



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 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.

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Signature Kenneth R. Tindall

Date December 7, 1977

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IONS IN MICROBIAL SYSTEMS

by

KENNETH RAYMOND TINDALL

A thesis submitted in partial fulfillment
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Approved:

Samuel J. Rogers
Chairperson, Graduate Committee

Edwin H. Abbott
Head, Major Department

Henry L. Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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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.

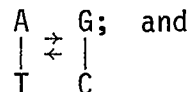
INTRODUCTION

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.

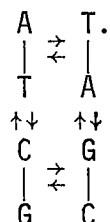
In general, proponents of this theory believe cancer may arise as a result of mutations within somatic cell lines such that normal control of processes regulating cellular proliferation is disrupted. As little evidence is available to either prove or disprove this theory, considerable interest has been 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 ultra-violet (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 *uvrA*, *uvrB*, *uvrC*, *polA* and *lig* genes in the following manner. The *uvrA* and *uvrB* 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 *uvrC* gene product prevents the resealing of the DNA by ligase and allows the excision process to take place (19). The *polA* 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 *uvrA*, 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 *uvrA*, B, C, *recA*, *recBC*, and *lexA* 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 *polA* mutants but rather was stimulated with the stimulation being dependent on the *uvrA* 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 *recBC* 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 *recA* and *lexA* 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 *polA* or *exrA* genes. Therefore, as data indicates the *recA*, *recB*, and *exrA* genes control a single branch of *uvr* gene-dependent excision repair, specifically the long-patch mode of repair and as the *polA* 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 *recA*, *recBC*, and *lexA* mutations and their role in recombinational repair. The *recA* gene is of particular interest since a mutation within this gene appears to block nearly all recombinational activity in *E. coli* (7,19) and *recA* as well as *lexA* 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 *recA* and possibly *recBC* 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 *recA*, *recBC* 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 *recA*⁺ and *lexA*⁺ genotype. A number of other cellular functions such as λ phage induction, septum inhibitor, protein x, and exonuclease V inhibitor production are also under concomitant

control of the *recA* and *lexA* alleles (60). Thus, a mutation in the *recA* 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 *lexA* allele does not affect the constitutive *recA*, *recBC* mediated recombinational repair pathway, although a mutation in the *lexA* 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

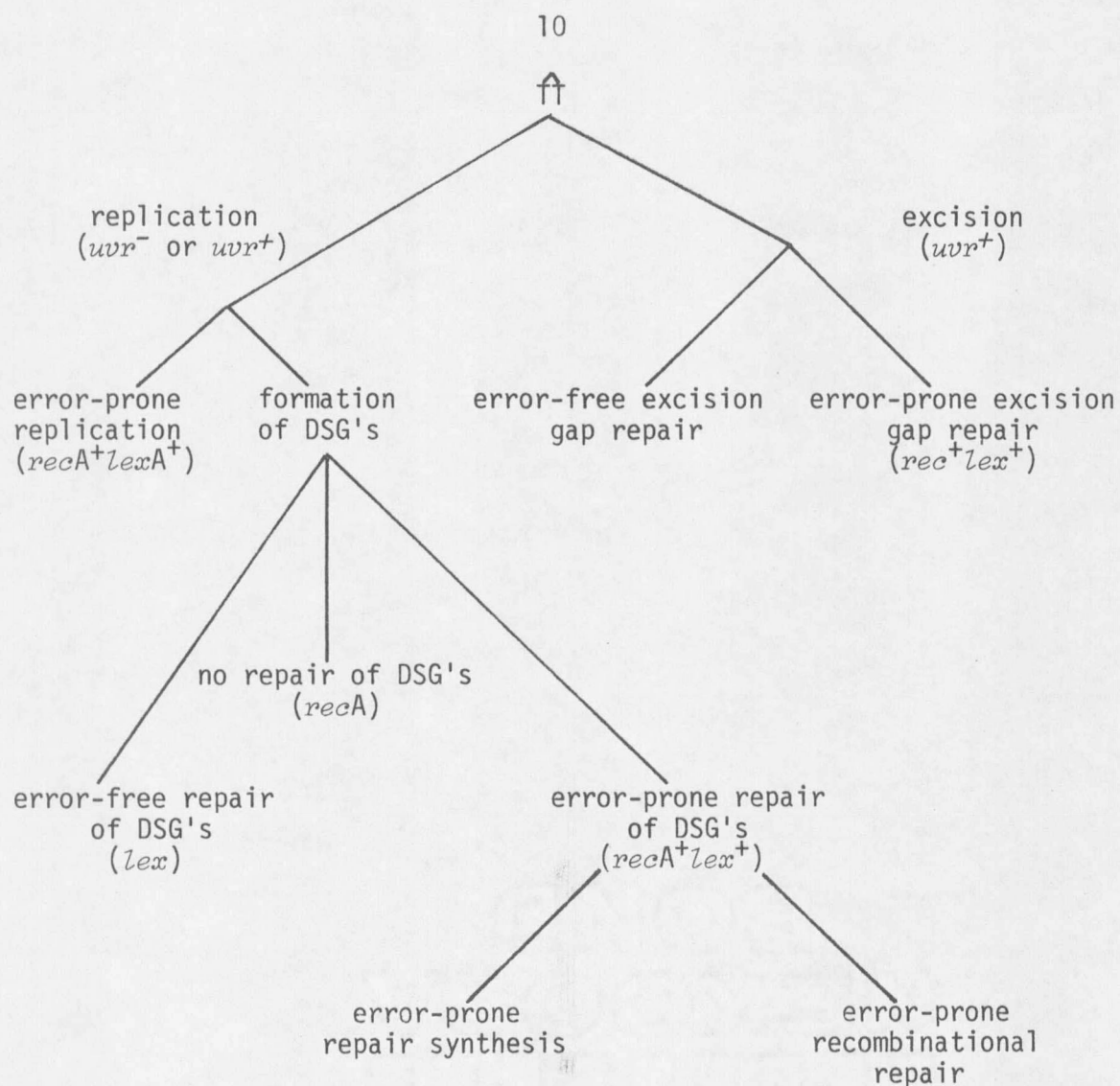


Figure 1. Repair of UV damage in *E. coli* strains of various genotypes and its possible relation to UV mutagenesis (61).

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

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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 congenita, 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 above-mentioned 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 H₂O 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 T₄ (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 CrO₄⁻² species was responsible for the induction of base pair substitutions specifically G → A 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 *recA* 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, FeSO₄, 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, *uvrA*⁻, and *polA*⁻ strains yet had no effect on the *recA*⁻ strain. These results would seem to imply that arsenic might play a role in the inhibition of a *recA*-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.

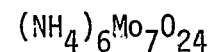
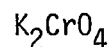
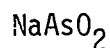
MATERIALS AND METHODS

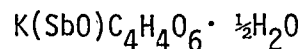
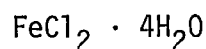
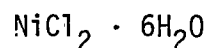
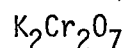
Spot Test Assay on *Salmonella typhimurium*

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).

Preparation of Metal Salts. .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*.

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





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
$(\text{NH}_4)_2\text{SO}_4$	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 frame-shift 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) ₆ Mo ₇ O ₂₄
CdCl ₂	NiCl ₂
K ₂ CrO ₄	SbCl ₃
HgCl	K(SbO)C ₄ H ₄ O ₆
MnCl ₂	ZnCl ₂

0.1 M metal salt solutions were prepared in either sterile doubly-distilled water or DMSO. Twenty microliters (20 λ) 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 ₂ O	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.

<u>Strain</u>	<u>Repair Deficiency</u>
AB 1157	wild type
P 3478	<i>polA</i>
AA 34	<i>exrArecA</i>
AB 2494	<i>lexA</i>
RH 1	<i>uvrArecA</i>
GW 801	<i>recA</i>
PAM 5717	<i>exrA</i>
AB 1886	<i>uvrA</i>

While these strains are not strictly isogenic, the differences are slight with the exception of P 3478, the *polA* 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 *exrA* and *lexA* genes are considered to be the same allele. In this series, the *exrA* mutation was originally isolated in *E. coli* B and transferred to the K12 species while the *lexA* 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 (*exrA*) and GW 801 (*recA*) 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^{Sb}_C and 801^{Sb}_C, indicating the antimony-induced resistant mutants.

Antimony Resistance in *recA* and *exrA* 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^{Sb}_C 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, *uvrA*, *recA* and *exrA* 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.

Growth of *recA* and *exrA* Strains in the Presence of DCS

Growth of *E. coli* K12 strains GW 801, 801_C, 801_C^{Sb}, PAM 5717, 5717_C, and 5717_C^{Sb} in the presence of DCS was assayed by spectrophotometric means at 550 nm on a Bausch and Lomb Spectronic 20.

Assay of the 801 (*recA*) Series. Six tubes per strain were prepared with 3 ml of nutrient broth plus 0.1 ml of the respective strain of *E. coli* (GW 801, 801_C and 801_C^{Sb}). Growth of the bacteria was monitored by measuring optical density at time intervals between 0 and 450 minutes. As in the growth assay in the presence of antimony potassium tartrate, one tube served as a control; no DCS was added thus allowing normal growth. The remaining five tubes contained DCS at concentrations of 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml, and 25 µg/ml.

Assay of the 5717 (*exrA*) Series. Five tubes per strain of the 5717 series were prepared with 3 ml of nutrient broth per tube plus 0.1 ml of the respective strain of *E. coli* (PAM 5717, 5717_C, 5717_C^{Sb}). Growth was monitored by measuring optical density at time intervals between 0 and 405 minutes. Again one tube per strain served as a control to monitor normal growth in nutrient broth. Growth was observed in the remaining four tubes containing 5 µg/ml, 7.5 µg/ml, 10 µg/ml, and 12.5 µg/ml DCS.

RESULTS

Spot Test Assay on *Salmonella typhimurium*

Five of the fifteen metal compounds tested (NaAsO_2 , K_2CrO_4 , K_2CrO_7 , MnCl_2 and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$) have been previously reported as mutagens in bacterial assays (14,30,35,58). A qualitative analysis for either basepair substitution or frameshift activity in the form of a spot test on the Ames *Salmonella typhimurium* histidine reversion assay produced positive results only with the chromates as shown in Table 1. Detection of chromate mutagenicity was found to be dependent upon the medium used. The Davis minimal revised (DMR) proved to allow greater sensitivity in detecting chromate mutagenicity; however, Dexon, the organic positive control, clearly exhibited positive responses on both types of media.

Both K_2CrO_4 and K_2CrO_7 exhibited basepair substitution and frameshift activity in the *S. typhimurium* strains (Table 1). Each was active in reversion tests detecting basepair substitution mutagenesis. Due to the presence of an R factor, TA 100 is the most sensitive of these basepair substitution detecting strains (1). Basepair substitution activity of both K_2CrO_4 and $\text{K}_2\text{Cr}_2\text{O}_7$ was detected at the 200 nmole concentration by TA 100 on DMR as well as at the 2000 nmole concentration on both Vogel Bonner (VB) and DMR media. The less sensitive strain, TA 1535, detected basepair substitution activity by K_2CrO_4 only on DMR at the 2000 nmole concentration. $\text{K}_2\text{Cr}_2\text{O}_7$ activity was detected by TA 1535 on both VB and DMR media, although again only at the 2000

Table 1. Spot test assay on *Salmonella typhimurium*.

Strain	Neg. Control	Dexon	$K_2CrO_4^*$		$K_2Cr_2O_7$	
			(nmoles/plate)		(nmoles/plate)	
			200	2000	200	2000
1535 VB	-	+	-	-	-	+
DMR	-	+	-	+	-	+
1537 VB	-	+	-	+	-	+
DMR	-	+	-	+	-	+
1538 VB	-	+	-	-	-	+
DMR	-	+	-	-	-	+
98 VB	-	+	-	-	-	+
DMR	-	+	-	-	+	+
100 VB	-	+	-	+	-	+
DMR	-	+	+	+	+	+

*All other compounds tested: $NaAsO_2$, $CdCl_2$, $CoCl_2$, $FeCl_2$, $FeCl_3$, $HgCl$, $MnCl_2$, $(NH_4)_6Mo_7O_{24}$, $NiCl_2$, $PbCl_2$, $SbCl_3$, $K(SbO)C_4H_4O_6$, and $ZnCl_2$ were negative.

nmole concentration.

Similarly, the frameshift sensitive R factor-containing strain, TA 98, detected $K_2Cr_2O_7$ activity at the 200 nmole concentration only on DMR but on both media at the 2000 nmole concentration. Strain TA 1538 is the genetic equivalent of TA 98 but lacks the sensitivity granting R factor. Thus, as would be expected, frameshift mutagenesis in TA 1538 is detected on both media although only at the 2000 nmole concentration.

