The mutagenicity of inorganic ions in microbial systems
by Kenneth Raymond Tindall

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biochemistry
Montana State University
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Abstract:
A number of inorganic ions were assayed as mutagens in a standard bacterial system, the Ames test. In this assay, only K2CrO4 and K2Cr2O7 proved to be mutagenic. Further studies in DNA repair deficient strains of E. coli K12 provided some information concerning interactions between these metals and mechanisms of DNA repair. The discovery of metal resistant mutants of E. coli K12 arising in specific repair deficient strains upon exposure to K(SbO)C4H4O6, SbCl3, NaAsO2, and K2CrO4 prompted further investigation of these metals as mutagens in a forward mutational assay, the induction of resistance to D-cycloserine. In this assay, both K(SbO)C4H4O6 and NaAsO2 proved to be mutagenic.

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Date December 7, 1977

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KENNETH RAYMOND TINDALL

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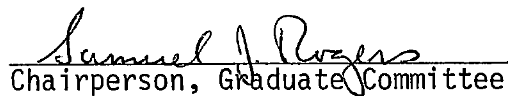
Approved: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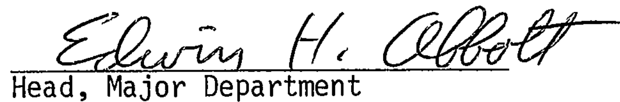
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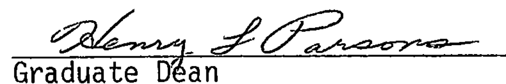
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ABSTRACT

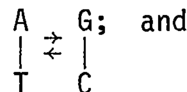
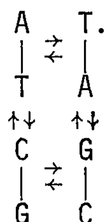
A number of inorganic ions were assayed as mutagens in a standard bacterial system, the Ames test. In this assay, only K_2CrO_4 and $K_2Cr_2O_7$ proved to be mutagenic. Further studies in DNA repair deficient strains of *E. coli* K12 provided some information concerning interactions between these metals and mechanisms of DNA repair. The discovery of metal resistant mutants of *E. coli* K12 arising in specific repair deficient strains upon exposure to $K(SbO)C_4H_4O_6$, $SbCl_3$, $NaAsO_2$, and K_2CrO_4 prompted further investigation of these metals as mutagens in a forward mutational assay, the induction of resistance to D-cycloserine. In this assay, both $K(SbO)C_4H_4O_6$ and $NaAsO_2$ proved to be mutagenic.

INTRODUCTION

Research in the fields of molecular biology and genetics during the last decade has provided a basic understanding of many of the functional and regulatory aspects of DNA. From this understanding has come an increased awareness and interest in disease as a result of genetic dysfunctions. The scientific community has begun to discern one disease, cancer, as a disorder which may be at least partially a result of alterations in these functional and regulatory aspects of DNA. Chemical and biochemical investigations of agents which affect DNA have provided some interesting concepts as to the mechanisms involved in permanently altering the DNA molecule. As a result, the somatic cell mutation theory of cancer, previously given little serious regard as a general theory of cancer induction, has received new acclaim.

In general, proponents of this theory believe cancer may arise as a result of mutations within somatic cell lines such that normal control of processes regulating cellular proliferation is disrupted. As little evidence is available to either prove or disprove this theory, considerable interest has been generated in elucidating basic mechanisms of mutation induction.

Classically, the word mutation has been used to define a heritable change in the genetic material; however, this thesis shall deal with only one sort of mutational event, the point mutation. Advances in biochemistry and molecular biology allow point mutations to be more accurately defined on the molecular level as a result of one

or more chemical changes in the base sequence of DNA. A number of these changes have been defined. Base pair substitutions, one source of mutations, may be separated into two classes: transitions, purine to purine and pyrimidine to pyrimidine base changes, i.e.,  and transversions, purine to pyrimidine and pyrimidine to purine base changes, i.e.,  Alkylating agents are common inducers of base pair substitutions and appear to specifically induce transitions, *in vivo* (16). Frameshift mutations are a second type of alteration in the DNA sequence which result in the literal shifting of a DNA base template reading frame by the insertion or deletion of one or more bases in a DNA sequence. These mutations may arise by a number of mechanisms such as errors induced by the replication enzyme, errors induced by misrepair (generally thought to generate frameshifts although basepair substitutions may occur as well) (16), and errors as a result of intercalating agents such as the acridine dyes (16). Mechanistically, the insertion or deletion of one or two bases results in a base sequence which alters the entire primary structure of the ultimate protein C-terminal to the lesion; whereas the insertion or deletion of three bases merely results in the addition or deletion of one amino acid in the ultimate protein

and such a protein may or may not retain its functionality. Finally, inversions of portions of a chromosome can also occur resulting in another mechanism by which the DNA sequence may be altered (28).

Reviews are available which deal with biochemical and genetic mechanisms of mutation fixation as well as reviews which discuss postulated mechanisms of DNA repair (16,38). Only recently, however, have investigators begun to integrate knowledge of the mutational processes with the present understanding of DNA repair mechanisms to suggest models which can explain mutational events as a function of the availability and/or lack of specific repair functions (57,60).

