Mechanisms of DNA damage by redox active Cr(III) complexes
by Kent Dennis Sugden

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Chemistry
Montana State University

Abstract:
Investigations of the mechanisms and interactions of chromium complexes with DNA are essential for the better understanding of how these complexes may induce carcinogenesis as well as their possible negative environmental impacts. The prevalence of chromium in the +3 oxidation state both environmentally and intracellularly implicate this oxidation state as a potentially important biologically active complex.

A series of biologically active and inactive Cr(III) complexes were synthesized and used to determine structure/activity relationships and the role that ligands have in conferring biological activity.

The biological activity of these complexes has been measured in a Salmonella reversion assay. Those complexes that tested positive were assayed in an anaerobic Salmonella assay to determine if mutagenic activity was dependent on oxygen. Loss of activity, upon assaying under anoxic conditions, implicates reactive oxygen species in the mechanism of DNA damage. Mutant frequencies were determined for the active complexes to give a relative reversion order irrespective of toxicity.

Cyclic voltammetry was employed to determine the redox kinetics of Cr(III) complexes and the relationship of this physical parameter with the generation of oxygen radicals. Cyclic voltammetry has shown that the ligands contribute to the formation of these radical species by "activating" the metal center.

Plasmid relaxation assays have been carried out to demonstrate that the complexes can produce a radical that uses DNA as a substrate. This radical can be shown to induce relaxation of supercoiled DNA consistent with a mechanism of oxygen radical generation.

Interactions of Cr(III) complexes with DNA are required for a radical mechanism of damage. The type of DNA interactions associated with mutagenic Cr(III) complexes have been shown using equilibrium dialysis, electrophoretic mobilities and UV-VIS spectrophotometry. Some of the mutagenic Cr(III) complexes show physical properties which implicate an intercalation mode of interaction with DNA.

A predictive model is proposed that allows ranking of efficacy of potentially mutagenic Cr(III) complexes based on their redox characteristics and interactions with DNA.

MECHANISMS OF DNA DAMAGE BY REDOX ACTIVE Cr(III) COMPLEXES

by

Kent Dennis Sugden

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Chemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

June 1992

APPROVAL

of a thesis submitted by

Kent Dennis Sugden

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, consistency, and is ready for submission to the College of Graduate Studies.

July 22, 1992 — Samuel J. Rogers

Date — Chairperson, Graduate Committee

Approved for the Major Department

July 22, 1992 — John R Crumrine

Date — Head, Major Department

Approved for the College of Graduate Studies

7/24/92 — R L Brown

Date — Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the library. I further agree that copying of the thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Request for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute copies of the dissertation in and from microfilm and the right to reproduce and distribute by abstract in any format."

Signature 

Date 7/22/92

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES-continued

LIST OF FIGURES-Continued

LIST OF FIGURES-continued

LIST OF FIGURES-continued

ABSTRACT

Investigations of the mechanisms and interactions of chromium complexes with DNA are essential for the better understanding of how these complexes may induce carcinogenesis as well as their possible negative environmental impacts. The prevalence of chromium in the +3 oxidation state both environmentally and intracellularly implicate this oxidation state as a potentially important biologically active complex.

A series of biologically active and inactive Cr(III) complexes were synthesized and used to determine structure/activity relationships and the role that ligands have in conferring biological activity.

The biological activity of these complexes has been measured in a Salmonella reversion assay. Those complexes that tested positive were assayed in an anaerobic Salmonella assay to determine if mutagenic activity was dependent on oxygen. Loss of activity, upon assaying under anoxic conditions, implicates reactive oxygen species in the mechanism of DNA damage. Mutant frequencies were determined for the active complexes to give a relative reversion order irrespective of toxicity.

Cyclic voltammetry was employed to determine the redox kinetics of Cr(III) complexes and the relationship of this physical parameter with the generation of oxygen radicals. Cyclic voltammetry has shown that the ligands contribute to the formation of these radical species by "activating" the metal center.