In contrast, K_2CrO_4 did not show activity on either TA 1538 or TA 98 but was shown to induce frameshift mutagenesis in TA 1537 at the 2000 nmole concentration range on both VB and DMR. This phenomenon may be indicative of either differences in equivalent concentrations of Cr in relation to the concentrations necessary for frameshift activity or different mechanisms of frameshift induction due to the differing DNA sequences in strain TA 1537 vs. strains TA 1538 and TA 98. The DNA sequences differ in the region of the *His* operon and allow detection of different kinds of frameshift activity (1).

Analysis of Table 1 indicates detection of mutagenic activity by both chromate and dichromate was favored by DMR media and the R factor bearing strains, TA 98 and TA 100.

Lethality Assay in Repair Deficient Strains of *E. coli* K12

A modification of the technique used by Nishioka (35) in his *B. subtilis* assay of lethality induced due to lack of *rec*-mediated repair was employed using a series of repair deficient strains of *E.*

coli K12. In this assay, chemically-induced lethality is compared between various strains of *E. coli* differing only in their capacity to repair DNA damage. Thus, if a metal induces DNA damage in a strain wild type for repair, mechanisms within the cell repair the damage and cellular processes function as normal. On the other hand, if damage occurs in a DNA repair deficient strain which requires repair via the missing pathway, it is assumed the result will be enhanced lethality to the cell. Thus the lethality assays measure the activity of a metal in relation to DNA metabolism; however, the differential zones of lethality are not necessarily a measure of mutagenic activity.

Nishioka's study involved a comparison of only *rec*-mediated repair in *B. subtilis*. The *E. coli* assay employed involves a series of repair mutations which delete several pathways of repair and thus allow a more comprehensive look at the interaction of metals and the available pathways of DNA repair. Data presented in Tables 2 and 3 illustrate the use of such a system.

These metals may be divided into two groups, i) those which have toxic effects independent of DNA repair mechanisms, i.e., the differential zones of lethality are minimal; and ii) those which have toxic effects that are markedly influenced by the absence of DNA repair pathways, i.e., the differential zones of lethality are easily distinguishable and markedly strain dependent. The first group includes MnCl_2 , $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, SbCl_3 and ZnCl_2 as these compounds did induce "rec"

Table 2. Lethality assay in repair deficient strains of *E. coli* K12.

Strain:	1157	P 3478	AA 34	2494	RH 1	801	5717	1886
Repair Character:	Wt	<i>polA</i>	<i>exrArecA</i>	<i>lexA</i>	<i>uvrArecA</i>	<i>recA</i>	<i>exrA</i>	<i>uvrA</i>
<u>Compound Assayed</u>								
NaAsO ₂	6	14	10	6	6	17	6	6
CdCl ₂	19	22	23.5	24	21.5	21	19	20
K ₂ CrO ₄	25	30	37	30	37.5	33	27	27.5
HgCl	37	35	40	41	43	35.5	37.5	40
MnCl ₂	6	8	8	6	6	9.5	9.5	8
(NH ₄) ₆ Mo ₇ O ₂₄	11	14	13	11	11	10	10	10
NiCl ₂	23	19.5	19	21	24	19.5	16.5	18
SbCl ₃	14	12	13	14	11	16	11	10.5
K(SbO)C ₄ H ₄ O ₆	22	32	31	30	22	30	26.5	25
ZnCl ₂	17.5	20	20	15.5	19.5	19.5	18	19.5

Above values are average diameters of the zones of lethality in millimeters. A total of six samples for each value reported were averaged to the nearest 0.5 mm.

Table 3. Lethality assay in repair deficient strains of *E. coli* K12.
Comparison to the wild type.

Strain:	1157	P 3478	AA 34	2494	RH 1	801	5717	1886
Repair Character:	Wt	<i>polA</i>	<i>exrArecA</i>	<i>lexA</i>	<i>uvrArecA</i>	<i>recA</i>	<i>exrA</i>	<i>uvrA</i>
Compound Assayed								
NaAsO ₂	0	++	+	0	0	+++	0	0
CdCl ₂	0	+	+	++	+	+	0	0
K ₂ CrO ₄	0	++	+++	++	+++	++	+	+
HgCl	0	-	+	+	++	0	0	+
MnCl ₂	0	+	+	0	0	+	+	+
(NH ₄) ₆ Mo ₇ O ₂₄	0	+	+	0	0	0	0	0
NiCl ₂	0	-	-	-	0	-	=	=
SbCl ₃	0	-	0	0	-	+	-	-
K(SbO)C ₄ H ₄ O ₆	0	+++	++	++	0	++	+	+
ZnCl ₂	0	+	+	-	+	+	0	+

5 $\leq$ = $\leq$ 10 zone of lethality in mm less than the wild type.

2 $\leq$ - < 5 zone of lethality in mm less than the wild type.

0 no difference from the wild type

2 $\leq$ + < 5 zone of lethality in mm greater than the wild type.

5 $\leq$ ++ < 10 zone of lethality in mm greater than the wild type.

10 $\leq$ +++ zone of lethality in mm greater than the wild type.

effects but in no case were the differences in the diameters of the zones of lethality greater than 5 mm from the wild type (Table 3). Of these metals, both MnCl_2 and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ have been shown to be mutagenic in other bacterial test systems (14,35,53); however, the mutagenic activity may not be influenced by the DNA repair activities represented by the strains of *E. coli* used in the lethality assay. For example, Mn^{+2} and Zn^{+2} have been shown to decrease the fidelity of Avian Myoblastosis Virus polymerase to a synthetic template (48,50). Such interactions with the DNA polymerase may provide another mechanism by which these metals may exert mutagenic effects.

It is interesting to note the differences between the trivalent form of antimony, SbCl_3 , and the pentavalent form, $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$. While SbCl_3 showed little activity in any of the strains, $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$ was quite active, perhaps reflecting differences in oxidation state (Sb^{+3} vs. Sb^{+5}), ease of transport ($\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$ is water soluble, SbCl_3 is not), as well as possible structural orientation of Sb in the chloride vs. the tartrate molecule in relation to the DNA. Antimony in either form is capable of conferring metal resistance (Table 4) and appears to act in this capacity as a function of repair pathways. This aspect of antimony activity shall be further discussed.

The second class of compounds mentioned, those which greatly affect cell viability in the presence or absence of repair pathways, include NaAsO_2 , CdCl_2 , K_2CrO_4 , HgCl_2 , NiCl_2 , and $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$ (Table 3).

Table 4. Metal induced mutants in repair deficient strains of *E. coli* K12

Strain:	1157	P 3478	AA 34	2494	RH 1	801	5717	1886
Repair Character:	Wt	<i>polA</i>	<i>exrArecA</i>	<i>lexA</i>	<i>uvrArecA</i>	<i>recA</i>	<i>exrA</i>	<i>uvrA</i>
Compound Assayed								
NaAsO ₂	-	-	-	+	-	+	+	-
SbCl ₃	-	-	+	+	-	+	+	-
K(SbO)C ₄ H ₄ O ₆	-	-	+	+	-	+	+	-
K ₂ CrO ₄	+	+	-	-	-	+	+	+

With the exception of NiCl_2 , all of these compounds in at least one repair deficient strain expresses significant lethality. NiCl_2 is an interesting example: Tables 2 and 3 show NiCl_2 in certain repair deficient strains to be less active thus allowing greater survival; that is, in all cases except RH 1, the *uvrArecA* double mutant, the zones of lethality were less than the zone observed in the wild type. This would seem to indicate that occasionally the lack of a specific pathway of repair may be beneficial to the survival of the cell. One might note that Hg, Sb and Zn all behaved similarly in various repair deficient strains as well.

NaAsO_2 and K_2CrO_4 , both demonstrable mutagens (30,35,58), exhibited extreme lethality in strains deficient in various modes of DNA repair. To rationalize the relationship between mutagenic activity and DNA repair, one might conceive of a mechanism where cell viability is maintained but at the expense of an increased mutation rate due to error-prone DNA repair synthesis. Thus data presented in Tables 3 and 4 are consistent, at least in the case of K_2CrO_4 and NaAsO_2 , with the views of Nishioka. His results reported both K_2CrO_4 and NaAsO_2 to be quite lethal in *rec⁻* strains of *B. subtilis* and mutagenic in *E. coli* with the mutagenic effect dependent upon the *recA* allele (35).

Of the remaining compounds inducing significant "rec" effects in the *E. coli* lethality assay (CdCl_2 , HgCl and $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$), all three have been shown to induce chromosomal damage in eukaryotic DNA,

and at least cadmium has been implicated as a carcinogen as well (55). As these compounds have been shown to interact with DNA in the above-mentioned assays, it is not too surprising that activity is observed in the *E. coli* system.

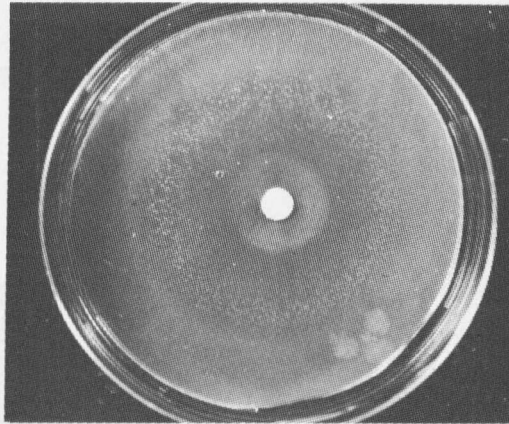
Thus a number of interesting possibilities of metal-repair pathway interactions exist based on the results reported in Tables 2 and 3; however, these interactions have not been fully elucidated. Certainly the data compiled here contain a wealth of information which deserves greater examination than this study has allowed.

Induction of Metal Resistance in *E. coli* K12

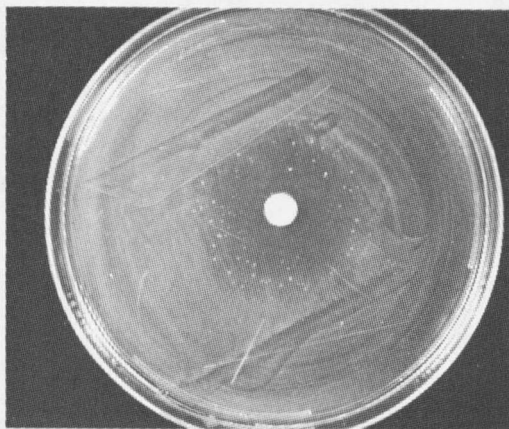
Some of the most active compounds in the lethality assay (K_2CrO_4 , $K(SbO)C_4H_4O_6$ and $NaAsO_2$) did, in this study, prove to induce mutations in bacterial systems as well; chromate expressing activity in the *S. typhimurium* assay (Table 1), antimony and arsenic inducing forward mutations to D-cycloserine resistance in *E. coli* (Table 9), and all three metals inducing forward mutations to metal resistance in *E. coli* (Table 4).

A totally unexpected result of the lethality assay was the occurrence of what appeared to be mutant colonies at the edge of the zones of lethality induced by $NaAsO_2$, $SbCl_3$, $K(SbO)C_4H_4O_6$ and K_2CrO_4 . Photographs of these apparent mutants induced by $K(SbO)C_4H_4O_6$ on GW 801 (*recA*) and PAM 5717 (*exxA*) are shown in Plate 1.

Three questions were asked upon initial observation of these



A



B

Plate 1. Antimony resistant colonies of *E. coli* K12 strains A) GW 801 and B) PAM 5717 arising in the presence of $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$.

colonies: i) are the colonies observed a result of mutagenic activity by As, Sb and Cr?: ii) if mutation induction is being observed, what is the nature of that mutation?; and iii) what limits the apparent specificity of this mutation induction process in relation to the available modes of DNA repair?

Colonies arising around the $K(SbO)C_4H_4O_6$ disc (Plate 1) were isolated and cultured from plates containing the parent strains, GW 801 and PAM 5717. The following evidence proves these cultures to be resistant to $K(SbO)C_4H_4O_6$; thus they were termed 801^{Sb} and 5717^{Sb}.

Table 5 clearly shows differences in zones of lethality between GW 801 and 801^{Sb} as well as between PAM 5717 and 5717^{Sb} at all three concentrations of $K(SbO)C_4H_4O_6$ (0.5, 1.0 and 2.0 μ moles/tab). In this assay, 5717_c, the spontaneous D-cycloserine resistant mutant of PAM 5717, and 5717_c^{Sb}, the antimony-induced DCS resistant mutant of PAM 5717, were also assayed for antimony resistance and found not to be resistant. This indicates DCS resistance and antimony resistance may arise from different mutations although one might expect to be able to select a colony which was doubly mutant, i.e., DCS and antimony resistant.

