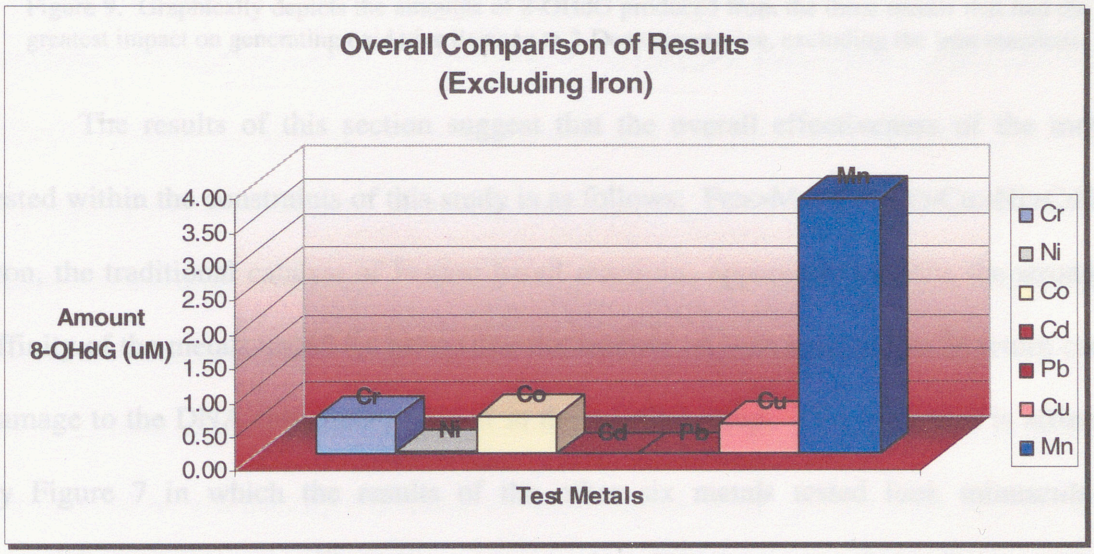


Figure 8. Graphically depicts the amounts of 8-OHdG produced from the test metals Manganese, Copper, Lead, Cadmium, Cobalt, Nickel, and Chromium, independent of the Iron results so that these results could be viewed in greater depth.

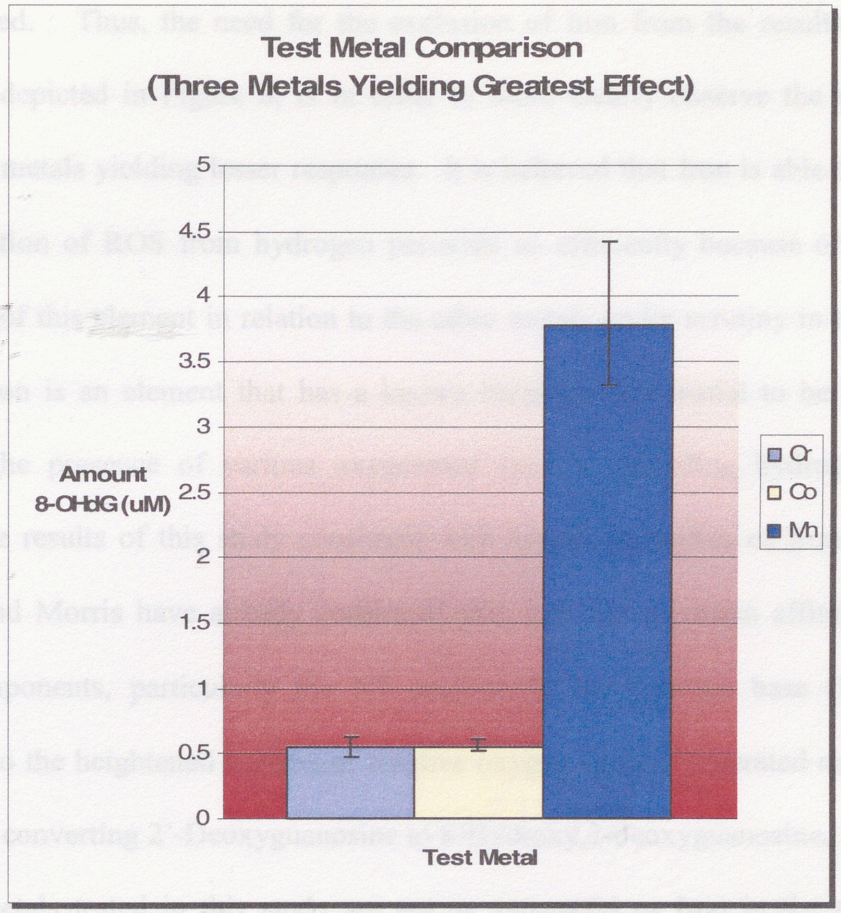


Figure 9. Graphically depicts the amounts of 8-OHdG produced from the three metals that had the greatest impact on generating oxidative damage to 2-Deoxyguanosine, excluding the Iron reactions.

The results of this section suggest that the overall effectiveness of the metals tested within the constraints of this study is as follows: Fe>>Mn>Co>Cr>Cu>Ni>Cd/Pb. Iron, the traditional catalyst of Fenton based reactions, apparently exhibits the strongest affinity of the metals tested for generating the reactive oxygen species that in return cause damage to the DNA constituent present in the reaction setup. This statement is affirmed by Figure 7 in which the results of the other six metals tested look minuscule in comparison to the overwhelming amount of 8-OHdG generated by Iron mediated reactions. Furthermore, according to the averages reported in Table 1, Iron was able to produce more 8-OHdG than the next closest metal, Manganese, by a factor greater than

one hundred. Thus, the need for the exclusion of Iron from the results of the other metals as depicted in Figure 8, is in order to more clearly observe the effects of the remaining metals yielding lesser responses. It is believed that Iron is able to accomplish the generation of ROS from hydrogen peroxide so efficiently because of the physical properties of this element in relation to the other metals under scrutiny in this study. In general, Iron is an element that has a known heightened potential to be very reactive when in the presence of various oxygenated species, including hydrogen peroxide, making the results of this study consistent with known properties of Iron. Studies by Colwell and Morris have already confirmed that Iron has a certain affinity to bind to DNA components, particularly the N7 position of the guanine base (2003). This attributes to the heightened success of reactive oxygen species generated near the site of damage in converting 2'-Deoxyguanosine to 8-Hydroxy,2-deoxyguanosine. It is believed that the metals tested in this study are not as successful as Iron in the production of oxidative damage because of 1) dissimilar or inadequate chemical properties of the less effective metals in binding to the nucleoside and ultimately directing the site specific damage of any ROS created in the reaction mixture, and 2) the efficiency of the other metals at inducing Fenton like reactions which cleave the hydrogen peroxide added to the reaction mixtures resulting in the hydroxyl radicals available to cause damage. Also, Iron in its 2^+ state appears to be a highly reactive ion that is easily oxidized, thus it is readily stripped of an electron by the strong oxidizing agent, hydrogen peroxide, present in the reaction mixtures. This aspect is later validated by the examination of the results in relation to the generation of 8-OHdG by the more successful metals of this experiment in Part 2 of this study.

