REPAIR AND MUTAGENESIS OF DNA DAMAGED WITH *cis* - or *trans* -DIAMMINEDICHLOROPLATINUM(II)

Sonya C. Popoff

A Dissertation

Submitted to the GraduateCollege of Bowling Green State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

May, 1987

Approved by Doctoral Committee:

Advisor
Department of Biology

Graduate College Representative

ABSTRACT

The purpose of this study was: 1) to determine which DNA repair pathways of *Escherichia coli* were operative in alleviating DNA damaged with the antitumor compound *cis* - diamminedichloroplatinum(II) (*cis* -DDP) or the isomeric *trans* -DDP; and 2) to determine which adducts were responsible for the mutagenic activities of *cis*- DDP. To delineate which repair mechanisms functioned in the repair of plasmids, DNA repair-proficient and various DNA repair-deficient cells of *E .coli* were transformed with plasmid pBR322 damaged with *cis* - or *trans* -DDP *in vitro* and the transformation frequencies were compared. These experiments indicated that *cis* -DDP damaged plasmids were repaired primarily by excision repair as mediated by the UvrA, UvrB, and UvrC proteins. Adducts produced in plasmid DNA by *trans* -DDP were less toxic and may be repaired by a number of DNA repair pathways, while the requirements for repair of *cis* -DDP adducts were more stringent.

uvrD mutants were used to establish whether the *uvrD* gene product functioned in repair of DNA damaged with DDP. *cis* -DDP caused a decrease in the survival of *uvrD* cells, but the survival of plasmids damaged with *cis* -DDP in *uvrD* mutants was similar to wild-type, isogenic bacteria. Variabilty in survival of *uvrD* mutants was noted when cells were exposed to *cis* - or *trans* -DDP, which suggests that the mutants are not null, but have some remaining *uvrD* gene

function. Decreased ability to repair chromosomal DNA treated with *cis* -DDP but not plasmid DNA, may be due to a different spectrum of adducts formed in DNA *in vivo* , compared to those formed *in vitro.*

In vitro mutagenesis assays were used to examine the frequencies and types of mutations induced with DDP in the tetracycline-resistance gene of plasmid pXf3. This was accomplished by treating a region of the *tet* gene of the plasmid with DDP *in vitro*, reconstructing the plasmid by ligation and using these molecules to transform competent cells of *E.coli.* The mutation frequencies of tetracycline-sensitive plasmids were greatest when DNA was damaged with *cis* -DDP. Base pair substitutions (A:T to T:A transversions) were induced with *cis* -DDP primarily at AG sequences except for in a very G:C rich region where both *cis* - and *trans* -DDP induced mutations at a GTG sequence.

ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge and thank all the people who have lent their support, guidance, time, effort, and equipment, which were all very much appreciated. Sincere appreciation is extended to my major professor, Dr. Doris J. Beck, for her encouragement, enthusiasm, guidance and friendship throughout the period of my studies. I would like to thank my dissertation committee members - Drs. Carol Heckman, Ronny Woodruff, Tsanyen Yang, and George Clemens - for their time and suggestions. Thanks are extended to Drs. W. Dean Rupp, of Yale University, for supplying the purified proteins for the UVRABC excision nuclease; Joseph Frizado, of the Geology Department at B.G.S.U., for the use of the Atomic Absorption Spectrophotometer; and Robert Blumenthal, of the Medical College of Ohio, for the use of the densitometer. Sincere graditude is expressed to my family and friends, particularily to my parents, for their help and guidance over the years and a very special thanks to Patrick Hobbs for his presence in my life and for his encouragement and understanding.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

INTRODUCTION

cis -diamminedichloroplatinum(II) (*cis* -DDP) was first tested as an antitumor agent in the treatment of Sarcoma - 180 and leukemia - L1210 cells in mice (Rosenberg et al., 1969). The success in treating these tumors initiated clinical trials with this compound in 1972 and to date it is now one of the most widely used drugs in chemotherapy (Rosenberg, 1980). One limiting factor with *cis* -DDP as a chemotherapeutic agent has been associated with nausea and nephrotoxicity. The associated renal toxicity has been largely overcome by hydration regimens during therapy (Comis, 1980). Gastrointestinal toxicity can be overcome by administering a thiol containing compound such as mesna (sodium-2-mercaptoethane-sulfonate) prior to giving *cis* -platinum (Allan et al., 1986).

Cisplatin is often used in combination chemotherapy regimens for the treatment of a number of cancers. Much of this success came from the initial reports of Einhorn and Donohue (1977) in treating testicular cancers with cisplatin in combination with vinblastine and bleomycin. Clinical trials have demonstrated antitumor activites for combination chemotherapy regimens of cisplatin and 5-flourouracil in treating colorectal cancers (O'Connel et al., 1986). Cisplatin is often used in combination with cyclophosphamide and doxorubicin in

treating some lung cancers (Eagan et al., 1986). Combination of cis-platin plus cytosine arabinoside appears to be active against squamous cell carcinoma of the head and neck (Stewart et al., 1986). It has been postulated that cytosine arabinoside inhibits repair of DNA damaged by cisplatin (Bergerat et al, 1981). Further, many analogs of *cis* -platinum have been synthesized and tested for antitumor activities in experimental animals (Muggia et al., 1980). The platinum analogs should prove to be useful in treating tumors which were resistant to cisplatin as well as reducing the associated side effects.

The interaction of *cis* -DDP with DNA appears to be responsible for its cytotoxic effects and antitumor properties. *cis* -DDP is a neutral, square-planar compound with two labile chloride ligands and two amine groups in the *cis*-configuration (Fig. 1). In aqueous solution, *cis* -DDP undergoes hydrolysis to form a reactive aquated species which can interact with nucleophilic molecules in the cell such as DNA, RNA, and proteins (Lippard, 1985a). *cis* -DDP may selectively inhibit DNA synthesis in cells that cannot correct damage to the DNA template. The isomeric *trans* -DDP and other platinum compounds have been tested for use in chemotherapy and it appears that the *cis* - configuration is essential for *cis* -DDP to act as an effective antitumor compound.