Analysis of these integrated models of mutation induction suggest mutational events may arise either as a result of i) constitutive activity, i.e., spontaneous induction, ii) non-chemical activity, i.e., UV induction, or iii) direct chemical activity. Of course, all of these may arise independently or as a function of misrepair of an induced DNA lesion; that is, DNA may be directly altered to produce a mutational event or that event may be the product of either constitutive or chemically induced DNA repair functions which, in an attempt to repair an induced DNA lesion, may result in misrepair of DNA and thus a mutational event. To understand how this might occur, one must consider the possible modes of DNA repair available to the cell.

DNA Repair

DNA repair mechanisms and their relationship to the survival of cells following DNA damage have been studied extensively using ultraviolet (UV) light to induce pyrimidine dimers, most commonly thymine dimers, in various strains of *E. coli*. One should be aware, however, that a number of other alterations involving DNA such as crosslinks (10) and those induced by alkylating, intercalating, or strand breaking agents are repaired via many of the same enzymatic pathways within the cell as repair UV induced damage (26). To facilitate an understanding of some of the basic concepts of DNA repair, a discussion follows concerning a few of the mechanisms by which cells repair UV induced DNA damage.

There are three mechanisms by which the cell may repair damaged DNA: i) direct enzymatic reversal of altered DNA to its original form; ii) specific removal of the damaged DNA by excision enzymes followed by resynthesis of the excised portion; and iii) the dilution of damaged DNA through a series of enzymatically-mediated recombinational events to produce at least one "good" copy of the DNA (19).

Kelner and Dulbecco were first able to demonstrate photoreactivation, the enzymatic reversal of UV induced pyrimidine dimers to the original monomeric form in the presence of 320-370 nm light (17,23). Subsequent attempts to isolate a photoreactivating enzyme in a variety

of organisms have been successful; such that, this particular enzyme is generally thought to be ubiquitous (56).

Concerning the excision repair processes, short-patch repair (19) in *E. coli* is mediated by the gene products of the *uvr*A, *uvr*B, *uvr*C, *pol*A and *lig* genes in the following manner. The *uvr*A and *uvr*B genes code for an endonuclease (correndonuclease II) which cleaves the damaged strand of DNA between the 5' phosphate of the pyrimidine dimer and the adjacent ribose. With the subsequent formation of a 3' hydroxyl on the remaining ribose, the *uvr*C gene product prevents the resealing of the DNA by ligase and allows the excision process to take place (19). The *pol*A gene product, Pol I, proceeds to excise the pyrimidine dimer along with approximately 20 nucleotides (3,47) via a 5'→3' exonucleolytic activity and restores the proper DNA sequence through its 5'→3' synthetic properties (19). Upon completion of the synthetic process the enzyme ligase reseals the nick to form a continuous strand of newly-repaired DNA. This *uvr*A, B, C, Pol I, ligase dependent mechanism of excision repair is generally thought to be an error-free repair process (60).

A long-patch pathway of excision repair is available to the cell as well, requiring the *uvr*A, B, C, *rec*A, *rec*BC, and *lex*A gene products, unwinding protein, Pol II, Pol III, and ligase (19,47). This type of repair reinserts a patch of nucleotides at least 100 times longer than the short-patch pathway of repair and was discovered by

Cooper and Hanawalt (12) who noticed nonconservative DNA synthesis was not limited in *pol*A mutants but rather was stimulated with the stimulation being dependent on the *uvr*A gene product (31). The long-patch pathway of repair requires the endonucleolytic properties of all the *uvr* gene products followed by ATP dependent double-stranded exonucleolytic activity directed by the *rec*BC gene product (exonuclease V) (19). Finally, the resulting gaps are filled by the polymerizing functions of Pol II and Pol III and resealed as before with ligase. The *rec*A and *lex*A gene products appear to have some regulatory function which may at least in part account for the fact that long-patch repair is generally considered to be inefficient and error prone (45,60,62).

Some evidence for a third branch of the *uvr* gene-dependent excision repair process has been elucidated by Youngs and Smith (62). This branch is postulated to operate independently of either the *pol*A or *exr*A genes. Therefore, as data indicates the *rec*A, *rec*B, and *exr*A genes control a single branch of *uvr* gene-dependent excision repair, specifically the long-patch mode of repair and as the *pol*A gene product, Pol I, is known to be functional in the short-patch repair pathway, this third branch of *uvr*-dependent excision repair becomes a very interesting branch of the DNA repair scheme. The efficiency of this third branch of *uvr*-dependent excision repair has not yet been determined and thus its capacity as an error-free or error-prone repair process is presently unknown.

As an added note, there exists another well-characterized endonuclease (19), correndonuclease I, which unlike correndonuclease II, is not sensitive to UV-induced damage but rather appears to act upon apurinic sites (20). This may be one mechanism by which damage induced by alkylating agents is removed as some alkylated purines may ultimately result in the formation of apurinic sites in the DNA (24). This finding is significant as supportive evidence that enzymes exist which specifically recognize chemically-induced damage.