Growth curves of AB 1157 (wt), GW 801 (*recA*), and 801^{Sb} in the presence of 0, 15, 30, 45 and 60 nmoles of $K(SbO)C_4H_4O_6$ provided corroborative evidence of the resistance of the 801^{Sb} strain to antimony as shown in Figures 2, 3 and 4 which correspond to the values presented in Tables 6, 7 and 8. Some differences were observed between the *recA*

Table 5. Plate assay of Sb resistance in *recA* and *expA* strains of *E. coli* K12

Strain	Conc. (μ mole/tab)		
	.5	1.0	2.0
801	35 ¹	39	40
Resistant mutants	+ ²	+	+
801 ^{Sb}	16	20	24
Resistant mutants	-	-	-
5717	34	37	41
Resistant mutants	+	+	+
5717 ^{Sb}	10	19	25
Resistant mutants	-	-	-
5717 _C	37	39	42
Resistant mutants	+	+	+
5717 ^{Sb} _C	37	38	39
Resistant mutants	+	+	+

¹Diameter of zones of lethality in millimeters. Average values of two experiments done in duplicate.

²+ denotes presence of metal resistant mutant colonies; - denotes absence of metal resistant mutant colonies.

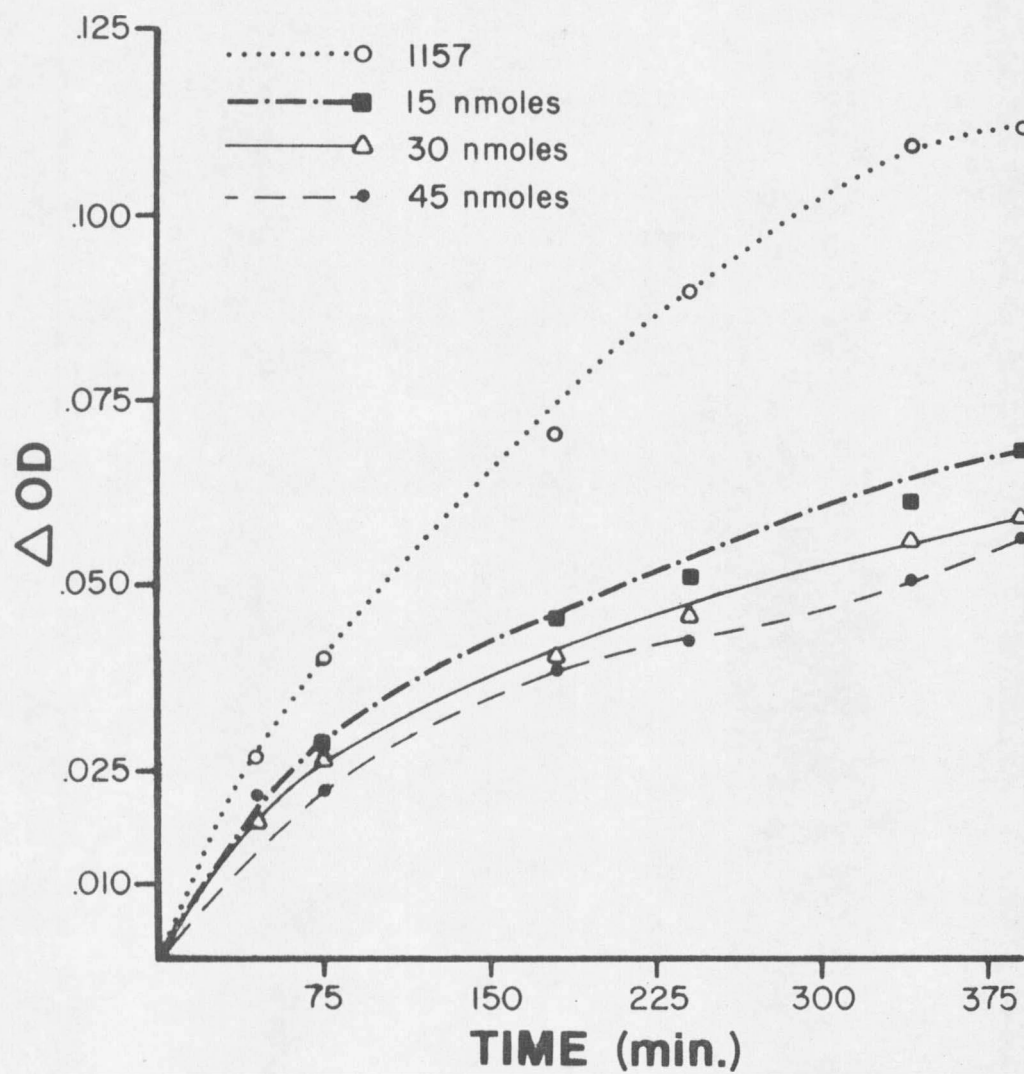


Figure 2. ΔOD vs. time. Growth curves of 1157 in the presence of $K(SbO)C_4C_4O_6$.

Table 6. ΔOD vs. time. Growth curves of 1157 in the presence of $K(SbO)C_4H_4O_6$

Time (min):	0	45	75	180	240	350	390
Conc. (nmoles)							
0	.000	.027	.040	.070	.089	.109	.112
15	.000	.020	.028	.045	.051	.061	.068
30	.000	.019	.027	.041	.046	.056	.059
45	.000	.021	.023	.039	.043	.051	.057
60	.000	.017	.024	.032	.037	.045	.047

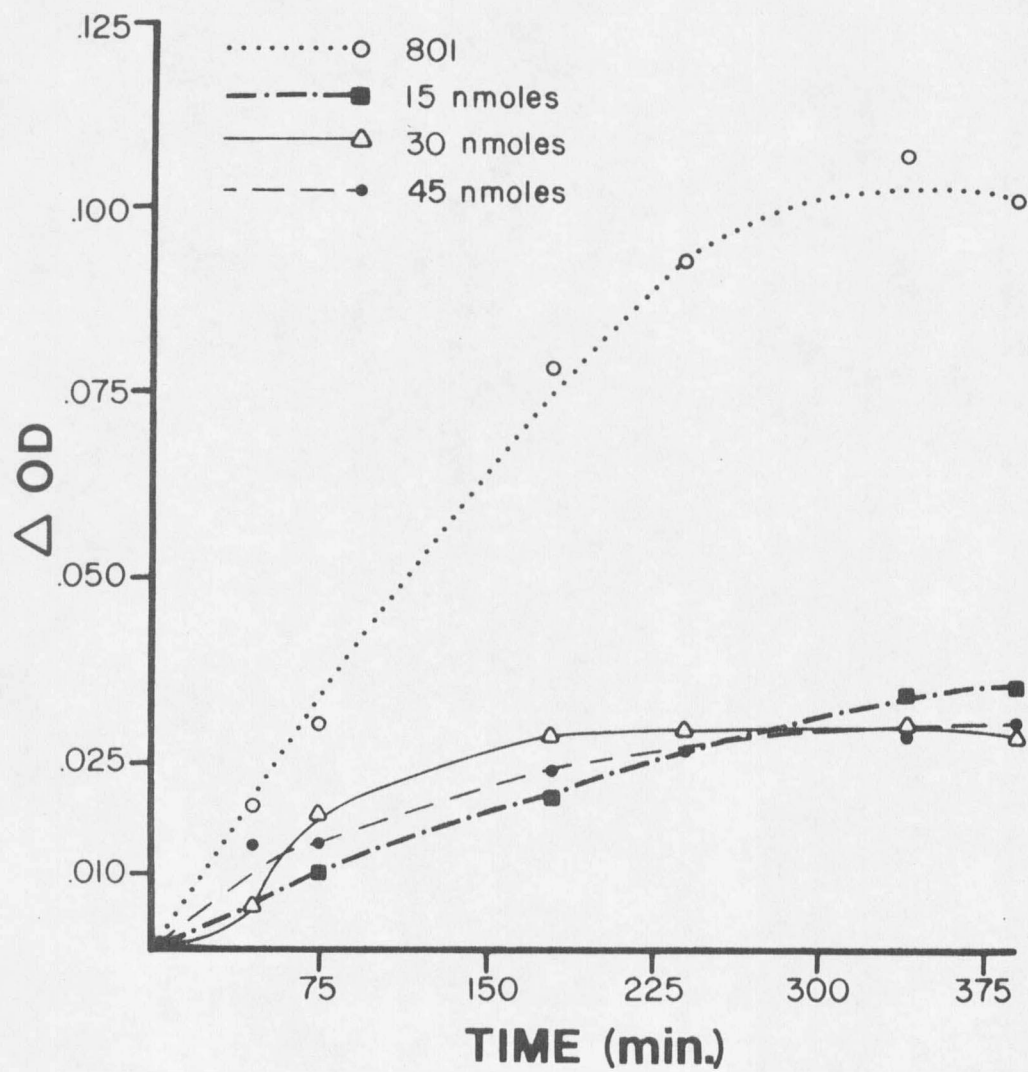


Figure 3. ΔOD vs. time. Growth curves of 801 in the presence of $K(SbO)C_4H_4O_6$.

Table 7. ΔOD vs. time. Growth curves of 801 in the presence of $K(SbO)C_4H_4O_6$

Time (min):	0	45	75	180	240	350	390
Conc. (nmoles)							
0	.000	.019	.030	.078	.093	.107	.101
15	.000	.006	.010	.020	.027	.034	.035
30	.000	.006	.018	.029	.029	.030	.029
45	.000	.014	.014	.024	.027	.029	.030
60	.000	.012	.014	.023	.025	.030	.030

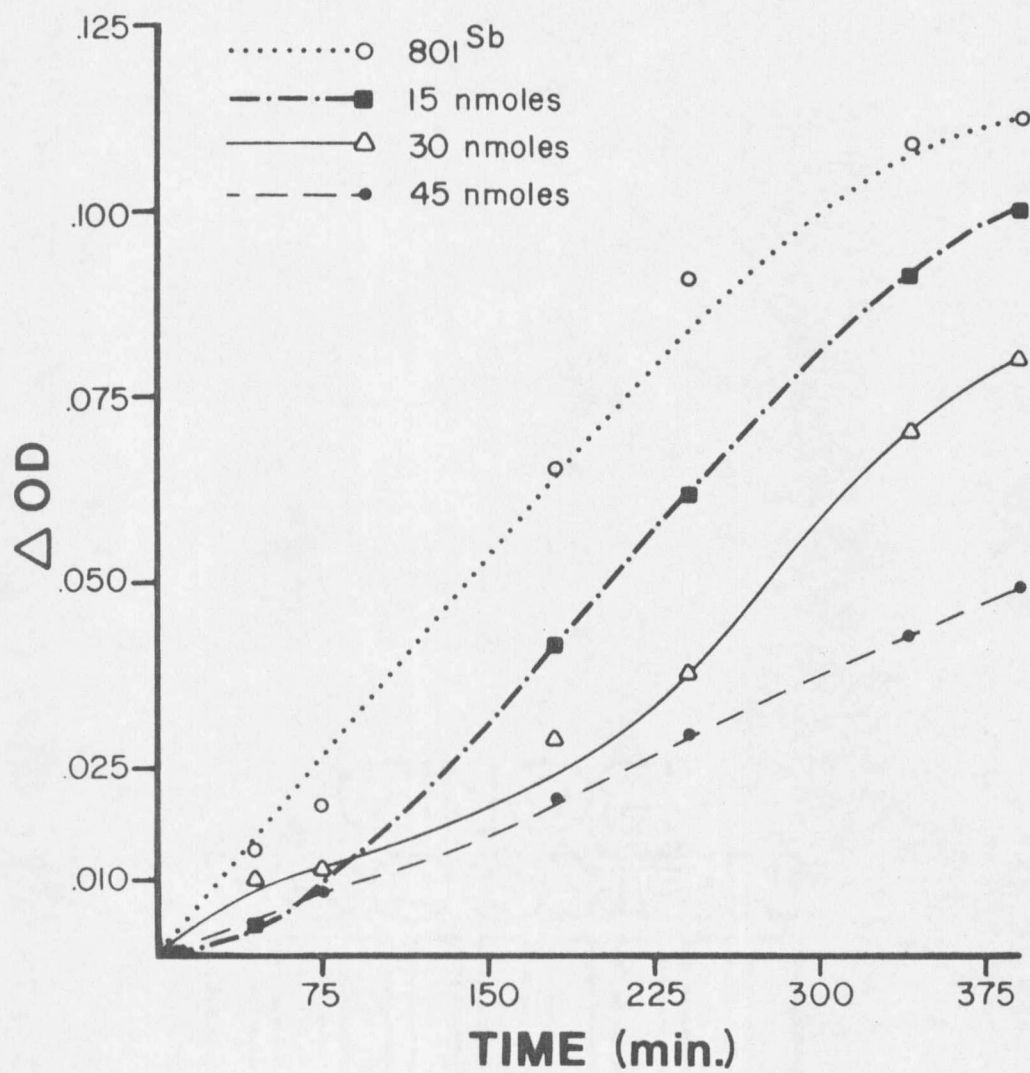


Figure 4. ΔOD vs. time. Growth curves of 801^{Sb} in the presence of $K(SbO)C_4H_4O_4$.