The effects of *cis* -DDP on biological systems have provided a substantial amount of evidence that the mechanism of cytotoxicity with *cis*- DDP is through its specific interaction with DNA. Moreover, differences between *cis* - and *trans*

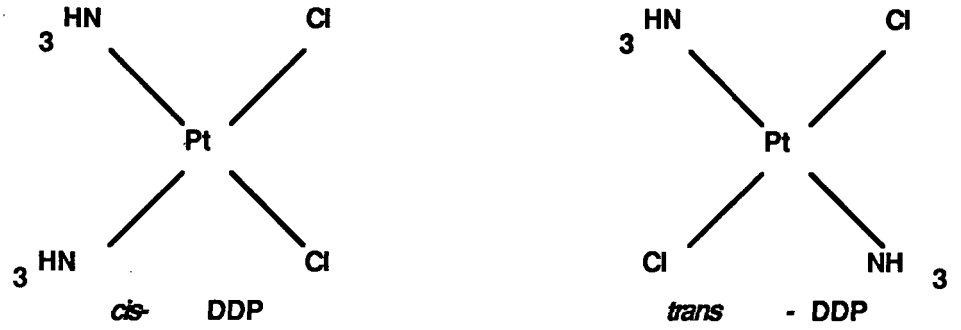


Figure 1. Configuration of *cis* - and *trans* - diamminnedichloroplatinum(II).

-DDP become more apparent when looking at biological systems. Strains of *Escherichia coli* deficient in DNA repair functions are more sensitive to *cis* -DDP than wild-type strains (Beck et al., 1985; Beck and Brubaker, 1973; Fram et al., 1986). For example, *uvrA*, *uvrB*, and *uvrC* mutants which are deficient in excision repair processes, were extremely sensitive to *cis* -DDP, but not *trans* -DDP (Beck et al., 1985). *cis* -DDP induces filamentous growth in *E. coli* (Markham and Brubaker, 1980), which will concomitantly inhibit cell division and is one manifestation of a diverse set of responses associated with SOS DNA repair (Walker, 1984). These responses occur after DNA damage or inhibition of DNA replication and are under the control of the *recA* and *lexA* gene products. Both excision and recombination repair appear to function in correcting *cis* -DDP damage in DNA. Induction of SOS repair serves to enhance both repair mechanisms and to provide other repair enzymes, some of which are error prone. *cis* -DDP is also mutagenic, while *trans* -DDP is neither a strong mutagen nor toxic to DNA repair deficient cells (Beck and Fisch, 1980; Beck and Brubaker,

1975; Fram et al., 1986).

Furthermore, evidence from eucaryotic systems provides additional evidence that deficiencies in DNA repair processes may be responsible for the selective cytotoxic effects of *cis* -DDP on tumor cells. The increased sensitivity of HeLa cells to *cis* -DDP in comparison to Chinese Hamster Ovary cells have been attributed to the presence of a functional post-replicative repair system in the less sensitive hamster cells (Roberts and Fraval, 1980). Human cells from individuals with the syndrome xeroderma pigmentosum are deficient in excision repair of DNA damaged with UV light. The XP cells were more sensitive to *cis* -DDP than normal foetus lung cells (Fraval et al., 1978). *cis* -DDP has also been reported to be mutagenic in a number of eucaryotic systems (Johnson et al., 1980; Zwelling et al., 1979; Taylor et al., 1979; Brodberg et al., 1983; Woodruff et al., 1980).

Although it is generally accepted that the target of the cytotoxic effects of *cis* -DDP is DNA, there is no consensus as to which DNA adduct is responsible for lethality or mutagenicity. Both *cis* - and *trans* -DDP have been shown to bind to DNA, preferentially at N-7 of guanine, to generate both monofunctional and bifunctional adducts (Fig.2; taken from Ficthinger-Schepman et al., 1985). Since *trans* -DDP cannot stereochemically bind to two adjacent bases, an intrastrand crosslink between two adjacent guanines has been hypothesized to be the unique and biologically active lesion associated with *cis* -DDP (Stone et al.,

1976) . Recent biochemical studies support the contention that the major adducts formed with *cis* -DDP are intrastrand crosslinks between two adjacent bases in the form of GG and AG adducts (Eastman, 1986; Fichtinger-Schepman et al.,1985). The GG adduct was the major adduct recognized by an excision repair enzyme complex reconstituted from purified *E.coli* UvrA, UvrB, and UvrC proteins (Beck et al.,1985). Further, the UVRABC excinuclease recognized adducts caused by *cis* - or *trans* -DDP differently, as a specific incision pattern both 5' and 3' to two adjacent guanines was observed when DNA was damaged with *cis* -DDP, while a nonspecific cutting pattern similar to the undamaged control was detected when DNA was damaged with *trans* -DDP.

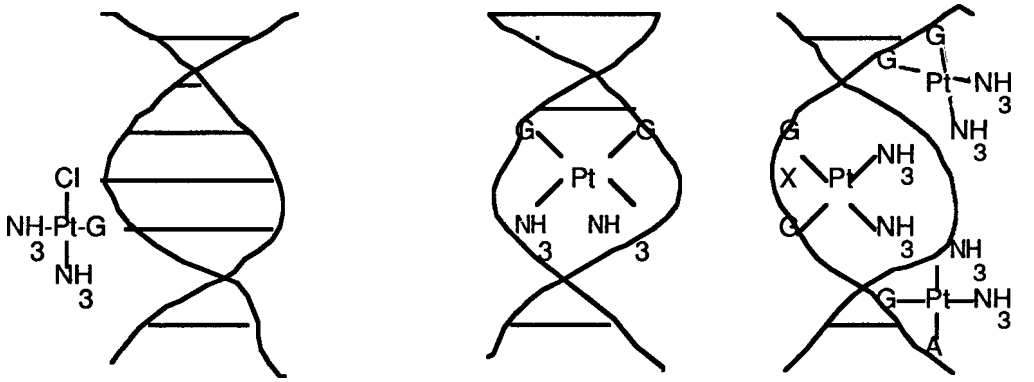


Figure 2. Schematic representation of adducts formed when DNA is reacted with diamminedichloroplatinum(II).

One objective of this research was to determine which DNA repair genes were requisite for alleviating damage in plasmid DNA damaged with *cis* - or *trans*

-DDP *in vitro.* This was of interest since it had been observed that DNA repair deficient mutants of *E.coli* were extremely sensitive to *cis* -DDP, so it seemed imperative to gain information on the repair processes operative on damaged plasmid molecules. Additionally, we had observed that plasmids damaged with the platinum compounds were incised by the UVRABC excinuclease *in vitro* (Beck et al., 1985), so it was of interest to see if excision repair functioned *in vivo* in repairing damaged plasmids. The platinum damaged plasmids were also used to quantitate the incision activity of the UVRABC excinuclease to determine whether damage produced with either of the two platinum isomers was different.