By far the most complex and least understood mechanism of DNA repair is recombinational repair. The comparatively more complicated mechanisms by which this type of repair takes place as well as the pleiotropic effects of mutations within genes involved in this type of repair have impeded the elucidation of mechanisms involved. Although there are a considerable number of genes which affect genetic recombination (see A. J. Clark's review (7) for an excellent analysis), this discussion will be limited to the effects of the *rec*A, *rec*BC, and *lex*A mutations and their role in recombinational repair. The *rec*A gene is of particular interest since a mutation within this gene appears to block nearly all recombinational activity in *E. coli*(7,19) and *rec*A as well as *lex*A gene function is required for UV mutability in *E. coli* (60). In addition, the repair pathways with which these genes are associated are generally thought to be error prone (see Figure 1) (61).

The recombinational repair process is generally thought to

occur postreplicatively following gap formation in the newly-formed daughter strand DNA due to a lesion in the parent strand past which the DNA polymerase was unable to replicate (21). One mechanism by which these gaps and lesions are repaired involves a series of crossing over events allowing the formation of one flawless copy of the DNA; or in the case of DNA containing multiple lesions, recombinational events may occur through a series of several replications and dilution of the lesions continues until a copy of the DNA is produced which lacks the original polymerase inhibiting lesion. This type of repair is thought to be a function of at least the *rec*A and possibly *rec*BC gene products all of which are considered to be constitutive enzymes (42).

In addition to the types of repair described above, the SOS repair pathway appears to be an inducible system of repair available to *E. coli*. Recently, both Radman and Witkin have published reviews of the SOS system of repair (42,60,61) and much of this discussion will be based upon their views. The SOS repair pathway appears to function postreplicatively and by recombinational mechanisms although under different control than the *rec*A, *rec*BC recombinational repair activity mentioned earlier. The repair process is presumably mediated by the production of a specific SOS repair protein(s), the induction of which is dependent upon the *rec*A$^+$ and *lex*A$^+$ genotype. A number of other cellular functions such as λ phage induction, septum inhibitor, protein x, and exonuclease V inhibitor production are also under concommitant

control of the *rec*A and *lex*A alleles (60). Thus, a mutation in the *rec*A gene serves to inhibit a host of cellular functions in addition to at least three pathways of DNA repair, that is, long-patch excision repair, constitutive recombinational repair, and SOS repair. Comparatively, a mutation in the *lex*A allele does not affect the constitutive *rec*A, *rec*BC mediated recombinational repair pathway, although a mutation in the *lex*A allele does inhibit SOS repair as well as the specific cellular functions mentioned earlier (60).

One can think of the repair pathways thus far discussed as mechanisms by which a cell may correct DNA lesions to allow survival. If the repair process is error free, the lesion is corrected and the proper DNA sequence merely restored; but if the repair process is error prone, the repair of the lesion is more likely to alter the sequence of the DNA and result in a mutational event.

Witkin's scheme of error-free vs. error-prone repair is diagrammatically represented in Figure 1 including the genotypes required for each type of repair (61).

Carcinogenesis

To extrapolate from the knowledge of mutation induction and its relationship with DNA repair mechanisms in bacterial populations to cancer induction in human populations, however, one must first attempt to understand the basis of the somatic cell mutation theory of cancer