Table 8. ΔOD vs. time. Growth curves of 801^{Sb} in the presence of $K(SbO)C_4H_4O_6$

Time (min):	0	45	75	180	240	350	390
Conc. (nmoles)							
0	.000	.014	.020	.065	.091	.109	.113
15	.000	.004	.010	.042	.062	.091	.100
30	.000	.010	.011	.029	.038	.070	.080
45	.000	.004	.009	.021	.029	.043	.049
60	.000	.009	.013	.022	.024	.032	.031

strains and the wild type strain although Figure 5 clearly shows 801^{Sb} to be more resistant to the presence of $K(SbO)C_4H_4O_6$ than either AB 1157 or GW 801 to concentrations of 30 nmoles.

Thus, the data indicate resistance to antimony toxicity is observed in strains 801^{Sb} and 5717^{Sb}; however, no evidence is presented to allow discrimination between the colonies shown in Plate 1 as resistant mutants arising as the result of induction of mutation or as the result of selection of previously existing resistant mutants within the population. Based on the number of previously existing mutants that would have to exist in the population as well as the fact that $K(SbO)C_4H_4O_6$ was shown to be mutagenic by inducing resistance to D-cycloserine in subsequent experiments within this study, the colonies shown in Plate 1 and referred to in Table 4 are suspected to be the product of induction as opposed to selection. In addition, the colonies arising as a result of exposure to As and Cr as reported in Table 4 are thought to be induced resistant mutants as well, although these colonies were not cultured and examined.

One other aspect of the genetic makeup of 801^{Sb} and 5717^{Sb} was investigated; that being the retention of the DNA repair deficiency (data not shown). In both cases the isolated cultures of 801^{Sb} and 5717^{Sb} behaved identically with parent strains GW 801 and PAM 5717 upon exposure to UV irradiation; that is 801^{Sb} and 5717^{Sb} retained their respective *recA* and *exrA* DNA repair deficiencies.

Finally, the question of the mutagenic activity of As, Sb and Cr on specific DNA repair deficient strains of *E. coli* K12. As one might expect, As and Sb behaved similarly (Table 4) probably owing to their similar S^2p^3 electron orbital configuration. The only other metal inducing this sort of mutation was Cr which has been shown to be mutagenic in the Ames test as well as elsewhere (30,35,58). While it is not too surprising that Cr might induce other sorts of mutations, the fact that As and Sb were active was somewhat surprising. Arsenic has been reported as a mutagen in at least one bacterial assay (35) and has long been known to be carcinogenic based on epidemiological evidence (55); however, while antimony has been shown to induce chromosomal aberrations in human leucocytes (39), it has not been previously documented as a mutagen in bacterial assays. Thus the activity of As and Sb as mutagens is quite novel.

Reexamination of Table 4 leads one to believe resistance to the As and Sb molecules is dependent on the *uvrA* gene function and the inhibition of *recA* or *exrA* mediated repair. Only one difference between arsenic and antimony activity was noted, that being the lack of activity of arsenic on AA 34, the *exrArecA* double mutant.

With knowledge of the lack of mutagenic activity of arsenic and antimony in a standard reversion assay, the Ames test and the somewhat serendipitous discovery of metal resistance to As, Sb and Cr in repair deficient strains of *E. coli* K12, another assay for mutagenic activity

was employed involving not back mutation as in the Ames test but rather forward mutation to resistance in a better characterized system than induction of metal resistance, that being the induction of resistance to the antibiotic D-cycloserine.

Induction of Resistance to D-Cycloserine

D-cycloserine, an analog of D-alanine (Figure 5), is known to competitively inhibit both alanine racemase, the enzyme catalyzing the racemization reaction from L-alanine to D-alanine and D-alanine:D-alanine synthetase, which catalyzes the ATP-dependent synthesis of the D-alanyl-D-alanine dipeptide as shown in Figure 6 (18). The inhibition of these enzymes results in blockage of peptidoglycan biosynthesis due to accumulation of a tripeptide derivative of the cell wall precursor, UDP-muramyl-NAc-pentapeptide, which lacks the terminal D-alanyl-D-alanine dipeptide. Due to the inhibition of cell wall biosynthesis bacterial growth will not be observed in media containing DCS unless a forward mutation to DCS resistance occurs.

Two types of DCS resistant mutants have been described. First, mutant strains with elevated levels of the enzymes normally sensitive to DCS (alanine racemase and D-ala:D-ala synthetase) (43); and second, DCS resistant mutants which have lost the ability to transport D-alanine and glycine. DCS appears to be transported into the cell via the D-alanine-glycine transport system which differs from the system which transports L-alanine. Wargel et al. (59) were able to show DCS activity in *E. coli*

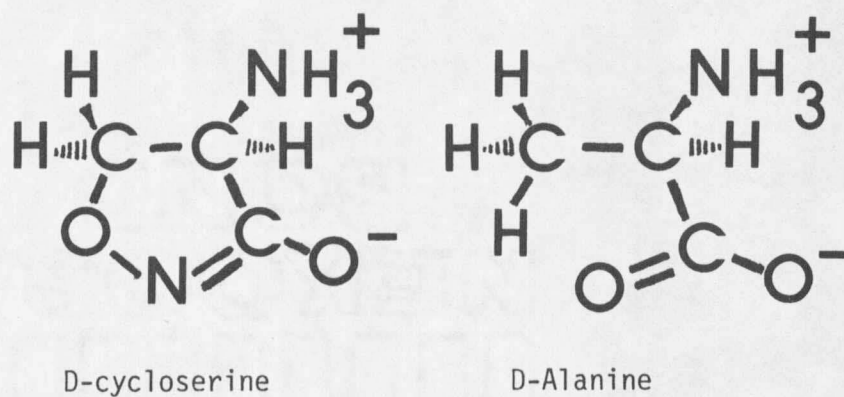


Figure 5. Structure of D-cycloserine and D-alanine.

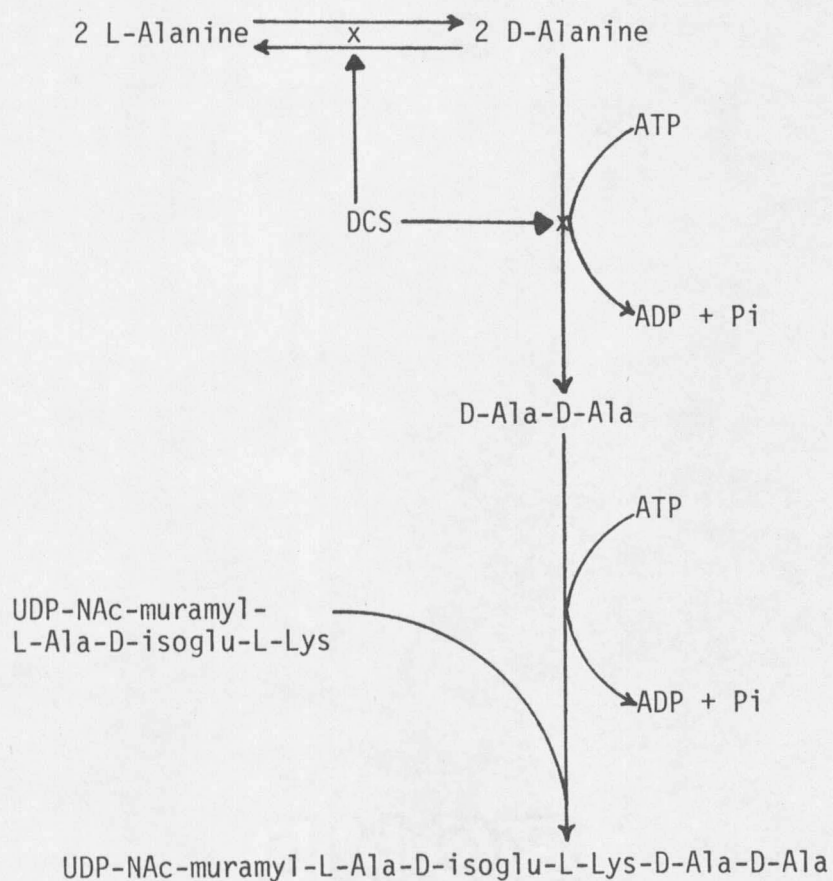


Figure 6. Reactions involved in the incorporation of alanine into the cell wall precursor, UDP-Mur-NAc-pentapeptide. The enzymes catalyzing the reaction converting L-alanine to D-alanine (alanine racemase) and the formation of the D-alanyl-D-alanine dipeptide (D-alanine:D-alanine synthetase) are inhibited by D-cycloserine (DCS) (18).

is absolutely dependent upon a functioning transport system for D-alanine and glycine but not L-alanine. Thus the mutant strains may be classified as either enzyme or transport mutants with the transport mutants, possibly the result of a membrane mutation.

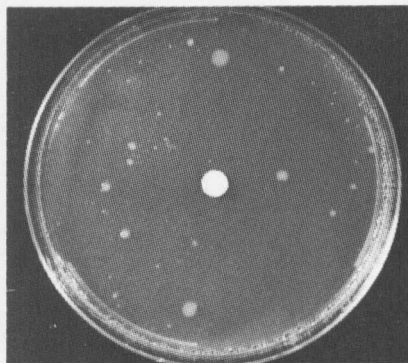
The three compounds which appeared to induce metal resistance, $K(SbO)C_4H_4O_6$, $NaAsO_2$ and K_2CrO_4 were assayed in selected repair deficient strains of *E. coli* K12 on media containing DCS (Table 9). Photographs corresponding to this data are shown in Plates 2 through 6.

Both antimony and arsenic were active in the induction of DCS resistant mutants with antimony inducing mutants on all strains and arsenic selective for the wild type, *recA* and *uvrA**recA* strains. It is interesting to note chromate was not active in this assay.

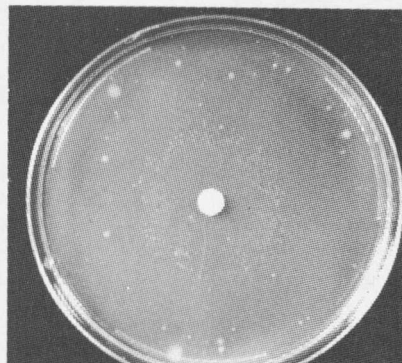
At appropriate concentrations of DCS in relation to the number of bacterial cells plated, one should assume growth on DCS containing plates would only occur if enzyme or transport mutations allowed resistance to the DCS; however, to absolutely establish metal-induced DCS resistance and therefore mutagenic activity of these metals, growth curves of the *recA* series, GW 801, the parent strain, 801_C, the spontaneously arising DCS resistant mutant, and 801_C^{Sb}, the antimony-induced DCS resistant mutant (Figures 7, 8 and 9) as well as the *exrA* series, PAM 5717, 5717_C and 5717_C^{Sb} (Figures 10, 11 and 12) were observed in the presence of DCS and compared to the growth curves in the absence

Table 9. Induction of D-cycloserine resistant mutants on repair deficient strains of *E. coli* K12

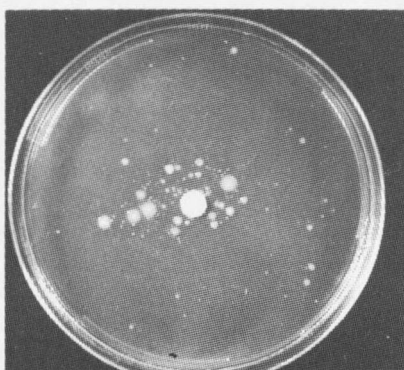
Strain	Control	K(SbO)C ₄ H ₄ O ₆	NaAsO ₂	K ₂ CrO ₄
1157 (wt)	-	+	+	-
5717(<i>exrA</i>)	-	+	-	-
301 (<i>recA</i>)	-	+	+	-
1886 (<i>uvrA</i>)	-	+	-	-
RH1 (<i>uvrArecA</i>)	-	+	+	-



A

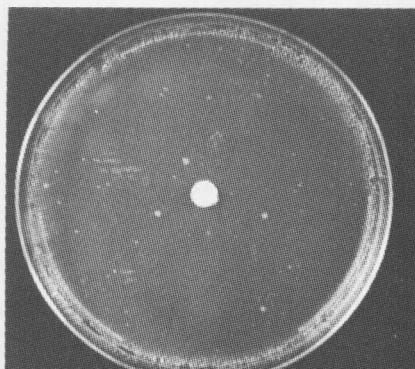


B

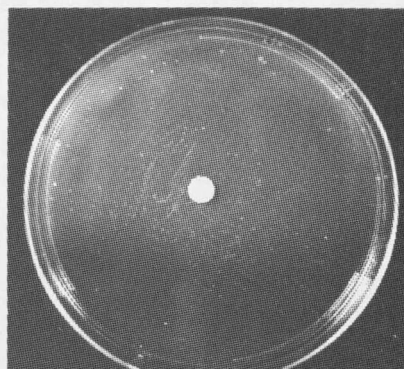


C

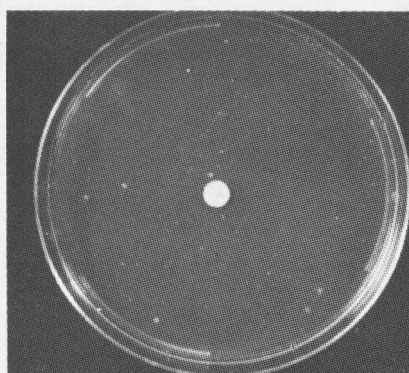
Plate 2. *E. coli* K12 strain AB 1157 on D-cycloserine containing plates. A) negative control. B) Induced mutants arising in the presence of $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$. C) Induced mutants arising in the presence of NaAsO_2 .