Since it had been reported that the *uvrD* gene product functioned in excision repair (Kuemmerle and Masker, 1980) and this repair pathway alleviated DNA damage with DDP; another objective was to establish if the *uvrD* gene product was involved in the repair of DNA damaged with *cis* - or *trans* -DDP. The incision step in excision repair is mediated by the UvrA, UvrB, and UvrC proteins (Sancar and Rupp, 1983; Yeung, et al.,1983), but later reports demonstrated that the *uvrD* gene product (helicase II) and DNA polymerase I were required for the turnover of the UVRABC excision nuclease and for the excision reaction (Husain et al., 1985; Caron et al., 1985). Studies presented here were thus undertaken to observe if the *uvrD* gene product was required in the repair of *cis* -DDP adducts. This was accomplished by testing *uvrD* mutant cells for senstivity to *cis* and *trans* -DDP, as well as assaying for the survival of plasmids damaged with either

platinum compound.

A final objective of this project was to determine what type of mutations were induced with the platinum compounds at the nucleotide level. It was of interest to establish if the major DDP adducts which had been identified biochemically or recognized by the reconstituted UVRABC excision nuclease of *E.coli* were also the sites of platinum mediated mutagenesis. Another goal was to determine if SOS repair and excision repair were involved in *cis* -DDP mutagenesis.

This dissertation is organized into three separate chapters, each of which treats the details of the objectives stated above, as separate problems. Each chapter will include separate subsections such as introduction, materials and methods, results and discussion. The chapters are presented such that they each describe the experiments, results and discussion of information in a context which contribute to the areas of DNA repair and mutagenesis of DNA damaged with platinum compounds. Chapter One covers aspects of the repair of plasmids damaged with *cis* - or *trans*-DDP and was taken from a manuscript that has just recently been published in Mutation Research (Popoff et al., 1987). Chapter Two focuses on how mutations in the *uvrD* gene affect the repair of chromosomal DNA and plasmid DNA damaged with *cis* - or *trans* -DDP. Finally, Chapter Three describes the use of an *in vitro* mutagenesis assay which allows for the examination of base sequences at sites of platinum mediate mutagenesis.

CHAPTER 1

Repair of Plasmid DNA Damaged *in vitro* with *cis* - or *trans* - diamminedichloroplatinum(II) in *Escherichia coli*

Introduction

In this study, we have treated plasmid pBR322 with *cis* - or *trans* -DDP and used these modified plasmids to transform *E.coli* strains with different DNA repair capabilities. Data from transformation assays were analyzed to determine which DNA repair gene products functioned *in vivo* to alleviate damage in plasmid DNA caused by either of the two platinum compounds. The effect of SOS functions on the survival of DDP-modified plasmids was analyzed. Assays measuring the UVRABC excinuclease incision activity on plasmids modified with *cis* - or *trans* - DDP further probed for differences in the adducts produced with the two platinum isomers. The data suggests that excision repair is the primary mechanism of alleviating damage in plasmids caused by *cis* -DDP. DNA damage introduced with *trans* -DDP was less toxic as adducts seemed to be repaired efficiently *in vivo*, but in a different fashion.

Platinum compounds, particularly *cis* -DDP, have been found to be useful as

antitumor drugs (Rosenberg et al., 1969; Roberts and Thompson, 1979; Pinto and Lippard, 1985a). The *trans* -isomer lacks the antitumor properties of *cis* -DDP, but both compounds have been shown to bind to DNA and generate a number of monofunctional and bifunctional adducts (Fichtinger-Schepman et al., 1985; Pascoe and Roberts, 1972; Stone et al., 1976; Pinto and Lippard, 1985b). The antitumor efficacy of *cis* -DDP appears to correlate with the presence of a pair of *cis* -labile chloride ligands which are able to interact with DNA to produce a unique adduct. The unique adduct generated with the *cis* -isomer has been hypothesized to be an intrastrand crosslink between two adjacent guanines (Stone et al., 1976) which is not stereochemically possilble when DNA is reacted with *trans* -DDP. Analytical studies have confirmed that this is the major adduct formed when DNA is reacted with *cis* - DDP (Eastman, 1983; Fichtinger-Schepman et al., 1985; Sherman et al., 1985). This adduct appears to be a substrate for the excision repair enzyme complex reconstituted from purifed *E.coli* UvrA, UvrB, and UvrC proteins (Beck et al., 1985).

In conjunction with its reactivity with DNA, *cis* -DDP has been found to be damaging to chromosomal DNA *in vivo* and to be toxic to strains of *E.coli* deficient in various DNA repair pathways (Beck et al., 1985; Beck and Brubaker, 1973; Markham and Brubaker, 1980; Alazard et al., 1982; Fram et al., 1985). In contast, *trans* -DDP although similarily reactive with DNA, is markedly less toxic than *cis* - DDP.

Materials and Methods

Bacterial strains and plasmids

The *E.coli* K-12 strains AB1157 (*uvr*$^+$, *rec*$^+$), AB1885 (*uvrB5*), AB2463 (*recA13*), AB2470 (*recB21*) and N14-4 (*uvrD3*) were obtained from Barbara Bachmann, *E.coli* Genetic Stock Center, Yale University. The strain DH1 was from D. Hanahan (Maniatis et al., 1982). Bacteria were routinely grown in Luria Broth (Maniatis et al., 1982) at 37°C with aeration. Plasmid pBR322 DNA was purified from cells of *E.coli* (DH1) by ultracentrifugation in CsCl-ethidium bromide gradients.

Platinum compounds

Platinum compounds were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Stock solutions of platinum compounds were made as previously described (Beck et al., 1985).

Treatment of plasmid DNA with platinum compounds

The concentration of DNA was determined spectrophotometrically by measuring the absorbance at 260 nm. pBR322 plasmid DNA (100 μg) in TEN 7.4 (10 mM tris-HCl, 1 mM EDTA, 10 mM NaCl, pH 7.4) was treated with 10 μg/ml DDP in a volume of 0.5 ml at 37°C. At various time intervals, 20-120 min, 100 μl aliquots were taken and added to 10 μl of 5 M NaCl. Sodium chloride was used to stop the reaction of DDP with DNA by shifting the equilibrium to the neutral

form of DDP rather than the reactive hydrolyzed species (Drobnik et al., 1975). Unbound DDP molecules were removed by spot dialysis (Marusyk and Sargent, 1980) of DNA samples on VM millipore filters (0.05 μm pore size) which were floated on the surface of 1 l TEN 7.4 with stirring at 4°C for 1 hr. This method for removing unbound platinum compound has been described by Scovell and Capponi (1984). Treated DNA was either stored at 4°C or used immediately.