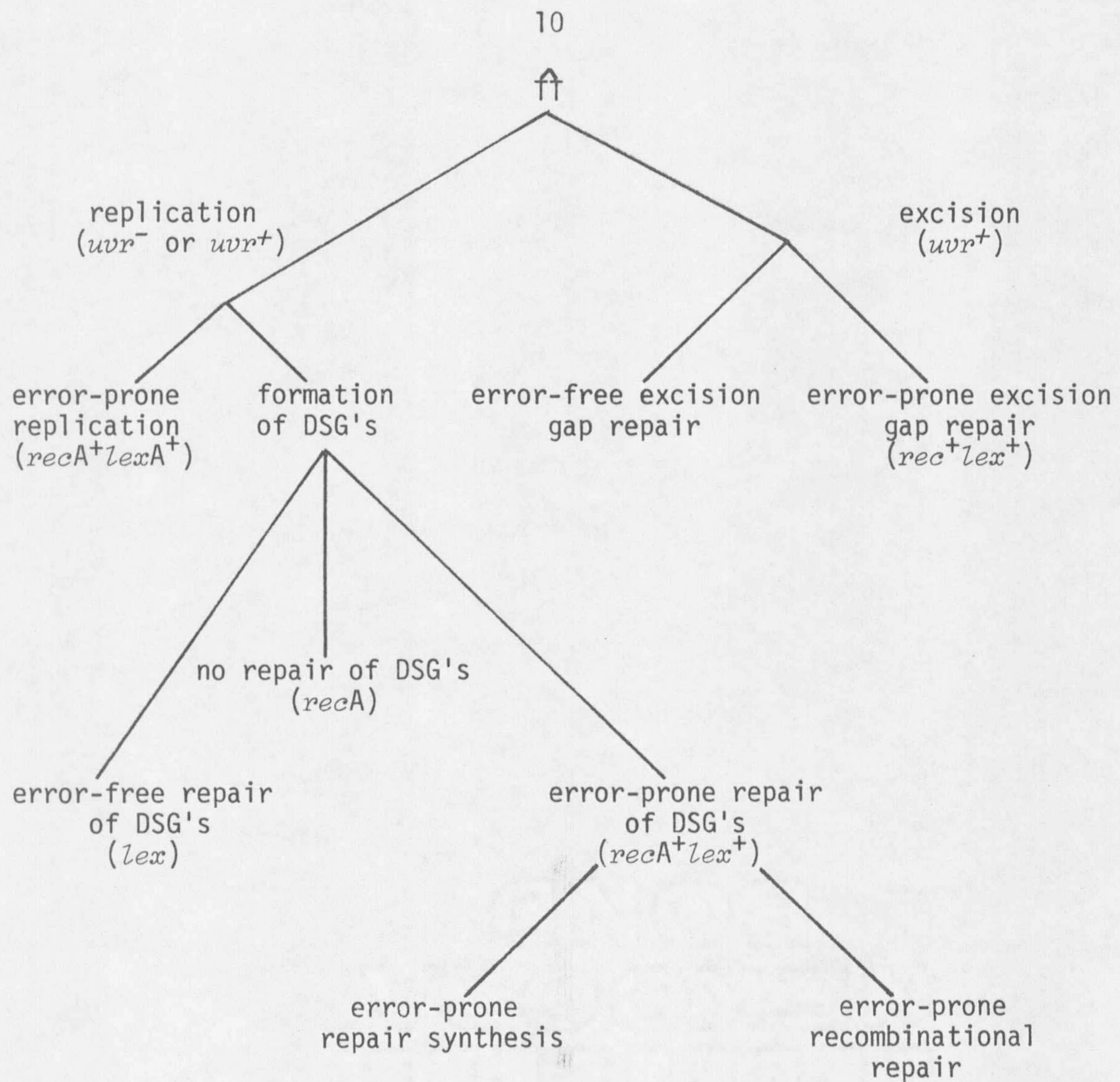


Figure 1. Repair of UV damage in *E. coli* strains of various genotypes and its possible relation to UV mutagenesis (61).

and the processes thought to underlie the physiological transformation of a normal cell to the cancerous state.

Certainly, cancer can arise by viral induction as a number of animal tumor viruses are under investigation (9). In fact, investigations in elucidating viral forms of cancer have been in progress since the early days of cancer research; however, the conclusive identification of human oncogenic viruses has met with limited success. As a result, investigators have begun to view cancer i) as a product of an external influence, i.e., chemical induction, and ii) as a function of a genetic predisposition.

Chemical induction of cancer has received attention since 1775 when Dr. Percivall Pott first described a high incidence of cancer of the scrotum in English chimney sweeps (2). Polycyclic aromatic hydrocarbons are now known to be the cause of Dr. Pott's initial observation. Indeed, some chemicals do induce cancer and yet a comprehensive study of the chemical induction of cancer must consider not only the chemicals involved but also the metabolic pathways within the cell which alter, transport and excrete a particular toxic chemical. If a chemical has successfully caused a DNA lesion, the repair processes available to the cell may then become important.

Although the mechanism by which normal cells are transformed to the cancerous condition is unknown, many chemical carcinogens can be shown to have some interaction with the DNA (13,34). Moreover, many

chemical carcinogens express mutagenic activity in test systems ranging from simple bacterial reversion assays to mammalian tissue culture. In 1975, McCann and Ames published a comprehensive evaluation of the mutagenic potential of 300 compounds, both carcinogens and noncarcinogens (32). In this study, 157/170, or approximately 90%, of the known carcinogens tested were shown to express mutagenic activity in the *Salmonella typhimurium* histidine reversion system developed by Ames (32,33). In the same study, less than 10% of the noncarcinogens were shown to exhibit mutagenic activity. This correlation between mutagenesis and carcinogenesis has now been corroborated in a number of laboratories using the Ames system as well as other microbial testing systems. One should not be too quick, however, to conclude that the induction of mutations within the somatic cell lines is the only mechanism by which chemicals induce the transformation process. That is, all carcinogens may not be mutagens; diethylstilbesterol (DES), a steroid analog, for instance, has not been shown to be mutagenic, yet has been shown to be a potent carcinogen. This should not be too surprising, however, as one might expect DES to act via alternate mechanisms in that hormones have been demonstrated to induce cellular hyperplasia and increase the probability of a tumor.

On the other hand, the somatic cell mutation theory of cancer would seem to indicate that all mutagens probably are carcinogens and models which attempt to further refine the somatic cell mutation theory

are currently being developed. Comings (11), for example, has suggested a mutation in the repressor region of a cell could lead to derepression of a latent genetic region (oncogene) and thus the subsequent transformation of a normal cell to the cancerous state. While the actual transformation process is almost certainly more complex than Comings' model, an acceptable mechanism of mutation based transformation is represented. Possibly, several mutations would be required which would be consistent with the fact that the incidence of cancer greatly increases with age. At any rate, such models are indicative of the heightened interest in investigations involving the biochemical basis of mutagenicity.