Plasmid relaxation assays have been carried out to demonstrate that the complexes can produce a radical that uses DNA as a substrate. This radical can be shown to induce relaxation of supercoiled DNA consistent with a mechanism of oxygen radical generation.

Interactions of Cr(III) complexes with DNA are required for a radical mechanism of damage. The type of DNA interactions associated with mutagenic Cr(III) complexes have been shown using equilibrium dialysis, electrophoretic mobilities and UV-VIS spectrophotometry. Some of the mutagenic Cr(III) complexes show physical properties which implicate an intercalation mode of interaction with DNA.

A predictive model is proposed that allows ranking of efficacy of potentially mutagenic Cr(III) complexes based on their redox characteristics and interactions with DNA.

INTRODUCTION

Chromium Mutagenesis

History

Chromium-related health problems have been the subject of many epidemiological studies in the past 100 years. Early recognition of chromium compounds as possible carcinogens due to occupational exposure has led to one of the most extensive investigations which continue to this day.[1,2] These studies have shown a direct relationship between chromium exposure and an increase in lung cancer. The major occupations affected by chromium exposure are the chrome-plating, leather tanning and pigment industries.[3-5] Epidemiological studies of workers in these industries have shown that the consequence of exposure to chromium can be cancer (mainly of the lung and digestive tract) as well as kidney failure, depression of the immune response and ulceration of the skin and nasal septa.[6] Chromium, in contrast to its carcinogenic potential, functions as an essential mineral in the body. The trivalent form of chromium, Cr(III), is a trace element that has a proposed enzymatic role as a cofactor in the metabolism of glucose.[7]

Chromium, in the environment, is found primarily in the form of chromite ore, $FeCr_2O_4$. Complexes of chromium can exist in a variety of oxidation states from minus 2 to +6 with the +6 and +3 states being the most stable and most common. These chromium complexes exhibit a wide range of geometries

including, square planar, tetrahedral, octahedral and various distorted geometries.

Chromium has the potential to be an economically important mineral in Montana since the only known exploitable ore deposits in North America are located in the Absaroka/Beartooth Wilderness Area.[8] Conversely, it is a major constituent in the hazardous mine tailings of the Berkely Pit Superfund site located in Butte, MT.[9] These opposing views of chromium as both an exploitable natural resource and an environmental hazard show the unique role that chromium plays in our region.

Theory

Activity Of Cr(III) Versus Cr(VI) The two major oxidation states of chromium that have been investigated for their biological activity are the hexavalent, +6, chromates and the trivalent, +3, chromic complexes. This focus on only two of the oxidation states is due to the stability of these two forms of chromium in the environment.[10] Historically, it was assumed that Cr(VI) was the only oxidation state of chromium that had a significant toxic and carcinogenic potential while the trivalent chromium species were generally considered to be biologically inert. Chromates have been shown to induce tumors in rats and mice,[11] cause mutations in bacterial, yeast and mammalian systems,[12] show differential lethality in a repair assay,[13] cause infidelity of *in vitro* DNA replication[14] and cause induction of lambda prophage.[15] Cr(III) salts, such as $CrCl_3$, have shown little or no activity in these same assays.[16-19] At the genetic level, Cr(VI) has been shown to produce a variety of lesions in DNA including single-strand

breaks, DNA-DNA and DNA-protein crosslinks.[20-22] Cr(III) salts have shown either no activity or a much lower degree of DNA lesion induction in these same assays. In contrast to the Cr(III) salts commonly used to assess activity of this oxidation state, hexacoordinate Cr(III) complexes with bidentate aromatic amine ligands have shown a significant degree of biological activity.[23,24] These complexes, possessing bipyridyl and phenanthroline ligands, have demonstrated mutagenicity in a variety of bacterial strains, genetic toxicity in a differential lethality assay and induction of the SOS response in *E. coli.*[25] In assays of mutagenicity against certain bacterial strains, these complexes have demonstrated activity comparable to that of the chromates.