UVRABC incision asays

Plasmid DNA which had been treated with *cis* -DDP or *trans* -DDP, as well as untreated control DNA, was digested with the purified UVRABC excinuclease as previously described (Beck et al., 1985). The reaction products were subjected to electrophoresis on 1% agarose gels (Maniatis et al., 1982). Gels were treated with ethidium bromide and photographed according to standard methods. Lanes in negatives of gels were scanned with a Shimadzu Dual Wavelength TLC Scanner CS-930 and the absorbances plotted. Areas under peaks of absorbances for relaxed and supercoiled bands were determined to calculate the amounts of each molecular form. The amount of remaining supercoiled DNA after excision nuclease digestion was calculated as the ratio of the amount of supercoiled DNA to the sum of the amounts of both relaxed and supercoiled molecules.

The presence of *cis* -DDP adducts in supercoiled DNA decrease its negative superhelicity more than *trans* -DDP adducts (Scovell and Collart, 1985). This

unwinding, consequently, decreases the amount of ethidium bromide which intercalates into supercoiled DNA, decreasing its fluorescence in UV light. Therefore, the amount of supercoiled DNA was normalized for the effect of platinum adducts by multiplying it by the ratio of the amount of supercoiled DNA in untreated DNA samples to the amount of supercoiled DNA appearing to be present after *cis* - DDP modification (amounts of each were determined as described by densitometry). The effect of *trans* -DDP on ethidium bromide intercalation was found to be negligible.

The relative remaining supercoiled DNA was determined as the ratio of the amount of supercoiled DNA in DDP treated samples relative to that in control untreated DNA after digestion of each with the excision nuclease.

Transformation assays

E.coli cells were made competent using a modified version of the procedure described by Rodriguez and Tait (1983). Bacteria were grown in Luria broth to a density of 2×10^8 cells per ml, harvested by centrifugation and resuspended in 0.50 volume of cold 0.1 M $MgCl_2$. Cells were centrifuged to remove $MgCl_2$, resuspended in the same volume of cold 0.1 M $CaCl_2$ and incubated on ice for 1 hr. Competent cells were centrifuged, resuspended in 0.10 culture volume of cold 30 mM $CaCl_2$, 15% glycerol. Aliquots of 300 µl competent cells were stored at -80°C.

In experiments where SOS functions were induced prior to making cells competent, logarithmically growing cells were treated upon reaching an absorbance at 550 nm of 0.20 with either UV light or *cis* -DDP. The cells to be treated with UV light were centrifuged and resuspended in one tenth volume of M9 buffer (Maniatis et al., 1982). The cell suspension was irradiated with UV light using a germicidal lamp at a distance of 40 cm for 30 sec. This UV dose resulted in 37% survival of wild-type cells. Cells were then added to fresh Luria Broth and incubated at 37°C for 30 min to allow for the expression of SOS functions. These procedures were performed in the dark to prevent photoreactivation of pyrimidine dimers. The SOS response was induced with *cis* -DDP by adding the platinum compound to logarithmically growing cells to yield a final concentration of 30 μg/ml. The cells were then incubated at 37°C for 30 min before treatment described above to render them competent.

Plasmid DNA (40ng) treated with *cis* - or *trans* -DDP or untreated control DNA was added to 300 μl of competent cells. Cells and DNA were incubated on ice 1 hr, "heat-shocked" at 42°C for 2 min, and incubated on ice an additional 5 min. The cells were then added to 5 ml of Luria Broth and incubated with shaking at 37°C for 3 hr. Bacteria were suitably diluted with 0.85% NaCl and plated on either Luria Agar to determine the number of viable cells or on Luria Agar with ampicillin (50 μg/ml) to quantitate the number of transformants. Transformation frequencies (number of transformants per number of viable cells per microgram

of DNA) are the averages of three experiments for all strains. The average transformation frequencies of strains transformed with untreated plasmid DNA were: 2.9 x 10^{-3} for AB1157 (*uvr*$^+$, *recA* $^+$), 2.0 x 10^{-3} for AB1885 (*uvrB5*), 2.3 x 10^{-3} for AB2463 (*recA13*), 2.0 x 10^{-3} for AB2470 (*recB21*), and 6.0 x 10^{-4} for N14-4 (*uvrD3*).

Results

Reactivity of platinum compounds with pBR322 DNA in vitro

The number of platinum adducts per molecule of pBR322 DNA modified with *cis* - or *trans* -DDP was determined using atomic absorption spectrophotometry. For both compounds, there was a linear relationship between the number of platinum adducts formed and the length of treatment over a 2 hr period (Fig. 1-1). Under these conditions of incubation, the rate of adduct formation was 6 per hr.

Plasmid DNA treated with either platinum compound was specifically incised by the UVRABC excinuclease (Fig. 1-2). The relative amount of supercoiled molecules remaining after digestion by the UVRABC excinuclease was determined as described in the Methods section. The relative amount of supercoiled DNA refers to that remaining in DDP treated samples with respect to that remaining in untreated DNA after the digestion of each by the UVRABC excinuclease. DNA containing *cis* - DDP adducts was more readily incised by

Figure 1-1. The number of platinum adducts per molecule of pBR322 DNA when plasmid DNA was incubated for 2 hr with 10 μg/ml of either *cis* - or *trans* -DDP at 37°C. Unbound platinum was removed by spot dialysis and the number of adducts determined using atomic absorption spectrophotometry.