In addition to the mounting evidence of chemical induction mechanisms, a number of clinical genetic disorders have been shown to predispose individuals toward cancer development. Patients afflicted with xeroderma pigmentosum, ataxia telangiectasia, dyskeratosis congentia, Faconi's anemia, Werner's, Bloom's, Chediak-Higashi and Down's syndromes all exhibit an extremely high incidence of cancer in addition to other specific syndrome anomalies (57). Of these disorders, xeroderma pigmentosum (XP) has been best characterized. XP has been the subject of a recent review (8) and biochemical evidence is accumulating which indicates XP patients lack specific DNA repair enzymes. Pyrimidine dimers formed upon exposure to UV light lead to the transformation of affected cells. Specifically, XP patients lack the ability to perform prereplicative excision repair of pyrimidine dimers, a function which,

as mentioned earlier, has been classically studied in *E. coli*. The *E. coli* studies have correlated the lack of this prereplicative excision repair process with an increased mutation rate upon exposure to UV light (60,61). Thus clinical evidence exists in human populations linking the lack of repair of a known mutation inducing source (UV induced pyrimidine dimers) and the transformation process. Clearly, cellular repair mechanisms play an important role in the cancer induction process and the somatic cell mutation theory of cancer induction again receives support.

Genetic Effects of Metals

This thesis project attempts to discern the mutagenic activity of various metal ions in microbial systems. Of particular interest are those metals which have been shown either epidemiologically or experimentally to be carcinogenic. Assuming carcinogenesis to be at least in part a function of mutagenesis and in turn mutagenesis to be a function of cellular DNA repair mechanisms, two questions become important: i) do these carcinogenic metals exert a mutagenic effect in bacterial testing systems and ii) how do these metals interact with known DNA repair pathways?

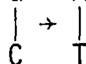
Sunderman (55) has recently reviewed the epidemiological and experimental evidence identifying those metals known to act as carcinogenic agents. In this review possible mechanisms by which these car-

cinogenic metals exert their effect are also discussed. Time and space do not permit a comprehensive discussion of metals as carcinogens; however, Sunderman's review signifies As, Be, Cd, Cr, Co, Fe-dextran complexes, Pb, Zn and Ni as the major carcinogenic metals. All the abovementioned metals have been clearly shown to induce cancers in animal assay systems with varying degrees of potency with the exception of arsenic. Arsenic is an interesting exception in that it exhibits little potential for carcinogenic activity in experimental animals while the epidemiological evidence of arsenic's carcinogenic potential is readily available.

Thus, the group of metals which are known or suspect carcinogens are of heuristic value for the investigation of metal ions as mutagens. While organic compounds are easily assayed as mutagens in the Ames test and one is 90% confident of detecting an organic carcinogen with this bacterial reversion assay, inorganic compounds and metals are not as easily assayed. Testing of metals in standard mutagenesis assay systems is difficult due to i) the insolubility of many metals in H_2O or phosphate based solutions, ii) their extreme cytotoxic effect in the bacteria employed, and iii) the accuracy of measuring a metal's activity in the presence of a large number of both physiological and environmental (media, buffer, etc.) metals. As a result, the list of metals which have been adequately assayed for their mutagenic potential is far from comprehensive.

Nevertheless, Demerec and Hanson (14) in 1951 first showed divalent manganese to exhibit mutagenic activity in *E. coli* by inducing forward mutations to streptomycin resistance. Continued efforts by investigators confirmed the work of Demerec and Hanson, in 1958, again by inducing resistance to streptomycin in *E. coli* (53); in 1964, by inducing mutations in the rII region of T_4 (37); and most recently by showing both nuclear and mitochondrial DNA of *Saccharomyces cerevesiae* to be affected by Mn^{++} (5).

Only within the last few years have scientists begun to study the DNA-damaging potential of a variety of metals. In 1974, Venitt and Levy published a study which demonstrated the induction of suppressor mutations in the *E. coli*, WP2, Trp^- series (58). Hexavalent chromium present in the CrO_4^{-2} species was responsible for the induction of base pair substitutions specifically



transitions. One year later, in 1975, Nishioka assayed a number of metals in a lethality assay with rec^-/rec^+ strains of *Bacillus subtilis* (35). Nishioka compared the effects of these metals on the viability of the cells as a function of their capacity to repair DNA damage by recombinational mechanisms. DNA damage which requires the *rec* allele for repair presumably results in increased lethality in the *rec*$^-$ strain. The same study attempted to further classify the damage induced by assaying As, Cr and Mo, the metals most active in the lethality assay, as potential mutagens in the

E. coli, WP2, Trp^- reversion system. All three metals were clearly mutagenic and appeared to require the *rec*A allele for the expression of mutagenic activity.

Other metals have been shown to express mutagenic activity specifically in the Ames bacterial reversion system; these include chromium, which induces both frameshift and base pair substitutions (30, 58), cis squareplanar Pt (II) compounds (27) and Se (VI) (30), which both appear to induce point mutations, and, finally, $FeSO_4$, which induces frameshifts (6). Thus, the metals which have been assayed for their mutagenic potential are few and of the carcinogenic metals tested, even fewer appear to induce mutations in bacterial DNA. Therefore, one might ask if bacterial reversion assays are reliable for determining the carcinogenic potential of metals and in addition if other *in vitro* assays might be developed which allow detection of metal carcinogens with a relatively high degree of efficiency.