The next highest mediators of 8-OHdG production, displayed in Figure 9, were Manganese(II), Cobalt(II), and Chromium(III). Manganese and Cobalt, the second and third most effective metals in this study, have properties very similar to Iron. Apparently, the mild differences between these metals and Iron are enough to severely retard the ability of Manganese and Cobalt to catalyze the decomposition of Hydrogen Peroxide; therefore, impeding their ability to generate the assumably large amounts of hydroxyl radicals needed to cause the same amount of oxidative damage accomplished by the Iron mediated reactions. While Cobalt and Manganese are known to be oxidized with relative ease, it is believed that neither has a strong affinity to bind as effectively to the nucleoside substrate in order to direct the path of the ROS generated. Also, Manganese tends to radiate a stronger ability to be readily oxidized, which contributes to explaining why the amounts shown in Figures 8-9 are greater than that of Cobalt since the efficiency of Manganese to mediate the generation of ROS would most likely be greater. Unlike Manganese and Cobalt, which may exude some potential for binding to the nucleoside present in the experimental reactions, it is believed that Chromium(III) would have even lesser capability, if any (Noblitt et al., 2007). This would mean that the effects of Chromium mediated oxidative damage would have to rely more heavily on its ability to create hydroxyl radicals, making it less effective at causing site specific damage. The same can be noted for the reactions involving Copper(II), even though it was not included in the top three producers of 8-OHdG (outside of Iron(II)).

The remaining metals tested, Lead, Cadmium, Nickel, Zinc, and Mercury, resulted in extremely low or no amounts of 8-OHdG production via the confines of this experimental setup. Two of these metals were eliminated during the preliminary phase of

this study due to findings during experimentation or information discovered during intense review of the literature compiled. Mercury, the first to be eliminated, was removed from the list of test metals because of solubility problems encountered during the making of the 100mM stock solutions. While two different salts of Mercury were used in experiments, Mercuric sulfate and Mercuric nitrate, both were observed to be mildly soluble in water making the known concentrations of the compound in solution indeterminable since a homogeneous mixture could not be attained. Zinc, on the other hand, was actually found to possess antioxidant effects during a literature search in the preliminary stages of this experimentation (Prasad et al., 2004). As a result, experimentation involving the metal was ceased because no amount of 8-OHdG was produced at the time, thus superficially agreeing with the findings of that journal article. As far as the remaining metals, it is believed that a limited amount to no 8-OHdG was produced as a result of the chemical and physical nature of the metals themselves. While these metals may be capable of generating reactive oxygen species and causing damage to DNA via some other pathway, the constraints of this experiment were not conducive to the generation of 8-Hydroxy,2-deoxyguanosine. As stated by Colwell and Morris, 8-OHdG formation from Fenton-based chemistry can be highly variable depending on the reaction conditions as well as the ability of the metal to bind to the original substrate being used (2003). Therefore, while Lead, Cadmium, and Nickel did not display an affinity for the generation of 8-OHdG in this reaction setup, other pathways or mechanisms may still involve these metallic species in generating metal mediated oxidative damage to DNA.

Part 2

The reactions performed were done in an attempt to observe the effects of temperature and time on the generation of 8-OHdG from the more effective test metals determined in Part 1 of this study. The metals included in this section were Iron, Manganese, Cobalt and Chromium. The following supplements (Tables 2-5 and Figures 10-13) depict the graphical results for the Part 2 experiments. Tables 2-5 represent the amounts of 8-OHdG generated from Iron, Manganese, Cobalt, and Chromium for each experiment at each time and temperature interval, respectively. Figures 10-1 and 10-2, represent the overall values and adjusted averages of the time and temperature data for experiments involving Iron. Figure 10-3 depicts the data from the previous image with the addition of trend lines to aid in understanding the tendencies of the data plotted. Figures 11-1 and 11-2 represent the overall values and adjusted averages of the time and temperature data for the experiments involving Manganese. The subsequent Figure, 11-3, illustrates the data from the previous image with the addition of trend lines to aid in understanding the tendencies of the data plotted. Next, the overall values and adjusted averages of the time and temperature data for the Cobalt reactions are plotted in Figures 12-1 and 12-2. The graph following the previous images, Figure 12-3, depicts the trending data for the Cobalt time and temperature results. Finally, the overall values and adjusted averages of the time and temperature data for the Chromium reactions are shown in Figures 13-1 and 13-2 while the subsequent graph, Figure 13-3, displays the same information with the addition of trending lines.

Iron					
	Ambient				
	T=0	t=20	t=40	t=60	t=80
Exp.1	517.1794	504.2292	488.2610	479.7305	470.9754
Exp.2	429.8506	410.4770	395.7793	385.5439	375.1784
Exp.3	521.6004	510.6979	502.3722	485.7420	483.2382
Average:	489.5435	475.1347	462.1375	450.3388	443.1307
Adj. Avg:	97.9087	95.0269	92.4275	90.0678	88.6261
	37°C				
	T=0	t=20	t=40	t=60	t=80
Exp.1	372.8877	316.3678	326.5326	325.3499	300.9551
Exp.2	587.0252	555.6530	518.6773	500.4044	487.7485
Exp.3	576.8394	534.8454	517.1453	493.3421	483.0888
Average:	512.2508	468.9554	454.1184	439.6988	423.9308
Adj. Avg:	102.4502	93.7911	90.8237	87.9398	84.7862

Table 2. Displays the overall results from experiments testing the time and temperature variants of experimental reactions involving the introduction of Iron in the standard reaction setup previously described. The data points in this table are the resultant concentration amounts (uM) of 8-OHdG produced from each experiment as well as the averages and adjusted averages of each set of reactions.