A

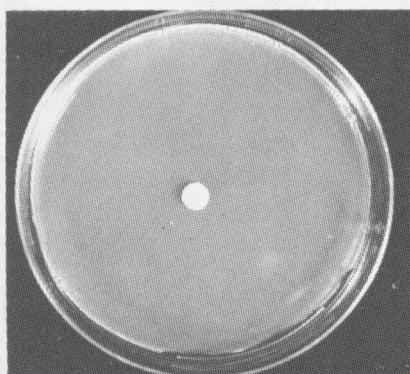


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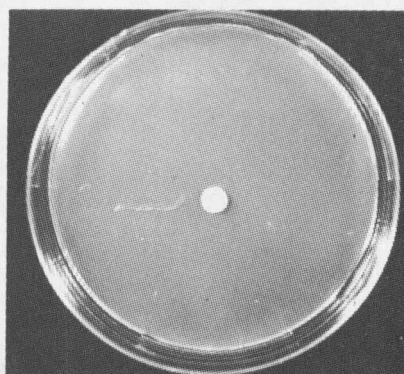


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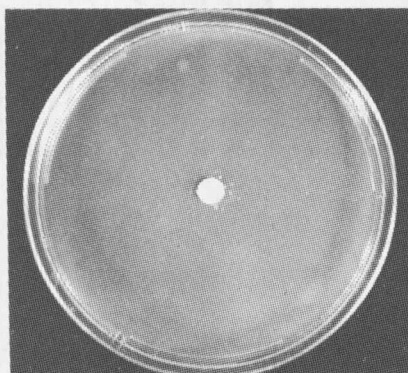
Plate 3. *E. Coli* K12 strain PAM 5717 on D-cycloserine containing plates. A) Negative control. B) Induced mutants arising in the presence of $K(SbO)C_4H_4O_6$. C) No induced mutants arising in the presence of $NaAsO_2$.



A

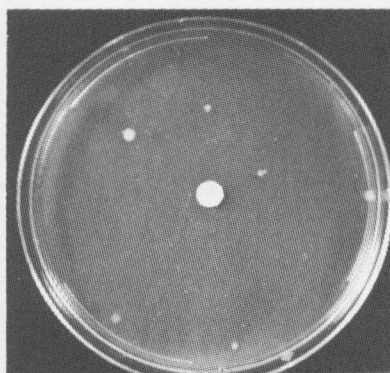


B

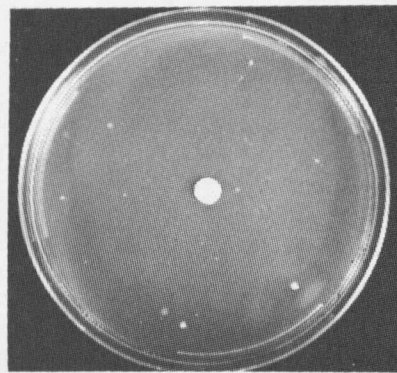


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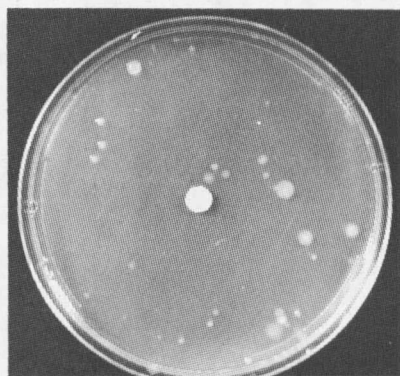
Plate 4. *E. coli* K12 GW 801 on D-cycloserine containing plates. A) Negative control. B) Induced mutants arising in the presence of $\text{K(SbO)C}_4\text{H}_4\text{O}_6$. C) Induced mutants arising in the presence of NaAsO_2 .



A

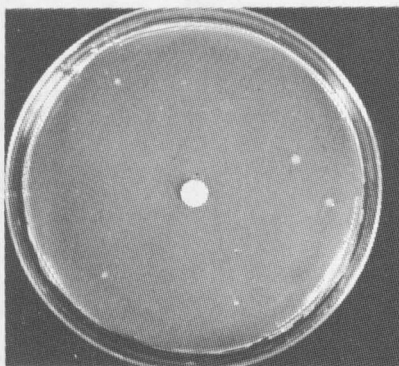


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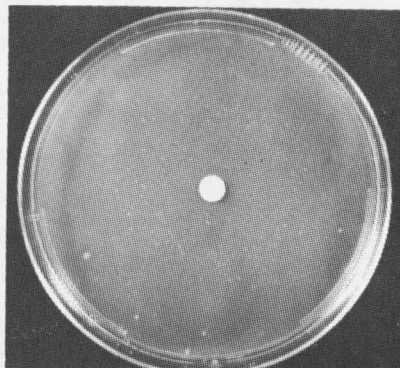


C

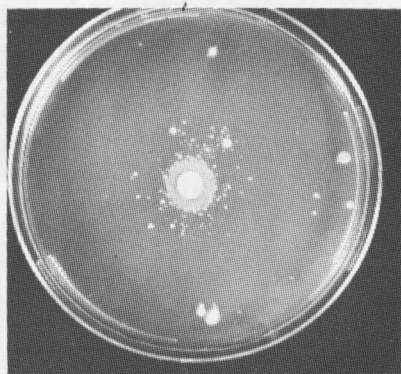
Plate 5. *E. coli* K12 strain AB 1886 on D-cycloserine containing plates. A) Negative control. B) Induced mutants arising in the presence of $\text{K(SbO)C}_4\text{H}_4\text{O}_6$. C) No induced mutants arising in the presence of NaAsO_2 .



A



B



C

Plate 6. *E. coli* K12 strain RH 1 on D-cycloserine containing plates.
A) Negative control. B) Induced mutants arising in the presence of $\text{K(SbO)C}_4\text{H}_4\text{O}_6$. C) Induced mutants arising in the presence of NaAsO_2 .

of DCS.

Figure 7 corresponds with the values reported in Table 10 and compares the normal growth curve of GW 801 with growth in the presence of increasing amounts of DCS. At a concentration of 5 $\mu\text{g/ml}$ DCS growth is severely inhibited until approximately 240 minutes when growth resumes but still shows marked inhibition. A possible explanation for the growth curve of GW 801 at the 5 $\mu\text{g/ml}$ concentration of DCS is that if the cell can overcome the inhibition of DCS in the spheroplast state, production of normal cell walls will allow proper growth. In summary, competitive inhibition of enzyme active sites by DCS requires one molecule of DCS per active site inhibited. As inhibition only affects the formation of the cell wall precursor, spheroplasts will form. These spheroplasts may continue to divide, producing more of the enzyme while the number of DCS molecules stays constant until enough of the enzyme is produced to overcome DCS inhibition and allow proper cell wall formation. The ability of spheroplasts to overcome DCS inhibition will only occur at low concentrations of DCS such that the balance between the DCS molecules and the enzyme active sites is upset to allow uninhibited enzyme active sites and thus normal enzyme function. At higher concentrations of DCS the spheroplasts may never produce enough enzyme to overcome DCS inhibition. Thus, at a DCS concentration of 10 $\mu\text{g/ml}$, growth of GW 801 is severely inhibited during the entire course of the study. In line with the argument presented above, this would

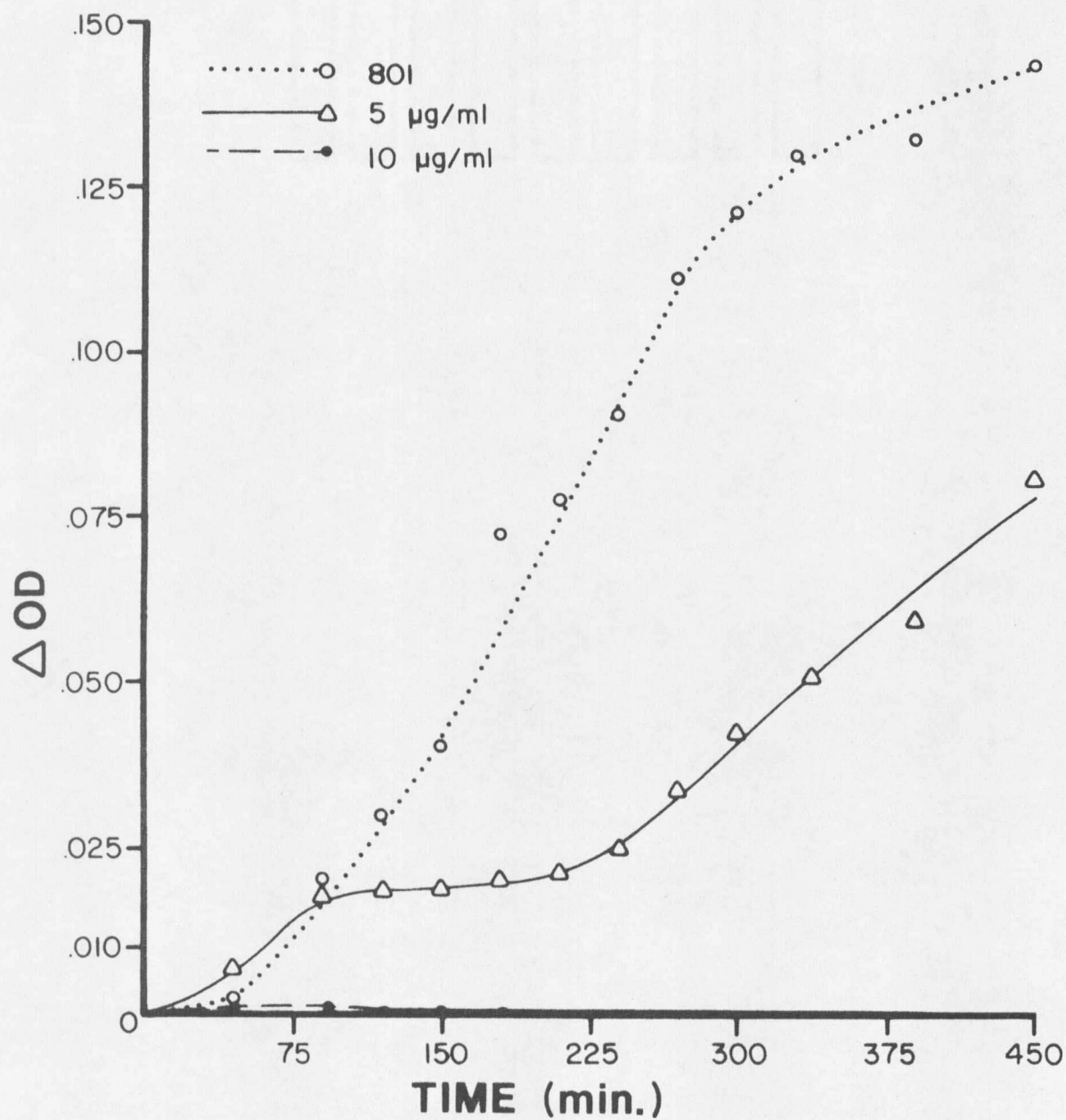


Figure 7. ΔOD vs. time. Growth curves of 801 in the presence of D-cycloserine.