The first question is not readily answered and is covered, at least in part, within the scope of this thesis. The answer to the second question has been recently investigated by Michael Sirover and Lawrence Loeb who have developed an *in vitro* screening system which measures the fidelity of avian myoblastosis virus (AMV) polymerase in the synthesis of complementary nucleotides to a synthetic template (48, 49,50). Metals have long been known to be associated with the DNA polymerase. Mg^{++} or Mn^{++} are required for catalytic activity (25,29) as

well as stoichiometric quantities of Zn^{++} (41,52). Sirover and Loeb's hypothesis involves metal ion induced infidelity of the polymerase enzyme but not necessarily by replacing magnesium or zinc at their respective binding sites. Their suggestion is that perhaps the environment that surrounds the replication complex influences the accuracy of the DNA replication process; one could then envision the momentary localization of a carcinogen inducing a base change. Of thirty-one metal salts tested in this assay system, Ag, Be, Cd, Co, Cr, Cu, Mn, Ni, and Pb, all decreased the fidelity of the AMV polymerase in the synthetic process. Certainly all of the metals which are active in this system have been implicated as carcinogens as well; although one should keep in mind the concentrations of these metals *in vitro* may far exceed the concentrations one might observe *in vivo*.

Finally, one must consider the role of metals as they are recognized by or perhaps interact with the DNA repair enzymes. Only Nishioka's study (35) attempts to look at a large number of metals and determine their activity as a function of the availability or lack of a specific repair process and even then only the lack of recombinational repair was compared to the normal functioning wild type. Considering the large number of repair pathways which have been elucidated in *E. coli*, one might think a more comprehensive evaluation of metals as they interact with DNA repair processes might be in order. Rossman et al. have investigated the effect of arsenic in repair deficient

strains of *E. coli* (44). Their results were interesting as the presence of arsenic increased the lethal effect of UV light in the wild type, *uvr*A$^-$, and *pol*A$^-$ strains yet had no effect on the *rec*A$^-$ strain. These results would seem to imply that arsenic might play a role in the inhibition of a *rec*A-dependent pathway of repair. Unfortunately, these two papers comprise the entire body of knowledge of metal interactions with DNA repair mechanisms.

The evolution of this thesis project has resulted in some interesting results concerning mechanisms of metal ion mutagenesis. Specifically, three aspects of metal ion-DNA involvement were investigated:

i) the mutagenic activity of a number of metal salts was determined in a standardized bacterial reversion system (the Ames test).

ii) the relationship between repair activity and metal salts was analyzed as a function of the lethal effects of these metals in repair deficient strains of *E. coli* K12.

iii) the effects of a specific metal, Sb, was analyzed as a mutagen in a forward assay system to D-cycloserine resistance in *E. coli* K12.

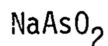
MATERIALS AND METHODS

<u>Spot Test Assay on *Salmonella typhimurium*</u>

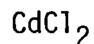
Metals of interest were first assayed as mutagens in the most standard of the bacterial mutagenesis assay systems, the Ames *Salmonella typhimurium* histidine reversion assay (1). The five *S. typhimurium* strains used (TA 1535, TA 1537, TA 1538, TA 98, TA 100) were kindly supplied by Dr. B. N. Ames, University of California, Berkeley. Each assay was completed on both Vogel-Bonner (VB) and revised Davis minimal (DMR) media. Bacteria were spread using the soft agar overlay technique described by Ames (1).

<u>Preparation of Metal Salts</u>. .01 M and 0.1 M metal salt solutions were prepared in either sterile distilled water or dimethylsulfoxide (DMSO) and filter sterilized samples of each concentration were then applied to separate, sterile 1/4" blank, antibiotic testing discs (Difco) such that 200 nanomoles and 2000 nanomoles, respectively, were applied to each of the plates in duplicate for all five strains of *S. typhimurium*.

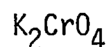
<u>Compounds Assayed</u>. The following metals were assayed for mutagenic activity in the spot test:

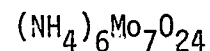

$NaAsO_2$

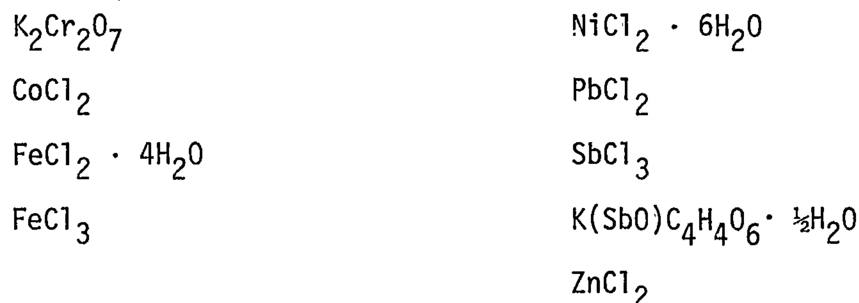
$HgCl$


$CdCl_2$

$MnCl_2$


K_2CrO_4


$(NH_4)_6Mo_7O_{24}$



$K_2Cr_2O_7$

$CoCl_2$

$FeCl_2 \cdot 4H_2O$

$FeCl_3$

$NiCl_2 \cdot 6H_2O$

$PbCl_2$

$SbCl_3$

$K(SbO)C_4H_4O_6 \cdot \frac{1}{2}H_2O$

$ZnCl_2$

All reagents were analytical grade and purchased from either Mallinckrodt Chemical Works or the J. T. Baker Chemical Company.