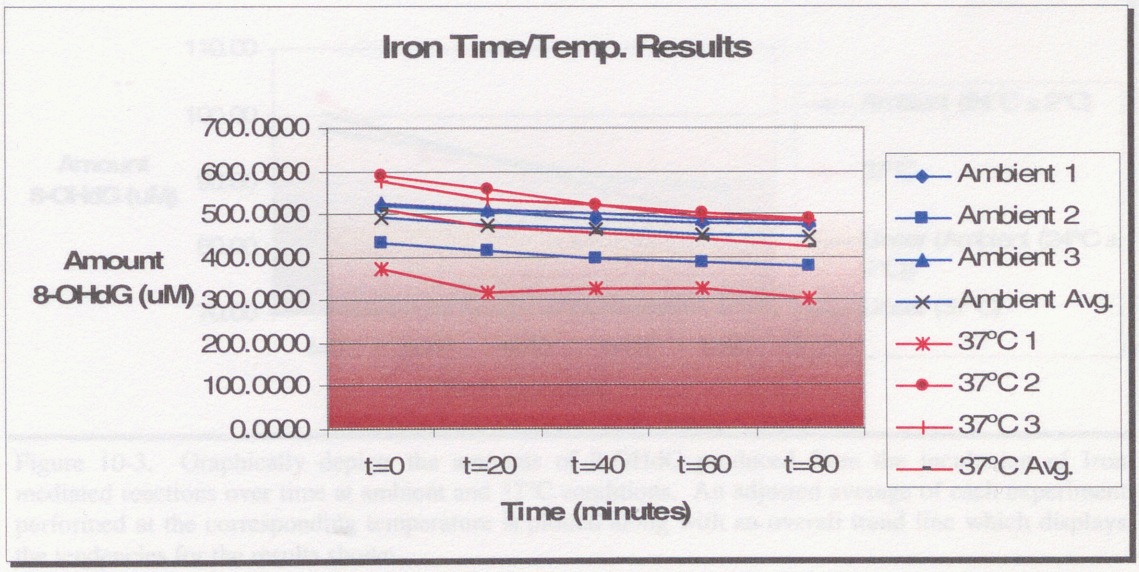


Figure 10-1. Graphically depicts the amounts of 8-OHdG produced from the incubation of Iron mediated reactions over time at ambient and 37°C conditions. Each experiment performed at the corresponding temperature as well as their average is plotted.

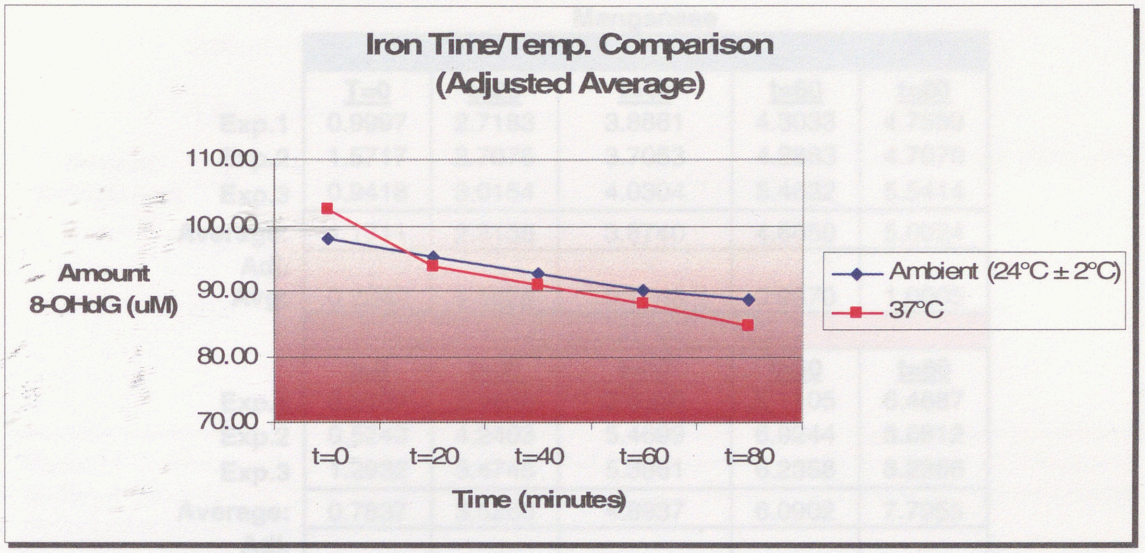


Figure 10-2. Graphically depicts the amounts of 8-OHdG produced from the incubation of Iron mediated reactions over time at ambient and 37°C conditions. An adjusted average of each experiment performed at the corresponding temperature is plotted.

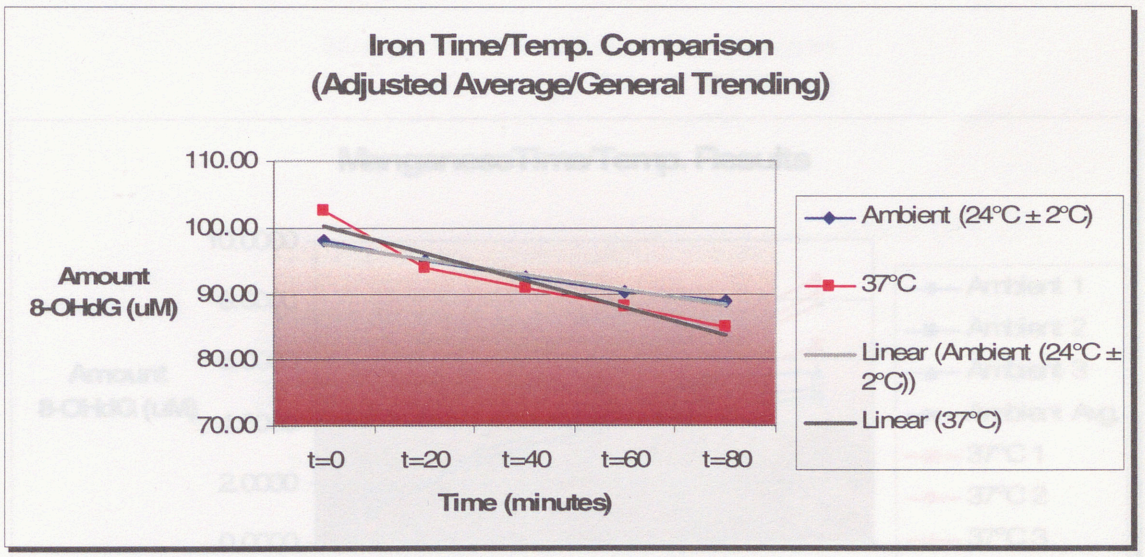


Figure 10-3. Graphically depicts the amounts of 8-OHdG produced from the incubation of Iron mediated reactions over time at ambient and 37°C conditions. An adjusted average of each experiment performed at the corresponding temperature is plotted along with an overall trend line which displays the tendencies for the results shown.

Manganese					
	Ambient				
	T=0	t=20	t=40	t=60	t=80
Exp.1	0.9997	2.7183	3.8861	4.3033	4.7589
Exp.2	1.5717	2.7076	3.7053	4.2883	4.7070
Exp.3	0.9418	3.0154	4.0304	5.4632	5.5414
Average:	1.1711	2.8138	3.8740	4.6850	5.0024
Adj. Avg:	0.2342	0.5628	0.7748	0.9370	1.0005
	37°C				
	t=0	t=20	t=40	t=60	t=80
Exp.1	0.5334	2.8632	3.8250	5.1105	6.4687
Exp.2	0.5243	4.2403	5.4699	6.9244	8.6812
Exp.3	1.2932	3.4745	5.3861	6.2358	8.2366
Average:	0.7837	3.5260	4.8937	6.0902	7.7955
Adj. Avg:	0.1567	0.7052	0.9787	1.2180	1.5591

Table 3. Displays the overall results from experiments testing the time and temperature variants of experimental reactions involving the introduction of Manganese in the standard reaction setup previously described. The data points in this table are the resultant concentration amounts (uM) of 8-OHdG produced from each experiment as well as the averages and adjusted averages of each set of reactions.