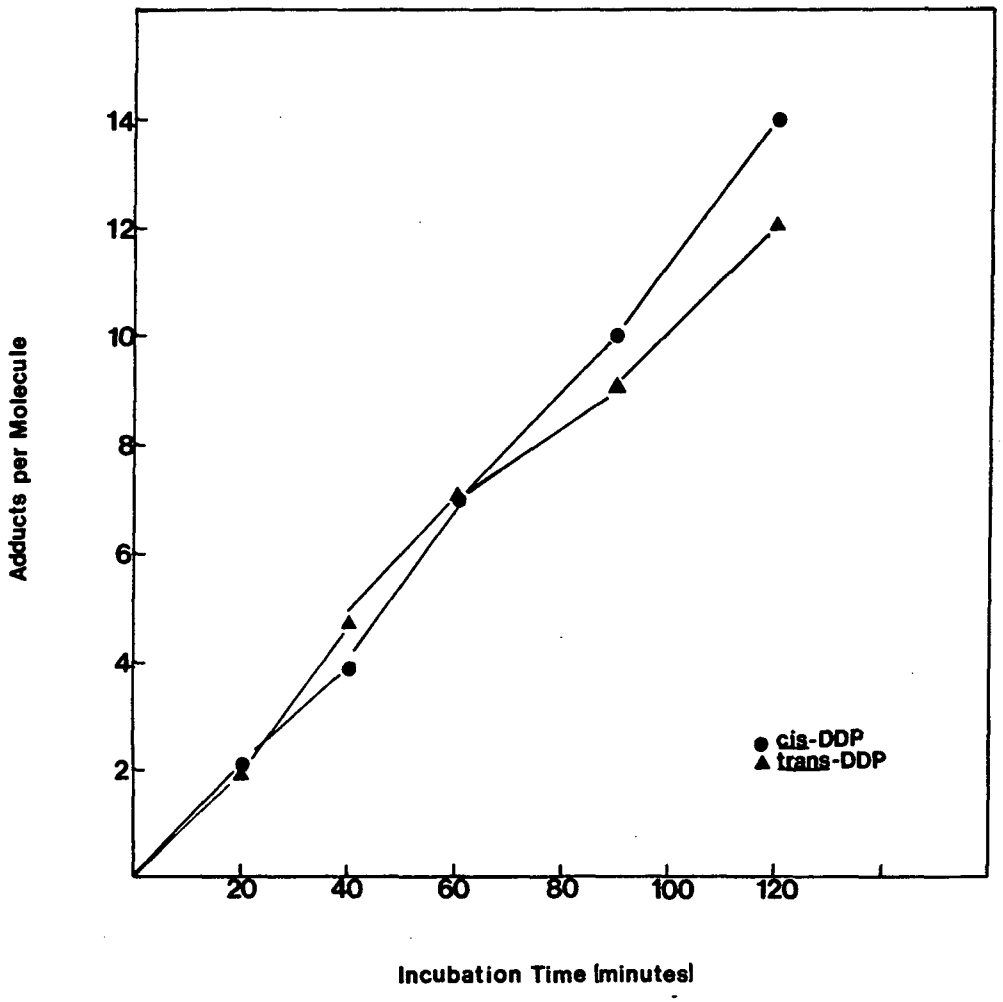
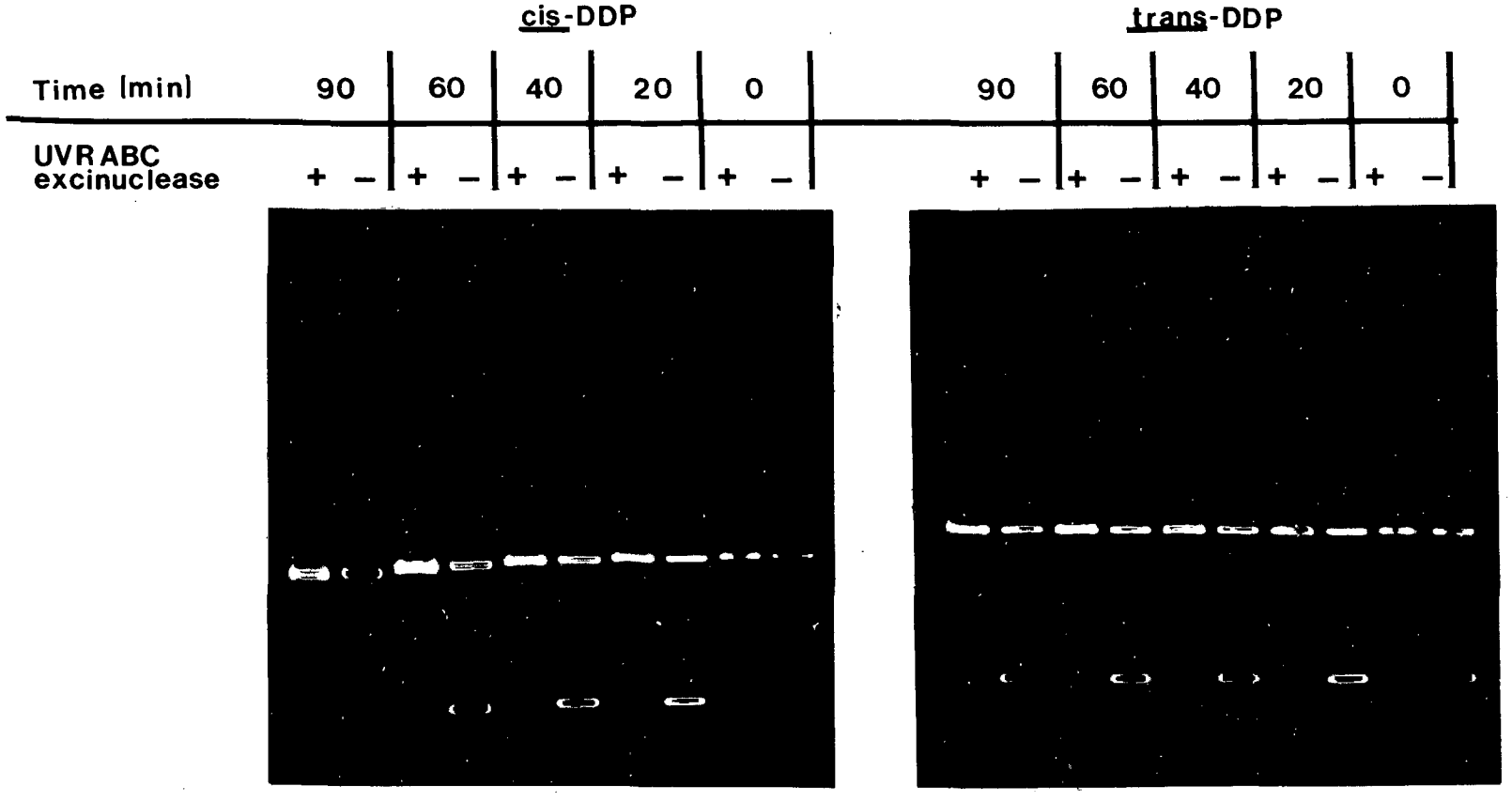

Adducts per Molecule
14
12
10
8
6
4
2
20
40
60
80
100
120
Incubation Time (minutes)
● cis-DDP
▲ trans-DDP

Figure 1-2. Agarose gels of pBR322 DNA treated with 10 μg/ml of *cis* - or *trans* -DDP before (-) and after (+) its digestion with the UVRABC excinuclease. Incubation times (min) for DNA treated with DDP is indicated above the adjacent lanes containing the DNA. Supercoiled molecules are the faster migrating form I DNA and relaxed molecules are the slower migrating form II DNA.


cis-DDP
trans-DDP
Time (min)
90
60
40
20
0
90
60
40
20
0
UVRABC
excinuclease
+ − + − + − + − + − + − + − + − + − + −

the UVRABC excinuclease than DNA containing an equivalent number of *trans* -DDP adducts (Fig. 1-3). The relative remaining supercoiled molecules was 90% for plasmid DNA with two *cis* -DDP adducts per molecule and decreased to 10% for 8 adducts. In contrast, plasmid DNA containing eight DDP adducts caused by the *trans* -isomer still had approximately 60% relative remaining supercoiled molecules.