A 5 mg/ml solution of the positive control Dexon, sodium [4-(dimethylamino)phenyl]diazenesulfonate, was prepared and again 20 λ applied to a blank 1/4" Difco disc. As Dexon induces both frameshift and basepair substitutions, all five strains were assayed for their mutability with this compound in spot test fashion.

Preparation of Media. Standard VB media was prepared according to the method described by Ames (1) and DMR media by dissolving the following in two separate solutions, autoclaving, and mixing these two solutions immediately before pouring.

Solution 1		Solution 2	
K_2HPO_4	7 gm	dextrose	1 gm
KH_2PO_4	2 gm	agar	15 gm
$(NH_4)_2SO_4$	1 gm	.5% CAA (casamino acids)	10 ml
$MgSO_4$	0.1 gm	10^{-3} M thiamine	1 ml
H_2O	500 ml	H_2O	500 ml

The revision in the Davis minimal medium involved the addition of 10 ml of .5% casamino acids. The inclusion of this supplement was used to stimulate transport mechanisms within the cells which might facilitate transport of metal ions across the cellular membrane.

Assay of Activity. Treated plates were incubated at 37° C for three days and visually assayed for a ring of histidine independent colonies surrounding the disc. Presence of such a ring is an indication of mutagenic activity as either a basepair substitution or a frameshift mutation must occur to revert the bacteria to the prototrophic state.

Lethality Assays on *E. coli* K12

Compounds Assayed. The following metals were assayed for their ability to induce differential zones of lethality in repair deficient strains of *E. coli* K12:

$NaAsO_2$	$(NH_4)_6Mo_7O_{24}$
$CdCl_2$	$NiCl_2$
K_2CrO_4	$SbCl_3$
HgCl	$K(SbO)C_4H_4O_6$
$MnCl_2$	$ZnCl_2$

0.1 M metal salt solutions were prepared in either sterile doubly-distilled water or DMSO. Twenty microliters (20λ) of each solution was then applied to separate sterile blank 1/4" Difco discs.

Preparation of Media. Salt-enriched complete growth media (JN) was prepared as follows:

nutrient broth	8 gm
Bacto-difco agar	15 gm
NaCl	5 gm
H_2O	1000 gm

Strains of *E. coli* K12 Used. The following strains of *E. coli* K12 used in the lethality assay were graciously supplied by Dr. G. Warren, Department of Chemistry, Montana State University. As described below, all strains are essentially isogenic except for the indicated repair deficiency.

Strain	Repair Deficiency
AB 1157	wild type
P 3478	*pol*A
AA 34	*exr*A*rec*A
AB 2494	*lex*A
RH 1	*uvr*A*rec*A
GW 801	*rec*A
PAM 5717	*exr*A
AB 1886	*uvr*A

While these strains are not strictly isogenic, the differences are slight with the exception of P 3478, the *pol*A strain which Dr. G.

Warren obtained from John Clark. All others are derived from AB 1157, the wild type and were constructed either by transduction or recombination techniques. In the case of the latter, no more than 15 minutes on the bacterial genome was allowed to undergo the conjugation process. The genetic character of strains AB 1157, AB 2494, and AB 1886 can be found in Bachman's review (4). Strains AA 34, RH 1, and GW 801 were constructed by John Donch, M. H. L. Green and Guylyn Warren, respectively. These strains contain an additional mutation making them auxotrophic for methionine (G. Warren, personal communication).

Concerning the above-mentioned strains, the *exr*A and *lex*A genes are considered to be the same allele. In this series, the *exr*A mutation was originally isolated in *E. coli* B and transferred to the K12 species while the *lex*A mutation was originally isolated in *E. coli* K12. There is reason to believe that although the mutations occur within the same gene the resulting effect on *lex*-mediated repair within the cell is slightly different (G. Warren, personal communication).

Procedure of Assay. Cultures of each strain of *E. coli* were inoculated in nutrient broth and incubated for 18 hours at 37° C. One hundred λ of each culture was applied to separate JN plates in duplicate. Difco discs containing the metal samples were applied to each plate in triplicate. Thus each strain of bacteria was assayed in triplicate (3 tabs/plate), twice (duplicate plates of each strain), for

each metal sample tested. The plates were incubated at 37° C for 24 hours and the diameters of the resulting zones of lethality were measured to the nearest millimeter, averaged, and values compared to those obtained on AB 1157, the strain wild type for repair.