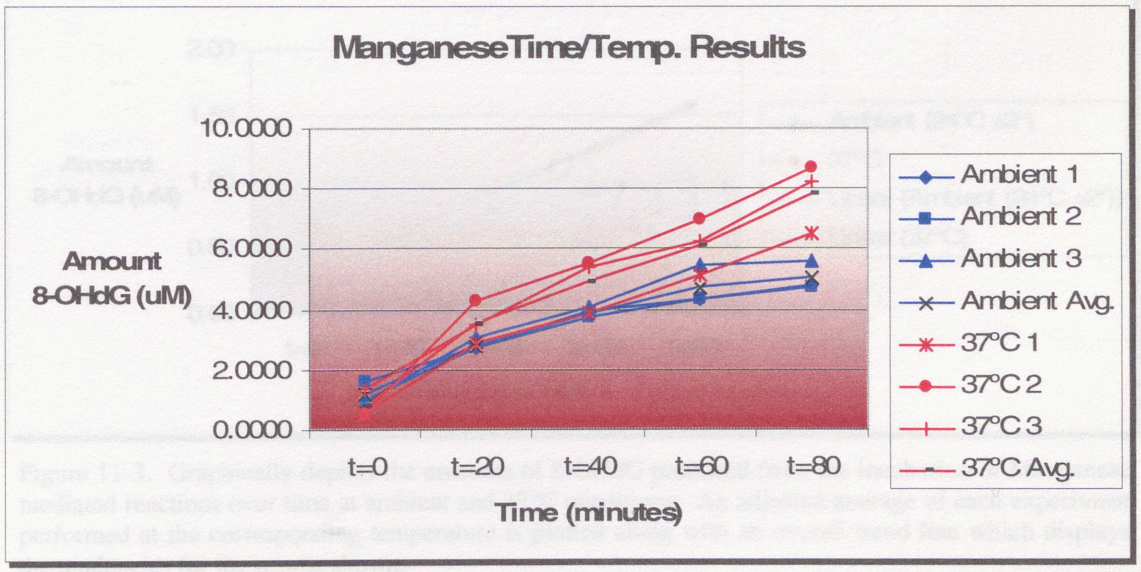


Figure 11-1. Graphically depicts the amounts of 8-OHdG produced from the incubation of Manganese mediated reactions over time at ambient and 37°C conditions. Each experiment performed at the corresponding temperature as well as their average is plotted.

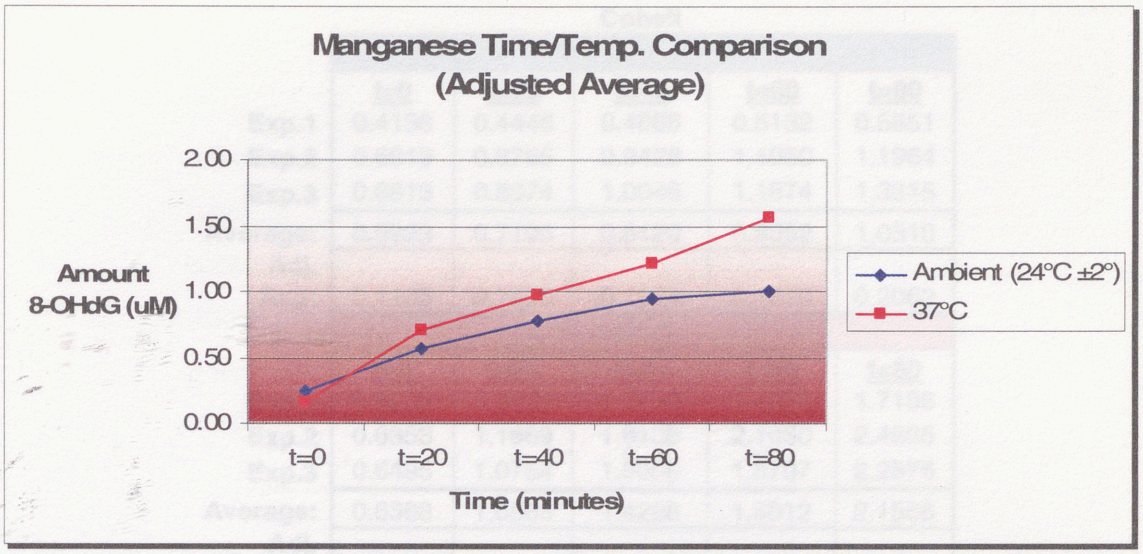


Figure 11-2. Graphically depicts the amounts of 8-OHdG produced from the incubation of Manganese mediated reactions over time at ambient and 37°C conditions. An adjusted average of each experiment performed at the corresponding temperature is plotted.

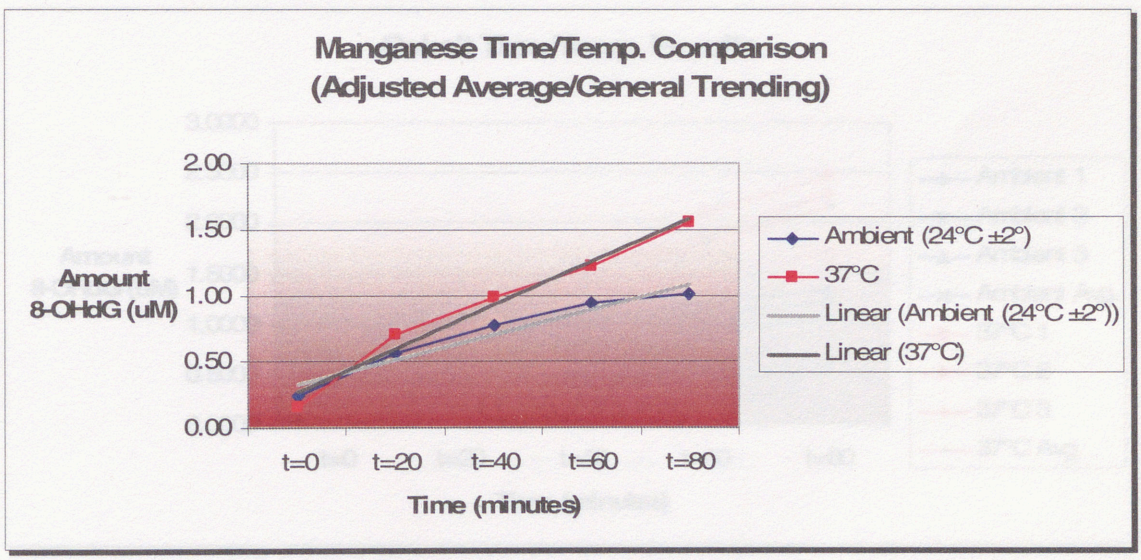


Figure 11-3. Graphically depicts the amounts of 8-OHdG produced from the incubation of Manganese mediated reactions over time at ambient and 37°C conditions. An adjusted average of each experiment performed at the corresponding temperature is plotted along with an overall trend line which displays the tendencies for the results shown.