Transformation frequencies by cis- or trans-DDP damaged pBR322 DNA in E.coli strains with different DNA repair capacities.

Adducts caused by treatment of plasmids with *cis* -DDP were more deleterious than those due to treatment with *trans* -DDP as determined by transformation assays. Plasmids treated with *cis* -DDP had lower transformation frequencies than those treated with *trans* -DDP at an equivalent number of bound platinum atoms (Fig. 1-4). The relative transformation frequency plotted here is the ratio of the average transformation frequency by damaged plasmids to the average transformation frequency by untreated plasmids. To facilitate comparison of the results obtained with the two platinum compounds, the relative percent frequency of transformation by plasmids with five DDP adducts per molecule in the various strains of *E. coli* is determined from Fig. 1-4 and listed in Table 1-1. This level of damage was chosen since it caused marked differences in transformation frequencies in bacteria according to their DNA repair capabilities. The transformation frequency for pBR322 with 5.6 *cis* - DDP adducts

Figure 1-3. Relative remaining supercoiled pBR322 DNA treated for 0-90 min with *cis* - DDP or *trans* -DDP and digested with the UVRABC excinuclease. This value was calculated by taking the ratio of the fraction of DNA appearing as supercoiled DNA in the DDP treated samples to the fraction of DNA appearing as supercoiled DNA in the undamaged plasmids after each of them had been digested with the UVRABC excinuclease.

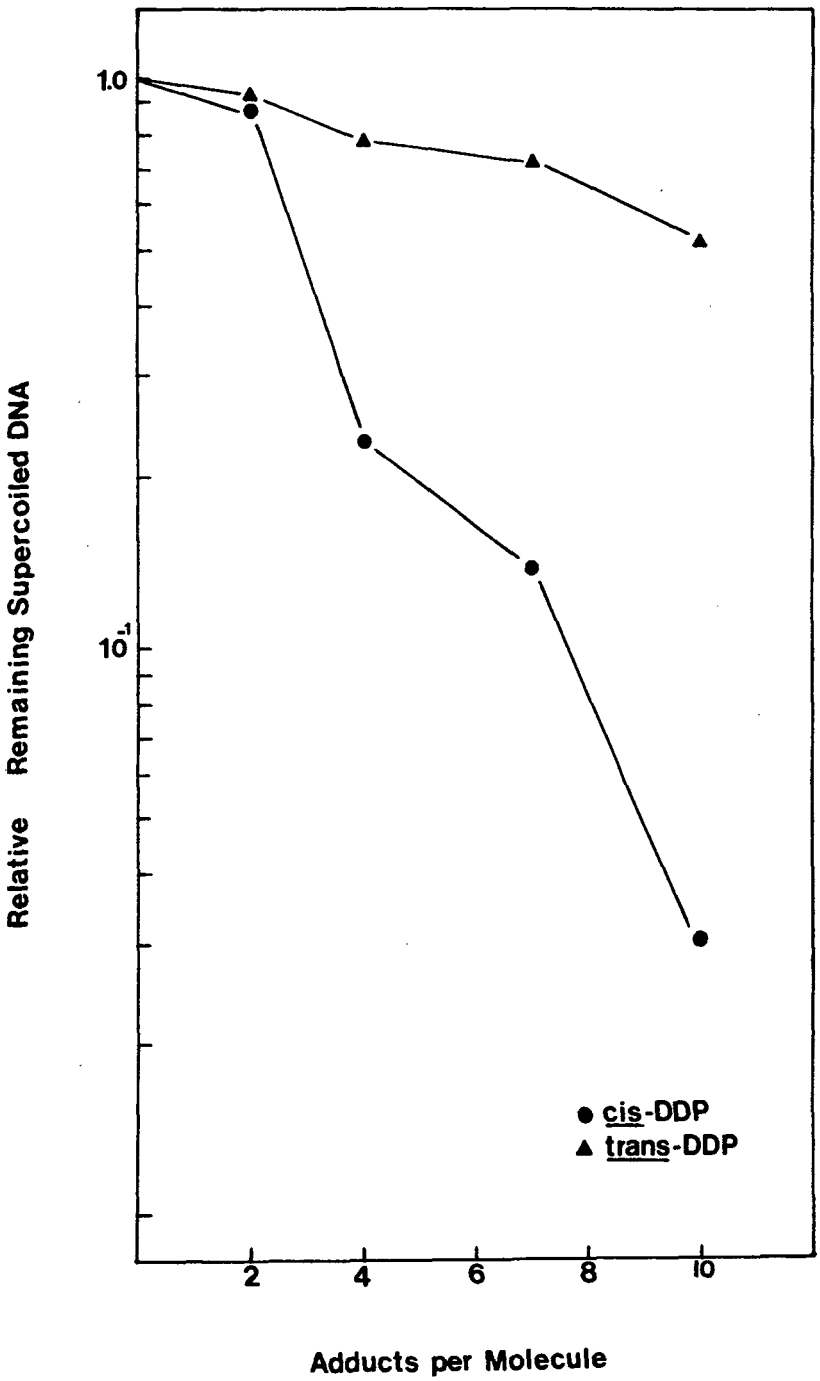

1.0
10⁻¹
Relative Remaining Supercoiled DNA
2
4
6
8
10
Adducts per Molecule
● cis-DDP
▲ trans-DDP

Figure 1-4. Relative frequency of transformation of pBR322 DNA modified with *cis* -DDP (a) or *trans* -DDP (b) in strains of *E.coli* with different DNA repair capabilities. Relative frequency of transformation is the ratio of the transforming capacity by plasmids damaged with DDP relative to the transformation frequency of untreated plasmids. The *E.coli* strains used were: AB1157 (*uvr*$^+$, *rec*$^+$) (●); N14-4 (*uvrD3*) (■); AB1885 (*uvrB5*) (▲); AB2463 (*recA13*) (◆); AB2470 (*recB21*) (⬢).