Assay of Repair Deficiencies in *E. coli* K12. The *E. coli* K12 strains were assayed for their DNA repair deficiencies by exposure to UV light. Each strain of *E. coli* K12 was streaked on NA plates and subject to exposure of 2.5, 5.0, 7.5 and 10 seconds of UV light at a distance of approximately 25 cm. Plates were then incubated overnight and visually assayed for survival. Due to the DNA repair deficiencies genetically inherent in each strain, one can verify the genetic DNA repair dysfunctions relative to one another. The strains listed in decreasing order of sensitivity to UV irradiation are as follows: RH 1 (*uvrArecA*) > AA 34 (*exrArecA*) > GW 801 (*recA*) > AB 1886 (*uvrA*) > PAM 5717 (*exrA*) ≃ AB 2494 (*lexA*) > P 3478 (*polA*) > AB 5717 (wt). These results correlate with published results (62) indicating lethality of various DNA repair deficient strains of *E. coli* upon exposure to increasing doses of UV irradiation.

These assays of the DNA repair markers were regularly performed while the *E. coli* strains were in use and were used to assay the repair character of the mutant strains, 801^{Sb}, 801_{c}, 801_{c}^{Sb}, 5717^{Sb}, 5717_{c}, 5717_{c}^{Sb}. The mutant strains proved to display the same sensitivity to UV irradiation as the parental strains, GW 801 and PAM 5717.

Isolation and Naming of Mutant Strains of *E. coli* K12

Metal Resistant Mutants. Colonies exhibiting apparent antimony resistance on PAM 5717 (*exr*A) and GW 801 (*rec*A) were isolated and incubated in nutrient broth. As these isolated cultures were eventually shown to be resistant to antimony (see data below) and because a reapplication of antimony potassium tartrate under the same conditions that induced the apparent mutants in Plate 1 subsequently produced no mutants in cultures of the isolates, these strains have been termed 5717^{Sb} and 801^{Sb}, respectively.

DCS Resistant Mutants. Similarly, colonies of the above strains isolated and cultured in the D-cycloserine (DCS) assay (see data below) which are resistant to DCS have been termed 5717_c and 801_c, indicating the spontaneously arising resistant mutants and 5717_c^{Sb} and 801_c^{Sb}, indicating the antimony-induced resistant mutants.

Antimony Resistance in *rec*A and *exr*A Strains of *E. coli* K12

Plate Assay. One hundred λ samples of 18-hour cultures of GW 801, 801^{Sb}, PAM 5717, 5717^{Sb}, 5717_c and 5717_c^{Sb} were applied to three sets of duplicate plates of NA. A 0.1 M solution of antimony potassium tartrate was prepared and samples of 5 λ (.5 micromoles), 10 λ (1.0 micromoles), and 20 λ (2.0 micromoles) were applied to sterile blank 1/4" Difco discs. Discs were then applied to the sets of duplicate

plates such that each strain was treated with all three concentrations of antimony in duplicate. The plates were incubated for 24 hours at 37° C and the diameter of the resulting zone of inhibition was measured to the nearest millimeter, averaged, and compared.

Growth Assay. Five tubes per strain were prepared containing 3 ml of nutrient broth per tube plus 0.1 ml of the respective strain of bacteria. One tube of the five served as a control and no antimony potassium tartrate was added thus allowing the normal growth process to be monitored. Increasing concentrations of antimony potassium tartrate were added to the remaining four tubes. Tube A contained 15 nmoles/ml antimony potassium tartrate, tube B contained 30 nmoles/ml, tube C contained 45 nmoles/ml, and tube D contained 60 nmoles/ml. Cultures were incubated at 37° C and the optical density read at 550 nm on a Bausch and Lomb Spectronic 20 at various time intervals between 0 and 390 minutes.

Assay of Forward Mutations to D-Cycloserine Resistance

D-cycloserine (DCS), D-4-amino-3-isoxazolidone, an alanine analog, may be incorporated into growth media and resistance to DCS observed by assaying for colony formation on the DCS containing plates. Optimum concentrations of DCS were determined experimentally for each strain (data not shown) and found to be 25 micrograms/ml DCS in NA for the wild type, *uvr*A, *rec*A and *exr*A strains, and 20 micrograms/ml DCS in

NA for the *uvrArecA* double mutant.

Because DCS is heat labile, it must be added to the nutrient agar after the media has been autoclaved. DCS was first dissolved in sterile doubly-distilled water and filter sterilized through a .4 micron millipore filter apparatus. The appropriate amount of sterile DCS solution was then added to the nutrient agar media immediately before pouring the plates. Plates were stored at 4° C and used within 3 days.

Metals on NA Plates Containing DCS. The three metals which were presumed to induce metal resistance in various strains of *E. coli* K12 were further assayed for their ability to induce resistance to DCS in the repair deficient strains of *E. coli* K12, AB 1157, PAM 5717, GW 801, AB 1886 and RH 1.

The *E. coli* to be used were incubated at 37° C for 18 hours and 100 λ samples of the resulting cultures were applied to NA plates containing the appropriate amount of DCS for that strain. Twenty λ samples of 0.05 M solutions of $K(SbO)C_4H_4O_6$, K_2CrO_4, $NaAsO_2$ were applied to sterile blank 1/4" Difco discs. Discs were then placed on duplicate NA plus DCS plates containing one of the repair deficient strains. Plates were assayed for colonies arising around the metal-containing discs and compared to the spontaneously-arising colonies on the control plates.