Cobalt					
	Ambient				
	t=0	t=20	t=40	t=60	t=80
Exp.1	0.4136	0.4446	0.4888	0.5132	0.5651
Exp.2	0.6819	0.8765	0.9428	1.1050	1.1964
Exp.3	0.6813	0.8374	1.0046	1.1874	1.3315
Average:	0.5923	0.7195	0.8120	0.9352	1.0310
Adj. Avg:	0.1185	0.1439	0.1624	0.1870	0.2062
	37°C				
	t=0	t=20	t=40	t=60	t=80
Exp.1	0.6255	0.9112	1.1750	1.4278	1.7186
Exp.2	0.6353	1.1889	1.6138	2.1050	2.4635
Exp.3	0.6495	1.0754	1.5005	1.8707	2.2876
Average:	0.6368	1.0585	1.4298	1.8012	2.1566
Adj. Avg:	0.1274	0.2117	0.2860	0.3602	0.4313

Table 4. Displays the overall results from experiments testing the time and temperature variants of experimental reactions involving the introduction of Cobalt in the standard reaction setup previously described. The data points in this table are the resultant concentration amounts (uM) of 8-OHdG produced from each experiment as well as the averages and adjusted averages of each set of reactions.

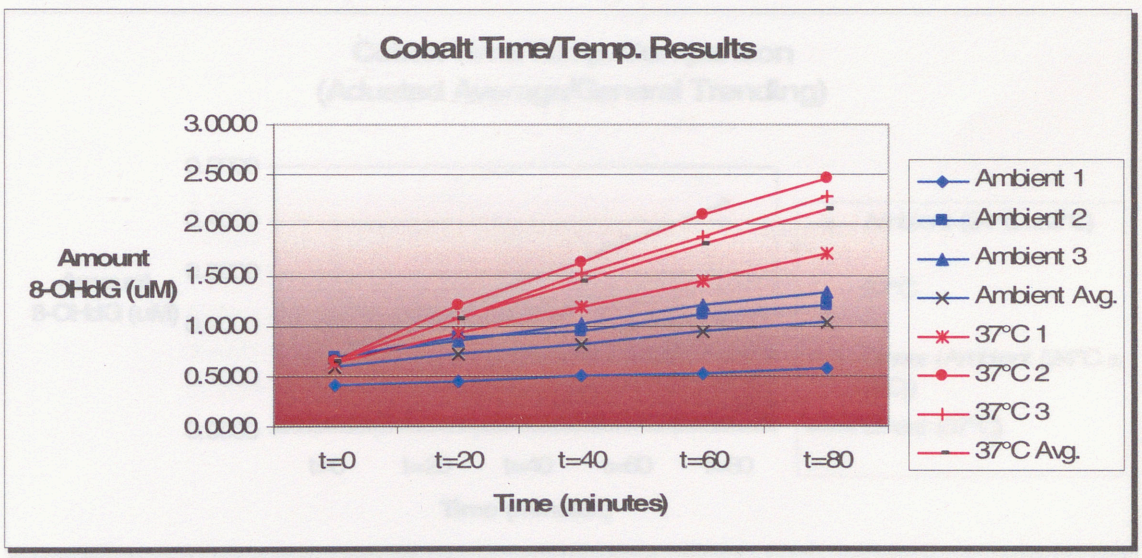


Figure 12-1. Graphically depicts the amounts of 8-OHdG produced from the incubation of Cobalt mediated reactions over time at ambient and 37°C conditions. Each experiment performed at the corresponding temperature as well as their average is plotted.

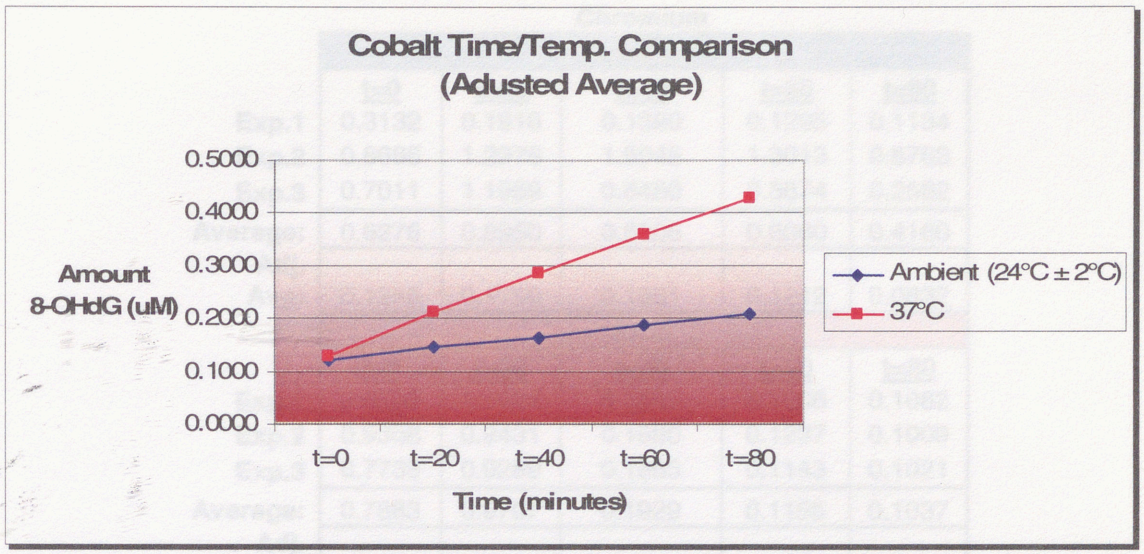


Figure 12-2. Graphically depicts the amounts of 8-OHdG produced from the incubation of Cobalt mediated reactions over time at ambient and 37°C conditions. An adjusted average of each experiment performed at the corresponding temperature is plotted.