Selective Uptake Reduction Model The mechanisms of DNA damage by chromium(VI) have been generally defined by the selective uptake/reduction model, figure 1.[16] This model proposes that the intracellular stable oxidation state of chromium is Cr(III) and it is this complex, or reactive intermediates formed in the reduction process of Cr(VI), that is the ultimate DNA damaging component *in vivo.* The inability of Cr(III) salts to induce mutagenesis or carcinogenesis directly has been attributed to a lack of membrane permeability. Whereas Cr(VI) readily traverses the plasma membrane of a cell via the sulfate anion transport system, Cr(III) salts have a limited plasma membrane permeability.[16] Cr(VI), once selectively taken up, can be reduced intracellularly by natural reductants such as flavonucleotides, microsomes, ascorbate, or sulfhydryls such as glutathione to generate the proposed biologically active intracellular Cr(III) complex and/or

produce reactive intermediates.[16,26] Once reduced, the active intracellular Cr(III) complex is unable to leave the cell because of this same membrane impermeability. This subsequent impermeability upon reduction of Cr(VI) leads to an intracellular accumulation of chromium.[16] Reactive intermediates, such as oxygen radicals, have been associated with Cr(VI) species and may account for some or all of the observed activity. Electron spin resonance (ESR) studies of Cr(VI) complexes *in vitro* have shown that a "stable" +5 oxidation state complex, tetraperoxochromate(V), Cr(V), can be formed that has the potential to generate oxygen radicals. In the presence of intracellular reductants such as ascorbate, reduced nucleotides and glutathione, the +5 oxidation state chromium complex can produce the short-lived hydroxyl radical that is captured using radical spin trapping compounds.[27-30] Some debate, however, has arisen as to whether the radical is generated by the Cr(V) alone or is an artifact of the system. Different systems have produced a variety of different radicals. Aiyar et al. and Shi and Dalal, using glutathione as a reductant, have detected the Cr(V) species and a glutathione thiyl radical but no hydroxyl radical.[31,32] It appears that the glutathione thiyl radical can react with molecular oxygen to generate the superoxide anion species. Cr(VI) in the presence of excess hydrogen peroxide will form the hydroxyl radical as shown by using DMPO, 5,5-dimethyl-1-pyrroline N-oxide as the radical spin trap.[28] No Cr(V) EPR signal was seen, however. Kawanishi et al. observed the formation of singlet oxygen, hydroxyl radical and the Cr(V) species using hydrogen peroxide as the reductant of Cr(VI).[33] It was proposed by Kawanishi et al. that the hydroxyl

radical could be formed by these products using iron as the catalyst between superoxide and hydrogen peroxide.[33] It should be pointed out, that all reactions were run in phosphate buffer that is known to have significant quantities of contaminating iron. Reduced nucleotides, such as NADPH reacted with Cr(VI) and hydrogen peroxide have shown significant production of the hydroxyl radical that was not significantly affected by addition of superoxide dismutase. This suggests that the iron-catalyzed reaction between superoxide and hydrogen peroxide is not the pathway through which hydroxyl radicals are generated. Jones et al. showed that a Cr(V) complex formed with glutathione, $Na_4Cr(GSH)_2$·(GSSG)·8H2O, in the presence of molecular oxygen can result in the production of the hydroxyl radical that is further increased upon addition of hydrogen peroxide.[34] This same complex, when run under anoxic conditions inhibited all radical formation. These same types of assays have been carried out with Cr(III) salts in the presence of hydrogen peroxide and have shown a limited production of hydroxyl radicals.[35] This, however, could only be accomplished using a pH 2 buffer which is an unlikely condition intracellularly and the significance of this work is questionable. The ability of certain hexacoordinate Cr(III) species to be biologically active by themselves may disturb the current model of Cr(VI) mutagenicity. If a redox active, or DNA binding Cr(III) compound is formed from the Cr(VI) reduction intracellularly, then it can be argued that this may be the actual DNA damaging complex formed *in vivo.* Until recently, little real evidence has been presented to uphold this theory.