Table 10. ΔOD vs. time. Growth curves of 801 in the presence of D-cycloserine

Time (min):	0	45	90	120	150	180	210	240	270	300	330	390	450
Conc. ($\mu\text{g/ml}$)													
0	.000	.003	.010	.030	.041	.073	.078	.091	.112	.122	.130	.133	.144
5	.000	.007	.018	.018	.018	.021	.022	.025	.034	.043	.051	.059	.081
10	.000	.001	.002	-- ¹	--	--	--	--	--	--	--	--	--
15	.000	--	--	--	--	--	--	--	--	--	--	--	--
20	.000	--	--	--	--	--	--	--	--	--	--	--	--
25	.000	--	--	--	--	--	--	--	--	--	--	--	--

¹ Absorbance reading less than reading at T_0 .

suggest an overwhelming number of DCS molecules available to inhibit enzyme activity.

Figures 9 and 10 correspond with the values reported in Tables 11 and 12 which show both 801_C and 801_C^{Sb} , respectively, to be clearly resistant to DCS at the 5 $\mu\text{g/ml}$ concentration with both strains beginning to overcome DCS inhibition at the 10 $\mu\text{g/ml}$ concentration. As 801_C^{Sb} behaved similarly to the spontaneous mutant 801_C and both strains were markedly different from the previously unexposed parent strain, GW 801, strain 801_C^{Sb} was considered to be a DCS resistant mutant induced by $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$.

Growth curves of the *exrA* series provided results similar to those observed in the *recA* series. Figure 10 corresponding to Table 13 shows the growth of PAM 5717 to be somewhat inhibited at concentrations of 5 $\mu\text{g/ml}$ DCS and clearly inhibited at concentrations of 7.5 and 10 $\mu\text{g/ml}$. As was noted previously in the 801 series, the growth curves of the spontaneous and induced DCS resistant mutants were comparable and quite different from the unexposed parent strain.

Figures 11 and 12 correspond to Tables 14 and 15 and show 5717_C and 5717_C^{Sb} , respectively, to be less sensitive than PAM 5717 to the 5 $\mu\text{g/ml}$ concentration, most clearly resistant to DCS at the 7.5 $\mu\text{g/ml}$ concentration and, like PAM 5717, sensitive to DCS at a concentration of 10 $\mu\text{g/ml}$. Thus 5717_C^{Sb} was also considered to be a DCS resistant mutant induced by $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$.

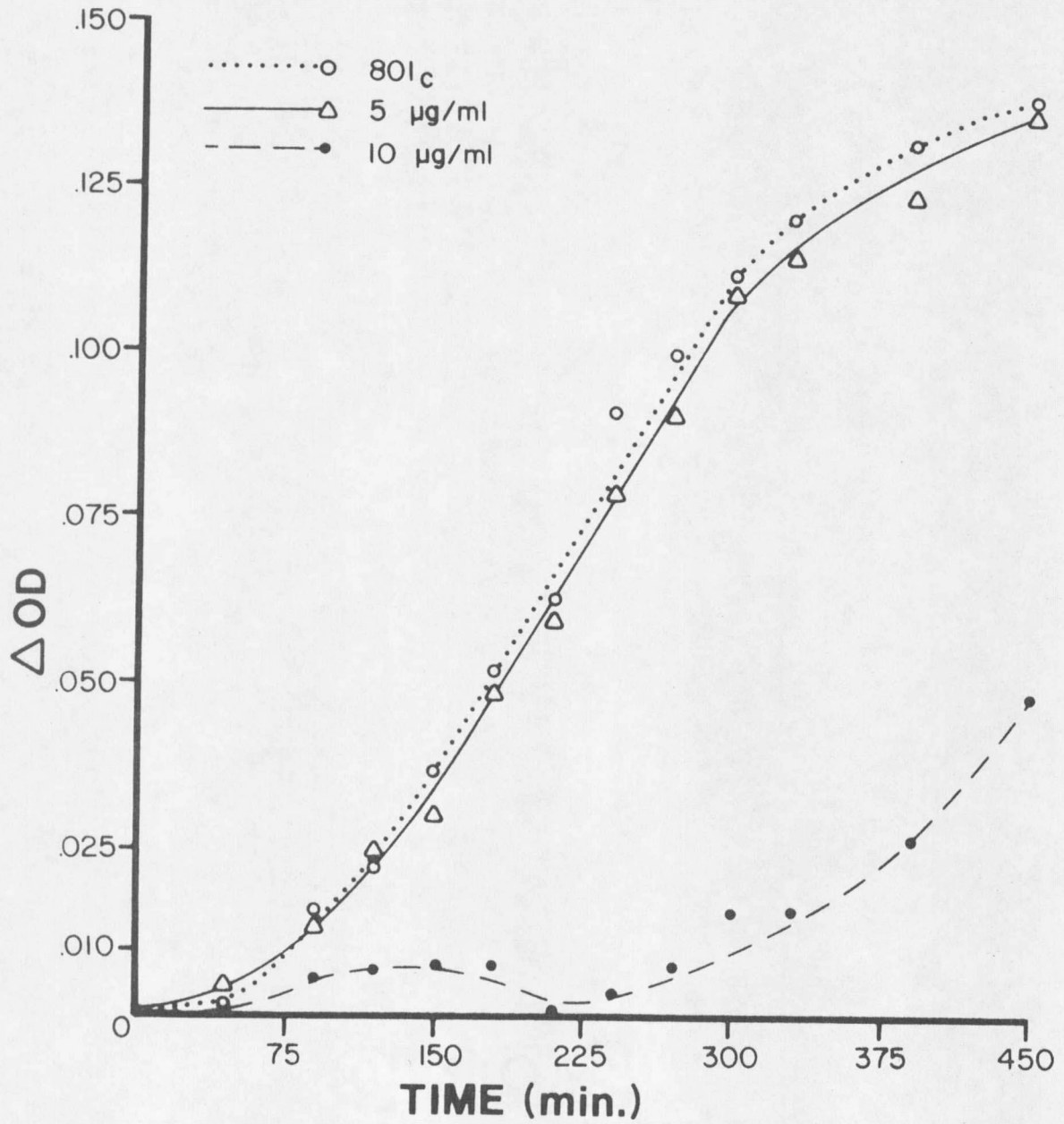


Figure 8. ΔOD vs. time. Growth curves of 801_c in the presence of D-cycloserine.

Table 11. ΔOD vs. time. Growth curves of 801_c in the presence of D-cycloserine

Time (min):	0	45	90	120	150	180	210	240	270	300	330	390	450
Conc. ($\mu\text{g/ml}$)													
0	.000	.002	.016	.023	.037	.052	.063	.091	.099	.112	.120	.132	.138
5	.000	.004	.018	.024	.030	.048	.059	.078	.090	.109	.114	.128	.137
10	.000	.000	.006	.007	.008	.008	.001	.004	.008	.016	.016	.027	.048
15	.000	-- ¹	.000	.000	.003	.004	--	--	--	.003	.000	.003	.001
20	.000	--	.002	.001	.001	.002	--	--	--	.001	--	.000	--
25	.000	--	--	--	--	--	--	--	--	--	--	--	--

¹Absorbance reading less than reading at T_0 .

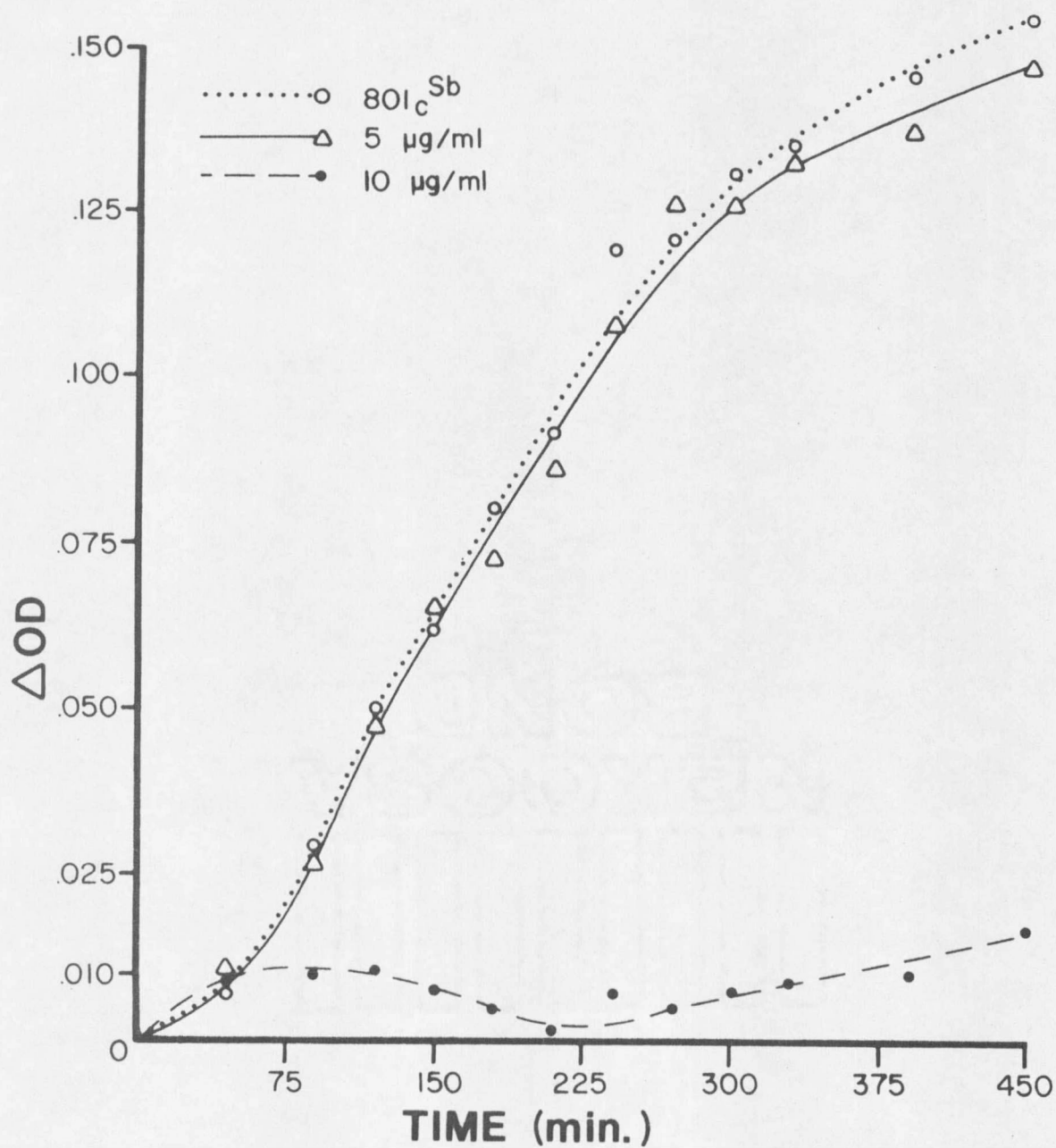


Figure 9. ΔOD vs. time. Growth curves of 801_c^{Sb} in the presence of D-cycloserine.

Table 12. ΔOD vs. time. Growth curves of 801_C^{Sb} in the presence of D-cycloserine

Time (min):	0	45	90	120	150	180	210	240	270	300	330	390	450
Conc. ($\mu g/ml$)													
0	.000	.008	.029	.050	.062	.080	.092	.119	.121	.131	.135	.146	.155
5	.000	.009	.027	.048	.065	.073	.086	.108	.126	.126	.133	.138	.148
10	.000	.010	.010	.011	.008	.005	.002	.008	.005	.008	.009	.010	.017
15	.000	.003	.001	.008	.000	-- ¹	--	--	--	.000	--	--	.000
20	.000	.001	.001	.005	.000	.000	--	--	--	--	--	--	.001
25	.000	.000	.001	.009	.001	--	--	.000	--	.001	.000	.000	.001

¹Absorbance reading less than reading at To.