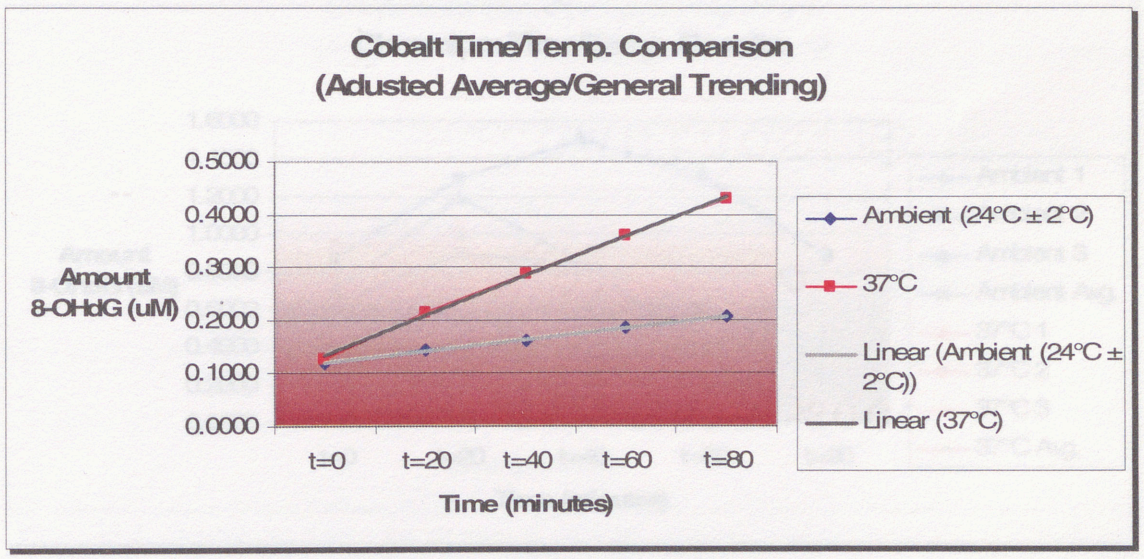


Figure 12-3. Graphically depicts the amounts of 8-OHdG produced from the incubation of Cobalt mediated reactions over time at ambient and 37°C conditions. An adjusted average of each experiment performed at the corresponding temperature is plotted along with an overall trend line which displays the tendencies for the results shown.

Chromium					
	Ambient				
	t=0	t=20	t=40	t=60	t=80
Exp.1	0.3132	0.1916	0.1390	0.1295	0.1134
Exp.2	0.8686	1.2976	1.5045	1.3013	0.8763
Exp.3	0.7011	1.1959	0.8480	0.3874	0.2582
Average:	0.6276	0.8950	0.8305	0.6060	0.4160
Adj. Avg:	0.1255	0.1790	0.1661	0.1212	0.0832
	37°C				
	t=0	t=20	t=40	t=60	t=80
Exp.1	0.6523	1.0401	0.2542	0.1206	0.1082
Exp.2	0.9388	0.9431	0.1560	0.1237	0.1009
Exp.3	0.7739	0.9289	0.1685	0.1143	0.1021
Average:	0.7883	0.9707	0.1929	0.1195	0.1037
Adj. Avg:	0.1577	0.1941	0.0386	0.0239	0.0207

Table 5. Displays the overall results from experiments testing the time and temperature variants of experimental reactions involving the introduction of Chromium in the standard reaction setup previously described. The data points in this table are the resultant concentration amounts (uM) of 8-OHdG produced from each experiment as well as the averages and adjusted averages of each set of reactions.

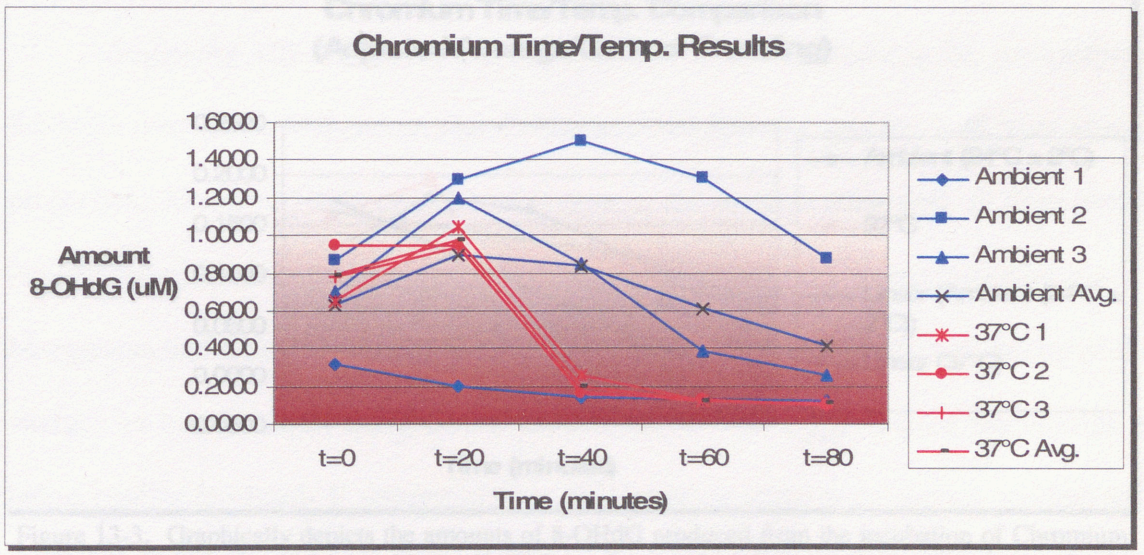


Figure 13-1. Graphically depicts the amounts of 8-OHdG produced from the incubation of Chromium mediated reactions over time at ambient and 37°C conditions. Each experiment performed at the corresponding temperature as well as their average is plotted.

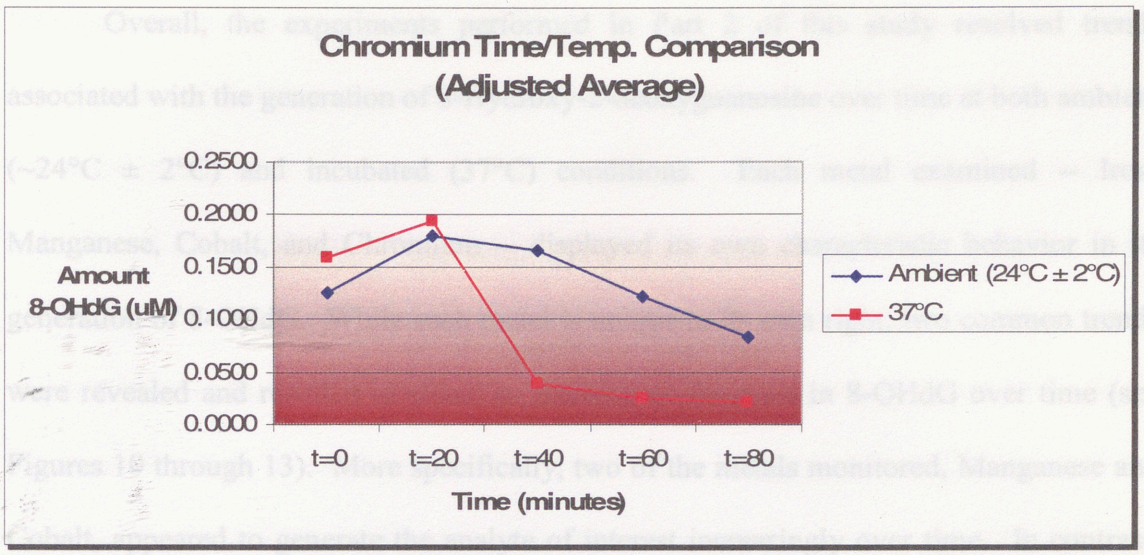


Figure 13-2. Graphically depicts the amounts of 8-OHdG produced from the incubation of Chromium mediated reactions over time at ambient and 37°C conditions. An adjusted average of each experiment performed at the corresponding temperature is plotted.