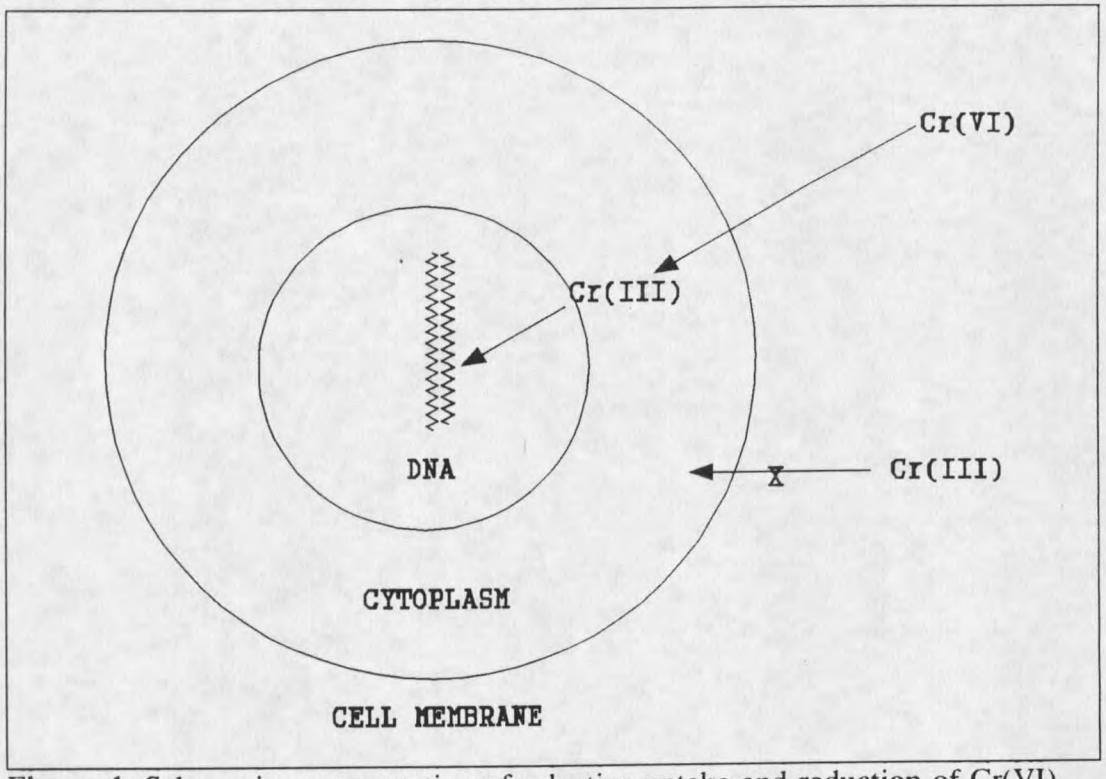

Figure 1: Schematic representation of selective uptake and reduction of Cr(VI).

Mechanisms Of DNA Damage By Other Compounds

While extensive studies have been carried out on Cr(VI) and Cr(III), the mechanism by which these complexes damage DNA have yet to be adequately elucidated. Research on a variety of other classes of mutagenic compounds have identified several mutagenic themes that serve as a basis for this study. While this does not preclude a wholly different method of DNA damage for these compounds, it does give us a rational starting point for this investigation. The different types of DNA damaging mechanisms that are most common are illustrated with examples on the following pages.