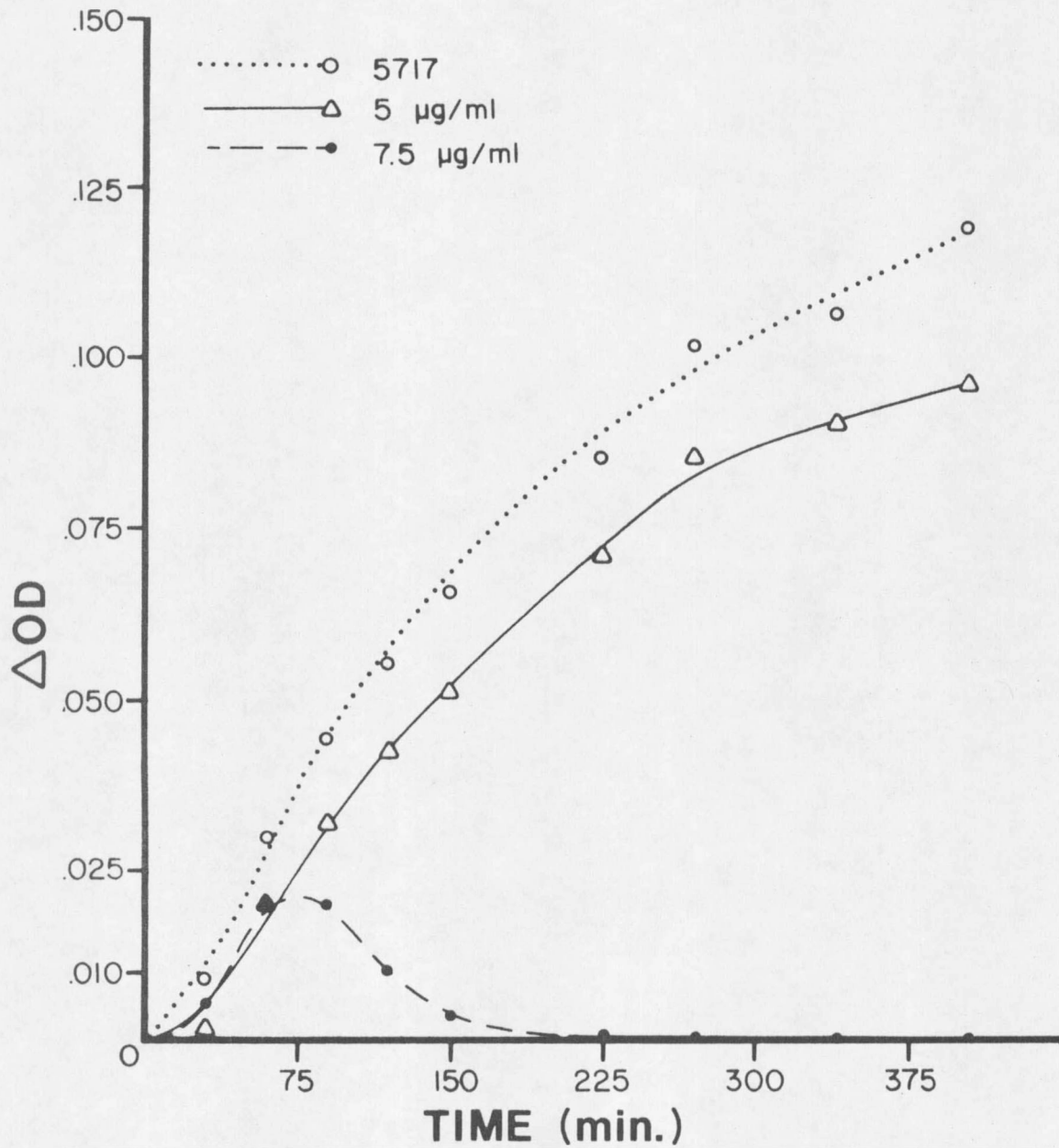


Figure 10. ΔOD vs. time. Growth curves of 5717 in the presence of D-cycloserine.

Table 13. Δ OD vs. time. Growth curves of 5717 in the presence of D-cycloserine

Time (min):	0	30	60	90	120	150	225	270	340	405
Conc. (μ g/ml)										
0	.000	.009	.030	.044	.055	.066	.085	.102	.106	.119
5	.000	.002	.020	.032	.043	.051	.071	.085	.090	.096
7.5	.000	.006	.020	.020	.011	.004	.000	.002	--	--
10	.000	.006	.014	-- ¹	--	--	--	--	--	--
12.5	.000	.010	.005	--	--	--	--	--	--	--

¹ Absorbance reading less than reading at To.

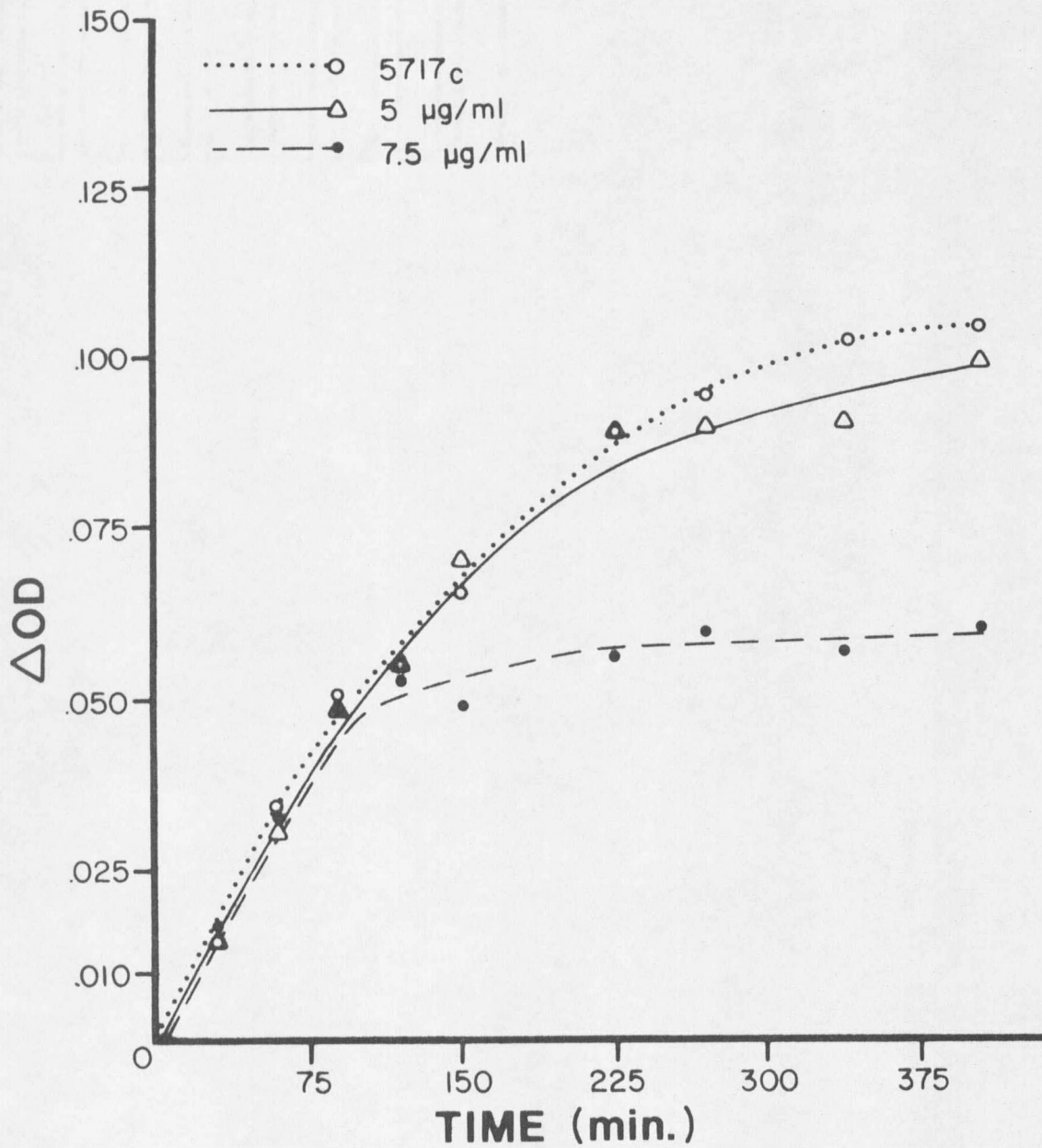


Figure 11. ΔOD vs. time. Growth curves of 5717_c in the presence of D-cycloserine.

Table 14. ΔOD vs. time. Growth curves of 5717_C in the presence of D-cycloserine

Time (min):	0	30	60	90	120	150	225	270	340	405
Conc. ($\mu\text{g/ml}$)										
0	.000	.014	.035	.051	.055	.065	.088	.094	.103	.104
5	.000	.014	.031	.049	.055	.070	.088	.089	.090	.099
7.5	.000	.015	.034	.049	.053	.048	.056	.059	.056	.060
10	.000	.015	.025	.018	-- ¹	--	--	--	--	--
12.5	.000	.015	.015	--	--	--	--	--	--	--

¹ Absorbance reading less than reading at To.

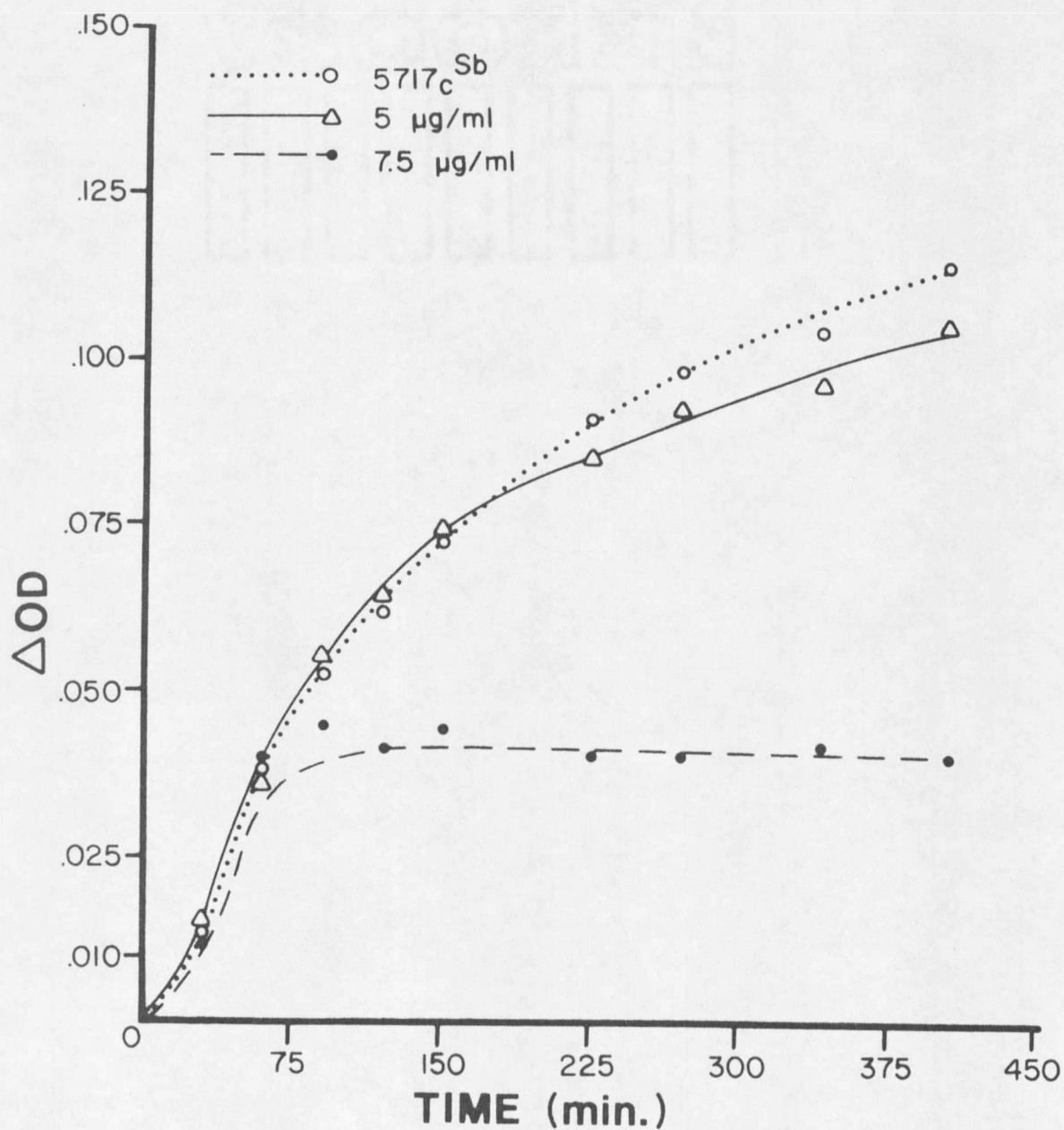


Figure 12. ΔOD vs. time. Growth curves of *5717_c^{Sb}* in the presence of D-cycloserine.

Table 15. ΔOD vs. time. Growth curves of 5717^{Sb}_C in the presence of D-cycloserine

Time (min):	0	30	60	90	120	150	225	270	340	405
Conc. ($\mu\text{g/ml}$)										
0	.000	.014	.038	.053	.062	.073	.091	.098	.104	.114
5	.000	.015	.037	.055	.064	.074	.085	.093	.096	.105
7.5	.000	.013	.039	.045	.042	.044	.040	.040	.042	.040
10	.000	.016	.033	.025	.007	-- ¹	--	--	--	--
12.5	.000	.018	.018	--	--	--	--	--	--	--

¹ Absorbance reading less than reading at To.

Thus these data provide sufficient evidence to confirm that the colonies arising on DCS containing plates are, in fact, DCS resistant mutants; and that $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$ is capable of mutagenic activity conferring DCS resistance in GW 801 and PAM 5717. Furthermore, it has been concluded that the colonies arising around the antimony and arsenic containing discs (Plates 2 through 6) are a result of the mutagenic effects of $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$ and NaAsO_2 .