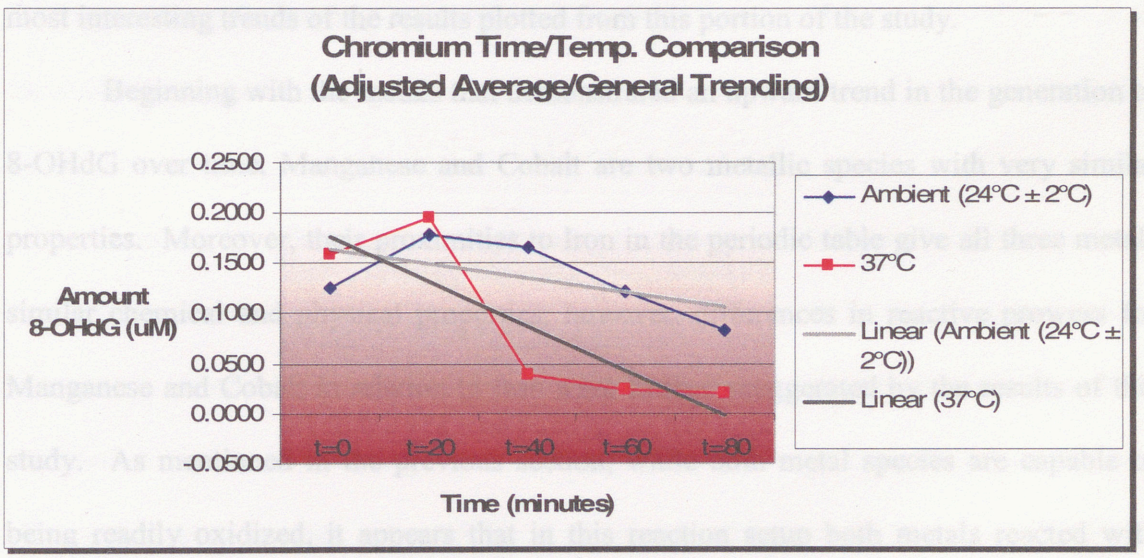


Figure 13-3. Graphically depicts the amounts of 8-OHdG produced from the incubation of Chromium mediated reactions over time at ambient and 37°C conditions. An adjusted average of each experiment performed at the corresponding temperature is plotted along with an overall trend line which displays the tendencies for the results shown.

Overall, the experiments performed in Part 2 of this study resolved trends associated with the generation of 8-Hydroxy-2-deoxyguanosine over time at both ambient (~24°C ± 2°C) and incubated (37°C) conditions. Each metal examined -- Iron, Manganese, Cobalt, and Chromium -- displayed its own characteristic behavior in its generation of 8-OHdG. While each metal is unique in its own right, two common trends were revealed and resulted in either an increase or decrease in 8-OHdG over time (see Figures 10 through 13). More specifically, two of the metals monitored, Manganese and Cobalt, appeared to generate the analyte of interest increasingly over time. In contrast, experiments involving the remaining metals, Iron and Chromium, appeared to have produced the highest amounts of 8-OHdG in the beginning minutes of each reaction with a decline over time. Out of the four metals, Cobalt and Chromium seemed to display the most interesting trends of the results plotted from this portion of the study.

Beginning with the metals that demonstrated an upward trend in the generation of 8-OHdG over time, Manganese and Cobalt are two metallic species with very similar properties. Moreover, their proximities to Iron in the periodic table give all three metals similar chemical and physical properties; however, differences in reactive prowess for Manganese and Cobalt in relation to Iron were further exaggerated by the results of this study. As mentioned in the previous section, while both metal species are capable of being readily oxidized, it appears that in this reaction setup both metals reacted with Hydrogen Peroxide in a steadily progressive manner. This is exemplified by the increasingly linear results plotted in Figures 11 and 12, with Cobalt exuding the most linear trends of all the metals tested (see Figures 12-2 and 12-3). Although the efficiencies of these metallic species at binding to the nucleoside constituent present in

the reactions are unclear, it is obvious by the data generated that the reactions involving Manganese and Cobalt result in a consistent increase of 8-OHdG over the 80 minutes that the reaction was allowed to occur. Furthermore, the addition of heat to these reactions only forced the outcomes of these experiments further into the direction of the pattern previously set at ambient conditions. These trends can be observed by comparing the ambient plots to the 37°C plots in Figures 11 through 12. Figures 11-3 and 12-3, clarify the overall trends in production of 8-OHdG from Manganese and Cobalt mediated reactions by the addition of linear lines extrapolated through plotting a generalized trend of each set of data points over time. Consequently, these results suggest that production of 8-OHdG from reactions involving Manganese and Cobalt mediated ROS do not instantaneously induce oxidative damage to 2-Deoxyguanosine upon the introduction of Hydrogen Peroxide. Instead, these reactions produce increased levels of 8-OHdG over an extended amount of time that is enhanced by the effects of temperature, most likely due to the chemical and physical properties of the elements used.

Iron, on the other hand, is an element that shows an ability to be quickly, almost instantly, oxidized upon addition of Hydrogen Peroxide in this reaction setup. This inference is made because the levels of 8-OHdG present in the Iron mediated reactions depicted in Figures 10-1 through 10-3 were highest at the initial time of the reaction and slowly decreased as time progressed. A similar trend is noticed with the Chromium reactions; however, in these reactions, a slight increase in 8-OHdG occurred at both temperatures tested between the times of t=0 and t=20 before an immediate decline occurred (Figures 13-1 through 13-3). It is between these time intervals that Chromium is believed to have undergone any reactions with H_2O_2 resulting in the oxidation of the

Chromium ions present as well as the generation of any ensuing ROS. This theory was accentuated by the observance of a severe decline in 8-OHdG from t=20 to t=40 minutes in the 37°C reaction. Also, it was observed that a dramatic change in color (not shown), from the characteristic green of Chromium(III) to a violet color, occurred between 0 and 20 minutes for the 37°C reactions. This is a further indication that the oxidative state of chromium has changed, since the Chromium(III) present in the initial reaction was most likely used up in a Fenton like reaction resulting in a change in oxidative state and hence the color of the entire reaction. A similar characteristic color change occurred in the Iron mediated reactions, but these changes are observed almost instantly upon the addition of Hydrogen Peroxide to the reaction mixture. The change of the Iron reactions from an almost clear color to the typical rust color is a classical indication of the conversion of ferrous species to ferric species. While it is unknown what the resultant oxidative state for Chromium is in this reaction, it would be fair to assume that Chromium(IV) would be a likely candidate. Unfortunately, while a review of the literature reports Chromium(IV) as a rare oxidative state of Chromium, no information was found on a species of Chromium that specifically has a characteristic violet or purple appearance. Further investigation into the actual identity of the Chromium species existing in the reactions following the addition and incubation of H_2O_2 is needed. In regards to the overall decline of 8-OHdG produced via Iron and Chromium mediated means, it is believed that the relatively quick and violent manner in which this compound is generated attributes to a certain level of instability. Therefore, molecules of 8-OHdG that were created from the original oxidation reaction are not stable enough to persist, thus, either reverting back to the original molecule or being oxidized to a more stable constituent. Recent

investigations into the hydroxylation of 2-dG to form 8-OHdG has lead certain scientists to believe that the –OH group that becomes associated with the C8 position of 2-Deoxyguanosine is actually an intermediate that is later converted to a more stable, double-bonded oxygen (Valavanidis et al., 2005). The proposed intermediate is represented in the reversible reaction of Figure 14 below.