Cis-Platinum

Cisplatin, *cis*-DDP or *cis*-dichlorodiammineplatinum(II), is a commonly used anti-tumor agent. The activity of this compound was first discovered, serendipitously, by Barnett Rosenberg when he noticed that *E. coli*, in the presence of a platinum electrode, showed the induction of filamentation.[36] Filamentation is a known bacterial response to stress, mediated by environmental conditions or DNA damage. Further studies revealed that the biologically active species of Pt included the *cis* isomer of diamminedichloroplatinum(II), as well as a tetraamminedichloroplatinum(IV) compound. The remarkable effectiveness of this compound as an anti-tumor drug has led to an extensive investigation into the mechanism of it's DNA damaging activity. Cisplatin has been shown to form dimeric crosslinks with a variety of substrates. These are interstrand DNA-DNA crosslinks, intrastrand DNA-DNA crosslinks and DNA-protein crosslinks.[37] The most common lesion, believed to be responsible for the majority of the activity with this compound, is an intrastrand crosslink between two adjacent guanine nucleotides, figure 2.[38] The genetic consequence of this type of lesion is at least two-fold. The guanine-guanine Pt crosslink must be repaired and an error prone repair could lead to mutation. Since repair of this lesion is quite slow, this same lesion could cause infidelity of replication by the DNA polymerase by blocking polymerase read through along the effected strand.[39-42]

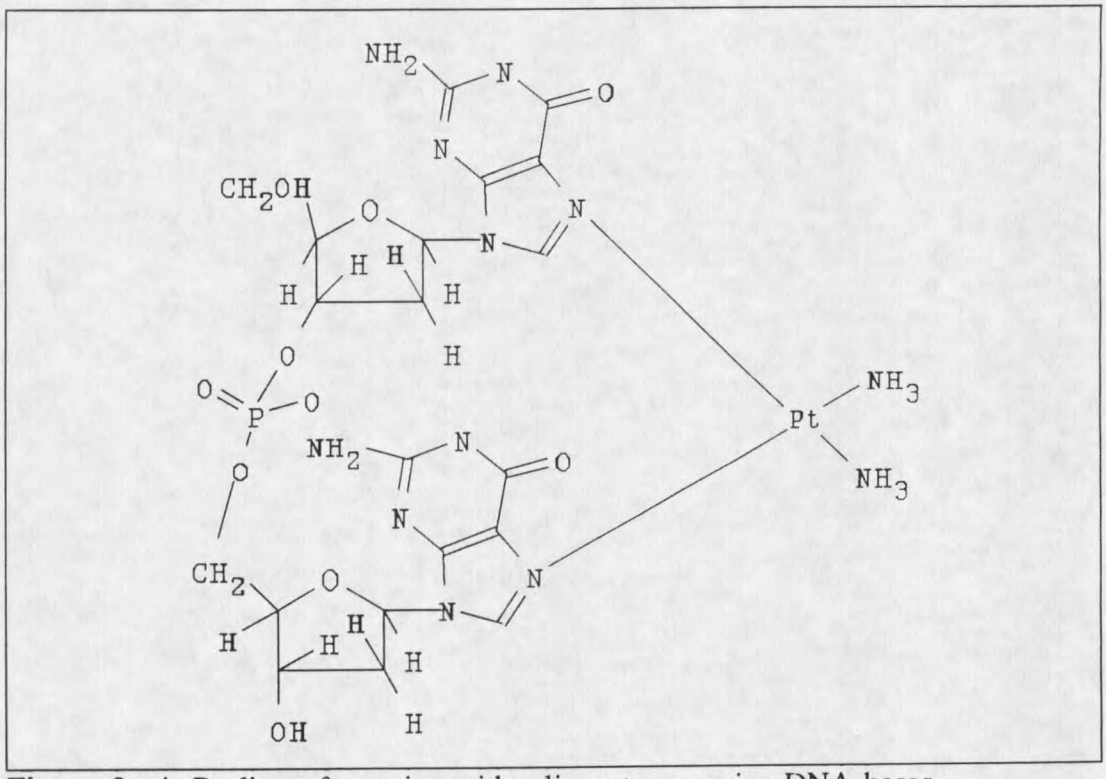

Figure 2: cis-Pt dimer formation with adjacent guanosine DNA bases.