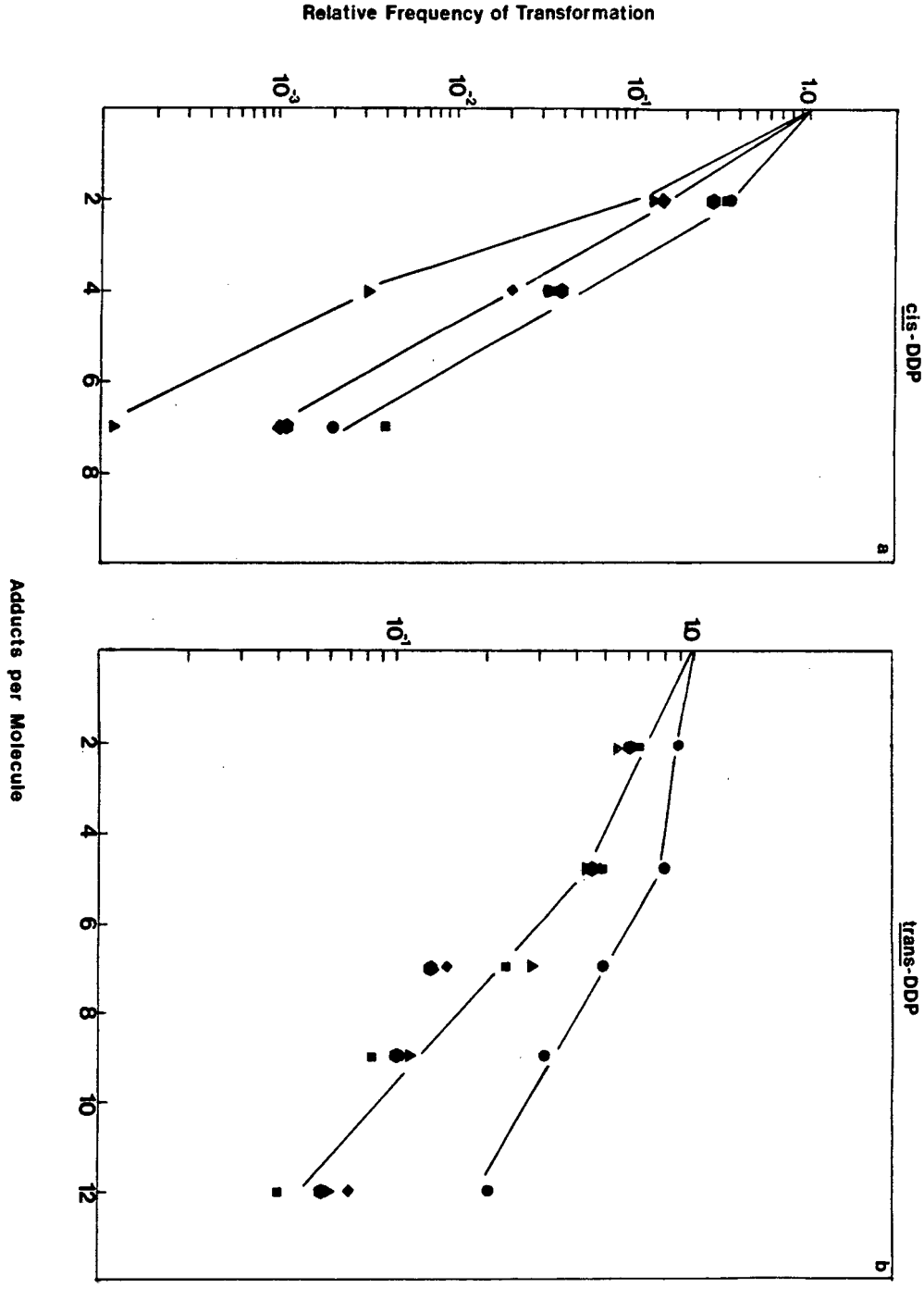

Relative Frequency of Transformation
Adducts per Molecule
cis-DDP
trans-DDP
a
b

Table 1-1. Relative percent frequency of transformation by pBR322 DNA with five DDP adducts per molecule in wild-type and DNA repair deficient strains of *E.coli*.

Strain	Relative percent frequency of transformation[a]			
	cis-DDP		*trans*-DDP	
AB1157 (*uvr*$^+$, *rec*$^+$)	2	(8)[b]	75	(75)
AB1885 (*uvrB5*)	0.1		40	
AB2463 (*recA13*)	0.7		40	
AB2470 (*recB21*)	2		40	
N14-4 (*uvrD3*)	2	(4)	40	(40)

a Relative percent frequency of transformation was calculated from transformation frequencies of damaged plasmids relative to undamaged plasmids. Data taken from figure 1-4.

b Numbers in parenthesis indicate the relative percent frequencies of transformation by plasmids with five adducts per molecule when the designated competent strains were induced for SOS functions. Data taken from figure 1-5.