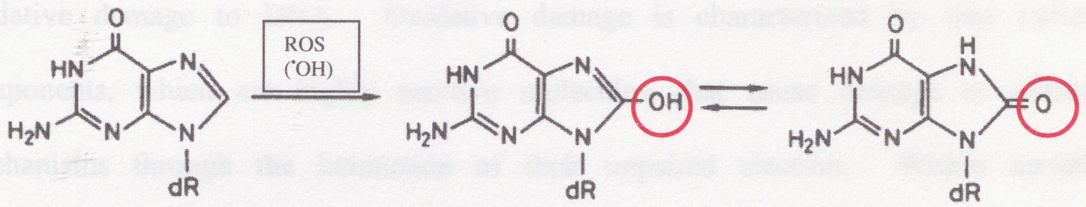


Figure 14. Represent the possible production of a reversible intermediate of 8-OHdG.

This example could be used to explain differences in production of 8-OHdG over the 80 minutes of the Iron and Chromium reactions, because of the development of possible inconsistencies made during the monitoring of 8-OHdG as the reaction mixture approaches equilibrium. Furthermore, the existence of unchelated ions of Iron(III) and Chromium(IV and higher), which are known contributors to the instability of 8-OHdG, could also account for the decline of levels of 8-OHdG. While Ethylenediamine Tetraacetic Acid (EDTA) is known to be highly efficient at chelating ions of the 2^+ and 3^+ oxidative states, ions of even greater oxidative state, such as 4^+ or even 6^+ in the case of Chromium, would not be chelated with as great efficiency. This would most likely account for why the decrease in 8-OHdG depicted in Figures 10 and 13 was not as dramatic for Iron as it was for Chromium. Most likely, this is the factor that attributed to the decline in 8-OHdG production.

CHAPTER 5

SUMMARY AND CONCLUSION

In efforts to understand the effects of foreign materials and energies on DNA structure, several types of genetic damage have been characterized. One type of genetic malfunction that has become popular among research professionals is the measure of oxidative damage to DNA. Oxidative damage is characterized by free radical components, which are highly reactive molecules, that cause damage to cellular mechanisms through the interaction of their unpaired electron. Within aerobic organisms, basic cellular metabolism involves the production of oxygen-based free radicals as well as non-radical reactive species better known as reactive oxygen species (Valavanidis et al, 2006). When situations of an imbalance occur between the creation and destruction of reactive oxygen species within an organism, a state known as oxidative stress ensues. For some time now, researchers, in identifying when these types of reactions, have begun to affect DNA and other cellular components, have used biomarkers in the detection of oxidative stress. Of the numerous adducts of DNA that are capable of being adequate biomarkers of oxidative stress, 8-Hydroxy-2-deoxyguanosine (8-OHdG) is the most studied oxidatively modified DNA base product because of its acute sensitivity and mutagenic potential (De Martins and Bianchi, 2002). 8-OHdG is considered to be a promutagenic compound because of its propensity to preferentially pair with adenine over cytosine during DNA replication, thus inducing G:C to T:A transversions (Bolin et al., 2004). One of the more common methods in which this compound is produced appears to involve metal dependant reactions yielding potent

hydroxyl radicals, one of many types of reactive oxygen species, which in return cause damage to DNA. Furthermore, several scientists have been able to demonstrate site specificity for metal ions on or near the structure of DNA. This determination makes the interaction of various metallic species with DNA an important mechanism for the indirect damage of one of the most important macromolecules utilized by living organisms.

In this study, a comparison examining the effectiveness of nine metals at generating oxidative damage to the DNA component, 2-Deoxyganosine, was investigated using a standard reaction setup incorporating Hydrogen Peroxide as the potential source of reactive oxygen species. Furthermore, a second component of this study involved the monitoring of the more successful metal species in their generation of the oxidative damage product, 8-OHdG, over predetermined time intervals at both ambient and heated conditions in attempts to understand the relationship of temporal and thermal variables on the creation of oxidative damage. Any resultant 8-OHdG generated was used as a way of monitoring oxidative damage to the DNA component. Two separate means of High Performance Liquid Chromatography (HPLC) was employed for the analysis of the experimental reaction. Both systems utilized very similar methodologies for the analysis of the experimental reactions, however, different analytical detectors were employed for each. The first phase of this series of experimentation utilized an HPLC coupled with an Ultra-Violet (UV) detector, while the second phase of this experimentation utilized an HPLC coupled with an Electrochemical Detector (ECD). Both instruments exuded excellent abilities at monitoring and detecting the analyte of interest, with the ECD detector ultimately displaying a more acute sensitivity for 8-OHdG over the UV detector.

The results of the first part of this study revealed Iron, the primary metal of Fenton reactions, to be the greatest mediator of ROS induced oxidative damage to 2-Deoxyguanosine. The remaining hierarchy of the metals tested was Manganese, Cobalt, Chromium, Copper, Nickel, Lead, and Cadmium respectively; Zinc and Mercury were eliminated because of various reasons in preliminary experiments. The results of the second part of this study revealed two general trends regarding temporal and thermal variants in the production of 8-OHdG from the top four metals resolved in previous analysis. First, reactions incorporating each metal tend to either increase or decrease in the concentration of 8-OHdG detected over time. Specifically speaking, the reactions involving Manganese and Cobalt tend to increase the concentration of 8-OHdG produced over time, and the reactions involving Iron and Chromium tend to decrease in 8-OHdG concentration over time. Next, the effects of increased temperature on the outcomes of the aforementioned trends seem to only push these reactions further in the directions in which they were already headed, causing either a noticeable rise or reduction in concentration of 8-OHdG beyond the results observed at the ambient condition. Ultimately, it was deemed that the results observed often occurred in response to the physical and chemical properties unique to the metal being tested. Certain metals tested had a huge affinity for being oxidized in the reaction setup used, while others had little or no affinity for being oxidized in the confines of this study. Moreover, it was very interesting to observe the generation of the 8-OHdG over time at the different thermal variations employed. Apparently, each metal, regardless of grouping or type, was able to react uniquely to the same conditions used within each reaction mixture.

In conclusion, the information elucidated by the means of this experimentation was very useful in understanding the effects of various metals in mediating oxidative damage to the DNA component, 2-Deoxyguanosine. While reactions involving Fenton-based chemistry can be highly variable depending upon the reaction conditions and binding abilities of each metal species, the primary determining factor for the effectiveness of metal mediated generation of ROS, and later oxidative damage, seems to be characterized by the chemical and physical properties of the metallic species involved. This simple but important statement can be supported by the data collected over the course of this study; as well as the fact that just as in any other chemical reaction, the product created is always determined by the characteristics of the reactants used.

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