Alkylating Agents (MNNG And MMS)

The alkylating agents, MNNG, N-methyl-N'-nitro-nitrosoguanidine, and MMS, methyl-methanesulfonate, are both powerful mutagenic agents. They potentiate their action by alkylation of DNA nucleotides.[43] While all nucleotides are susceptible to this type of alkylation reaction, the best substrate for this reaction is the guanine nucleotide. More specifically, the alkylation is shown to occur at the O-6 or N-7 position of guanine, figure 3.[44,45] The consequence of this type of lesion leads is lack of read through by the DNA polymerase and subsequent poor fidelity of replication.[46,47] Unlike Cisplatin, these type of lesions are readily repaired by endogenous alkyltransferase enzymes.[48]

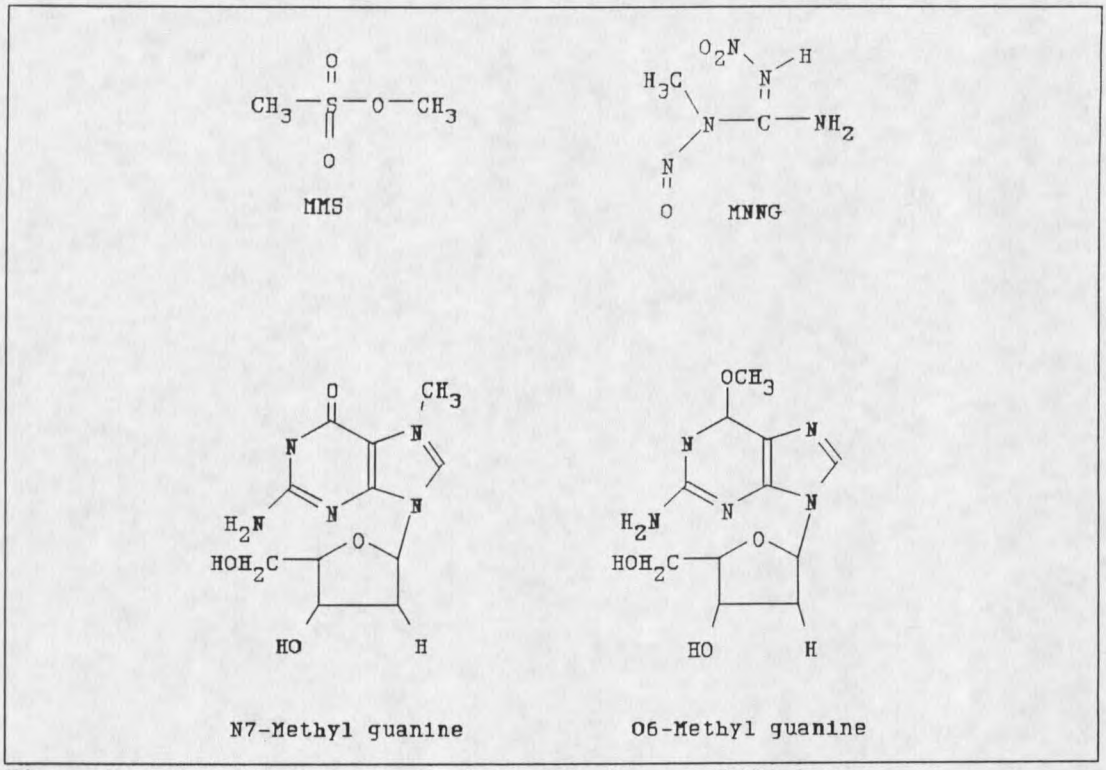

Figure 3: Alkylating agents and alkylated lesions on DNA bases.

Bleomycin

Bleomycin is a metalloglycopeptide that is an effective anti-tumor drug currently in clinical use, figure 4. The biological activity of this drug can be attributed to it's ability to induce strand-cleavage of DNA. Bleomycin consists of a glycopeptide, a small peptide with attached sugar units, that can interact with DNA by intercalating between the purine and pyrimidine bases to allow the active redox center of bleomycin, consisting of Fe(II), to generate oxygen radicals through a Fenton reaction.[49] These active oxygen radicals generated at the DNA surface interact with the DNA to induce strand scission. This iron catalyzed strand cleavage is the proposed mechanism by which bleomycin potentiates its mutagenic