DISCUSSION

Results obtained in the *Salmonella* assay were somewhat disappointing as the system proved to be sensitive only to chromate mutagenesis. Is the lack of sensitivity to metal ion mutagenesis a function of inherent problems within the *Salmonella* assay or a function of problems involved in testing metals as mutagens? The answer to this question is, of course, multi-faceted; such that, both the Ames test and testing procedures for metals may have to be modified to allow adequate testing of metal ions as mutagens. One should keep in mind the Ames test has been primarily designed to assay for organic mutagens; as previously discussed, the test is 90% effective (32,33) in detecting the mutagenic potential of organic carcinogens. Yet the enigma remains; a number of metals are carcinogenic. In accordance with the somatic cell mutation theory of cancer one might assume that at least some of these carcinogenic metals act via mutagenic mechanisms. If so, some general conclusions based on the results reported in this study may be drawn, particularly in relation to the activities of antimony and arsenic.

Physiological and environmental differences in the testing procedures may account for some of the problems encountered. Consider, first, the differences observed in detecting chromate mutagenesis on VB and DMR media. DMR allowed greater sensitivity due to the lack of the ligand citrate, the inclusion of a slight amount of casamino acids or more likely a combination of both. The question one must ask is

why positive results occur on these minimal media only with the chromates while metals that have been shown to be mutagenic in other bacterial assays (NaAsO_2 , MnCl_2 , and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$) were negative. This question is not easily answered; however, several possible explanations exist.

First, the spot test is only a qualitative measure of mutagenic activity. The positive results obtained with the chromates and Dexon, the positive control, are perhaps an indication of the potent mutagenic activity of these compounds. One could rationalize negative results with NaAsO_2 , MnCl_2 , and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ as a function of their comparatively weaker mutagenic potency. Certainly dose response data should be obtained on these compounds for a more comprehensive evaluation of the Ames test as an assay for mutagenic metals. On the other hand, Nishioka's data (35) provided evidence that NaAsO_2 , $\text{K}_2\text{Cr}_2\text{O}_7$ and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ were all mutagenic to the same order of magnitude. At a treatment level where approximately 30-40% of the cells were viable after exposure to the compound assayed in the *E. coli* B tryptophan auxotroph, WP2uvrA, which is comparable to the *Salmonella typhimurium* base pair substitution strain TA 1535, NaAsO_2 induced 35.5 trp^+ cells/ 10^8 survivors, $\text{K}_2\text{Cr}_2\text{O}_7$ induced 92.8 trp^+ cells/ 10^8 survivors, and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ induced 30.1 trp^+ cells/ 10^8 survivors. These are compared to a spontaneous reversion rate of 4.83 trp^+ / 10^8 cells observed in WP2uvrA. $\text{K}_2\text{Cr}_2\text{O}_7$ is the most active of the three but is not

significantly more active than the other two which provides some rebuttal to the thought that the chromates represent significantly more mutagenic activity.

Perhaps a better explanation may be provided through analysis of the activities of metals tested in the repair deficient strains of *E. coli* K12. As previously discussed, the lethality assay results clearly provide an indication of metal-induced lethality as a function of the availability or lack of modes of DNA repair. From this data, one can make the assumption that metals which induced strong "rec effects" are i) entering the cell and ii) interacting with DNA in such a way as to be recognized by repair enzymes. Subsequent data provided show two compounds other than K_2CrO_4 , specifically $NaAsO_2$ and $K(SbO)C_4H_4O_6$, to be mutagenic by presumably inducing metal resistance and by inducing resistance to D-cycloserine. In this case, metals which would have been classified as nonmutagens based on the results of the Ames spot test actually appear to be quite active inducers of resistance mutations as detected in the D-cycloserine assay and as indicated by the isolation of metal resistant mutants. Thus, differences in the assays obviously are a factor in detecting mutagenic activity induced by these metals.

One notable difference in the systems is that the Ames test, a reversion assay, is necessarily run on minimal medium while the metal resistant and D-cycloserine resistant mutants, results of forward

mutations, were only observed on complete media. Media differences have been shown to play a role in the expression of various pathways of DNA repair in *E. coli* (51). With this in mind, one might imagine a situation involving a premutagenic lesion induced by a metal requiring error-prone DNA repair for the expression of mutagenic activity. If that pathway were blocked due to physiological effects involving the media, either the lesion would be repaired via more efficient repair mechanisms or the lesion would result in death to the cell. In either case, the potential mutagenic event would not be detected.

Another difference involves the availability of DNA repair mechanisms within the *Salmonella* assay as opposed to those available within the repair deficient series of *E. coli*. The Ames strains contain a deletion through the *uvrB* region which should block the excision repair pathways leaving any repairable damage to be dealt with post replicatively and thus via the error-prone pathways of repair. This line of reasoning has met with great success in detecting organic mutagens.

The *E. coli* series, however, allow a number of repair interactions not available with the standard Ames *uvrB* strains. The *E. coli* strains shown in Table 4 appear to require *uvrA* gene function for expression of resistance to the antimony and arsenic compounds. Perhaps the expression of mutagenic activity by metals, in general,

requires the availability of pathways of repair not present in the *uvrB* strains of *S. typhimurium*. The fortuitous finding of antimony and arsenic resistant colonies arising on repair deficient strains of *E. coli* in what appears to be a consistent pattern could evoke speculation that reversion assays with *S. typhimurium* might possibly detect arsenic and antimony mutagenesis if other repair mutations were made available within that system. Specifically, if the excision repair capacities were restored and the *recA* and *errA* functions deleted.

Thus, modification of the assays may be necessary to allow consistent detection of mutagenic metals in microbial systems. These data seem to indicate possible starting points for the streamlining of these microbial assays to detect metallic mutagens by indicating possible media alterations as well as considerations of available or deleted repair capacities. Most evident is the fact that, as presently designed, microbial testing systems are probably not the best assays of metal-induced mutational activity; however, with some relatively minor changes, these assays could conceivably approach the efficiency Ames has obtained in detecting organic mutagens with the *S. typhimurium* testing system. In addition, the interesting interactions observed between metals and DNA repair may be of use to help more clearly characterize mechanisms of action of some of these repair pathways.

In this study, the apparent induction of As, Sb, and Cr resistant mutants followed by the induction of D-cycloserine resistance

by As and Sb was most interesting. Such mutations involving metal or antibiotic resistance in bacteria are not uncommon. In *Staphylococcus aureus*, plasmid-linked resistance to arsenate, arsenite, lead, cadmium, mercury and bismuth was demonstrated to involve separate genetic loci with resistance to antimony and zinc also found but these not separated genetically from resistance to arsenite and cadmium, respectively (36). Plasmid-linked resistance to cobalt, nickel, cadmium, arsenic, and mercury has been demonstrated in *E. coli* as well (22,54). The fact that some of these plasmids carrying metal resistance may also confer resistance to various antibiotics could serve to indicate the mechanism by which resistance to metals and some antibiotics might arise. For example, if resistance to a metal involved a membrane mutation which inhibited transport of that metal into the cell, such a mutation conceivably could inhibit transport of other molecules, such as antibiotics, as well.

Consider the case at hand involving antimony-resistant and antimony-induced D-cycloserine-resistant colonies. As discussed earlier, the antimony-resistant mutants most likely are induced and the DCS-resistant colonies definitely are induced upon exposure to antimony. The mechanism of DCS resistance was discussed as being a function of increased enzyme levels or a transport mutation. Suppose the transport mutation were more likely; this mutation inhibits DCS activity while still allowing the transport of Sb. Table 5 indicates

5717_C and 5717_C^{Sb} both to produce colonies as seen in Plate 1-B. This would seem to indicate induced Sb resistance and induced DCS resistance involve separate genetic loci although both may involve transport mutations.

Thus, the genetic locus mapped in *S. aureus* common to As and Sb could be a transport/membrane mutation and similar to the metal resistance mutation observed in this study, although probably different from the mutation conferring chromate or DCS resistance. Corroborative evidence of separate mutations and perhaps separate mutational mechanisms becomes apparent upon examination of the pattern of induction of metal resistance vs. DCS resistance on the repair deficient strains of *E. coli* (Tables 4 and 9). The fact that induction of resistance to antimony and arsenic occurs in such a definite pattern and different from the pattern induced by chromate may be indicative of the phenomenon observed in *S. aureus* (36). That is, antimony and arsenic could induce resistance at the same genetic locus by inducing a mutation that no longer allows transport of these metals. It seems likely both antimony and arsenic would be transported by the same transport mechanism due to their similar electron configurations and chemical activity. Finally, the fact that different patterns of mutation induction involving As and Sb resistance vs. As and Sb induced DCS resistance were observed on the repair deficient strains of *E. coli* may indicate different modes of mutation induction, although no explanation for this phenomenon seems

seems readily apparent.

What, then, are some plausible mechanisms by which antimony and arsenic might exert their mutagenic effect? Realizing induction of metal resistance to be a repair specific process and medium dependent as well, one might be somewhat surprised by the fact that mutations of any sort arose on the *recA* and *exrA* strains. Mutations at these alleles serve to inhibit the expression of UV mutagenesis (60) and would seem likely to inhibit the expression of any sort of repair dependent mutagenic activity due to the requirement of the *recA* and *exrA* gene products in the repair pathways generally considered to be error-prone (Figure 1) (61).

Witkin (60) notes, however, spontaneous mutations as well as mutations induced by agents believed to cause only replication errors may arise in *recA* and *exrA* strains. Arsenic and antimony could induce metal and DCS resistance at the point of replication. This thought would seem to be consistent with the hypothesis of Sirover and Loeb (48,49,50) that certain metals interact with the DNA polymerase to decrease polymerase fidelity to the template thus allowing mispairing. Although this hypothesis does not readily include DNA repair involvement in the mutation induction process, one might imagine mispaired DNA to be recognized by repair enzymes following replication. An error-prone form of repair attempting to reconstitute the mispaired region could result in fixation of that mutational event.

Based on the phenomenon cited in Table 4, if repair is involved in As and Sb mutagenesis, it would appear to be *wvr* gene dependent and independent of the *recA* and *exrA* functions. Dependency on *wvr* gene function would imply involvement of excision repair mechanisms. Of the possible branches of excision repair, short-patch repair would seem unlikely due to its efficient, error-free nature. The error-prone long-patch excision repair pathway would tend to be excluded as well due to the requirement of the *exrA* and *recA* gene functions. Thus, data in Table 4 may serve to indicate the error-prone nature of the aforementioned third branch of excision repair (62) which is *wvr* gene-dependent and independent of *exrA* and, by implication, *recA* function.

Finally, a hypothesis involving a previously-unreported pathway of repair could function to explain the pattern of metal resistance as well as the differences in observed repair-dependent mutation induction with As and Sb in the DCS assay.

One should keep in mind DNA repair mechanisms have been primarily investigated as they relate to UV-induced DNA damage. There are almost certainly a number of repair enzymes which deal with types of chemical damage as yet unstudied. An indication of such activity was discussed concerning the excision specificity of correndonuclease I for apurinic sites, i.e., damage induced by alkylating agents. Another example was recently reported by Cairns and Samson (46) in which they were able to show induction of a repair pathway sensitive to N-methyl-

N-nitro-nitrosoguanidine activity. Thus, it would not be too surprising to find a pathway of repair which specifically dealt with metal-induced DNA damage or, at least as indicated by this study, a pathway which involved repair, possibly in an error-prone fashion, of As and Sb induced damage.

In conclusion, the findings of this study are indicative of the complex mechanisms of mutation induction and DNA repair. Antimony-resistant colonies as well as antimony-induced DCS resistant colonies were demonstrated. These antimony-resistant colonies are thought to be a product of induction as opposed to selection of previously existing antimony resistant mutants as mentioned earlier due to the high numbers of resistant mutants observed (Plate 1) and the subsequent induction of DCS-resistant mutants by antimony. Chromate, too, induced mutations in the Ames test as well as presumed chromate-resistance mutations but not resistance to DCS. Further studies should be initiated to allow elucidation of mechanisms of As, Sb and Cr mutagenesis. Possible mechanisms of such activity involving As and Sb were discussed above, yet corroborative evidence is clearly required. As such, differences in As and Sb specific induction of metal and DCS resistance on the repair deficient *E. coli* strains is not explained; nor is the lack of chromate's ability to induce forward mutations to DCS resistance. In addition, media effects and their involvement in the expression of metal mutagenesis should also be investigated. Perhaps

such investigations could provide mechanistic explanations of metal mutagenesis and thus help to explain the activity of these metals as carcinogenic agents.

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Tindall, Kenneth R

The mutagenicity of inorganic ions in microbial systems

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