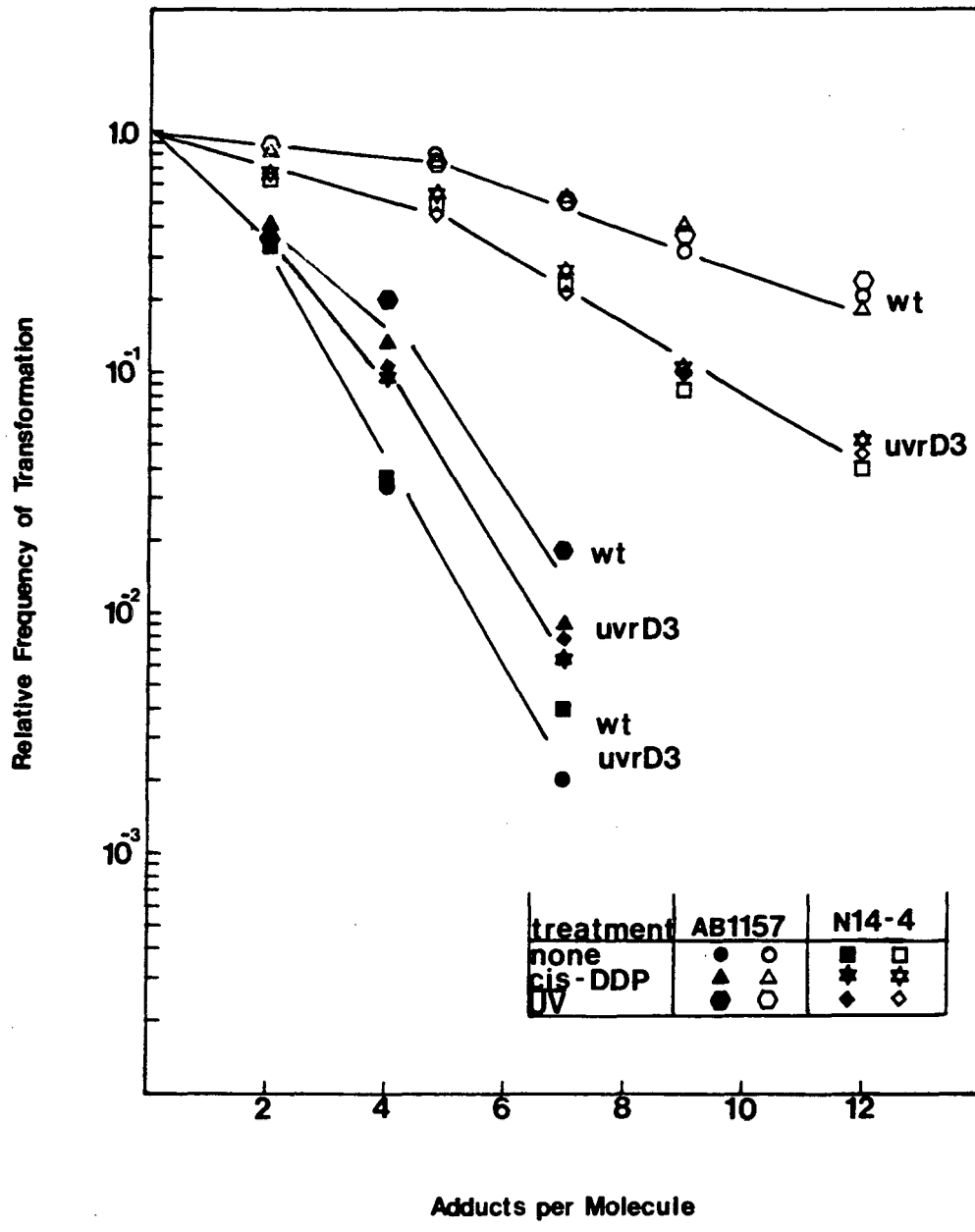
was reduced to 1% of that obtained by control untreated DNA in wild-type cells (strain AB1157).

Mutants at the *uvrD* or *recB* loci were as proficient as wild-type bacteria in the repair of adducts caused by *cis* -DDP; plasmids containing two adducts per molecule had a transformation frequency 37% of that obtained with untreated plasmids (Fig. 1-4-a). Damaged plasmids with five adducts also had similar survival rates in the three strains (Table 1-1). Mutants at the *uvrB* locus were deficient in their ability to repair *cis*- DDP adducts since one adduct per moleclue reduced their relative transformation frequencies to 37%. Few (0.3%) plasmids harboring 4 adducts survived and a greater number of adducts nearly eliminated plasmid survival. Intermediate ability to repair *cis* -DDP adducts was characteristic of *recA* mutants; the transformation frequencies by plasmids with five adducts was decreased to 0.7% of that observed with untreated plasmids.

There was a four-fold difference in the toxicity of adducts caused by *cis* -DDP and *trans* -DDP. Plasmid DNA with 8.6 *trans* -DDP adducts had a relative transformation frequency of 37% in wild-type strains whereas 2 *cis* -DDP adducts caused a similar effect (Fig. 1-4). The inactivation of pBR322 by *trans* -DDP was similar in all mutant strains and about 5.4 adducts reduced plasmid survival to 37% (Fig. 1-4-b). Similarily, plasmid DNA molecules containing 5 *trans* -DDP adducts had about a two-fold lower relative percent frequency of transformation in all mutant bacteria when compared to the wild-type (Table 1-1).

In line with these results, expression of SOS functions was effective in repair of adducts caused by *cis* -DDP but not those caused by *trans* -DDP. The transformation frequencies of plasmids damaged with *trans* -DDP were not increased by prior treatment of competent cells with either UV or *cis* -DDP (Fig. 1-5). The induction of SOS repair by *cis* -DDP increased the relative frequenceis of transformation by plasmids having 4-6 *cis* -DDP adducts, four-fold in wild-type and two-fold in *uvrD* mutant strains of *E.coli*, in comparison to uninduced cells (Fig. 1-5). Induction of SOS repair by UV was similarily effective in enhancing survival of *cis* -DDP modified DNA.

Figure 1-5. Relative frequencies of transformation by DDP damaged plasmids in *E.coli* cells induced for SOS functions. Closed or open symbols represent pBR322 DNA damaged with *cis* -DDP or *trans* -DDP, respectively.


Relative Frequency of Transformation
1.0
10^{-1}
10^{-2}
10^{-3}
2
4
6
8
10
12
Adducts per Molecule
wt
uvrD3
wt
uvrD3
wt
uvrD3
treatment
AB1157
N14-4
none
cis-DDP
UV

Discussion

Both *cis* - and *trans* -DDP had similar reactivites with DNA *in vitro* as measured by the number of adducts formed in DNA over time. However, the DNA adducts caused by the two isomers appeared to be repaired differently. We had previously observed that the UVRABC excinuclease incised *cis* -DDP damaged end-labeled linear DNA specifically both 5' and 3' of adjacent GG's, whereas *trans* -DDP exhibited a nonspecific cutting pattern similar to that of control unmodified DNA (Beck et al., 1985).

The experiments described here extend our earlier observations on the incision activity of the UVRABC excinuclease on DDP damaged supercoiled DNA. The frequency of incision by the UVRABC excinuclease was greater for *cis* -DDP adducts than for *trans* -DDP adducts as noted by quantitating the conversion of supercoiled molecules to the relaxed forms. Three *cis* -DDP adducts or ten *trans* -DDP adducts caused a decrease in the remaining supercoiled molecules to 50% that of control untreated DNA after reaction of the DNA samples with the repair nuclease.

Results obtained with *cis* -DDP damaged plasmids in transformation assays were similar to those obtained with plasmids modified with *cis* -1,2-diaminocyclohexldichloroplatinum(II) [(*cis* -DACH)$PtCl_2$] by Husain et al., (1985a). Adducts caused by *cis* -DDP were more toxic than those caused by

[(*cis* - DACH)$PtCl_2$] in *E.coli* as judged by numbers of adducts requisite for a lethal hit. The primary pathway of repair for these adducts appears to be excision repair. Mutations in the *uvrB* gene block the excision repair pathway and hence cause extreme sensitivity of these mutants to *cis* -platinum(II) compounds. Repair functions of the *recA* gene product are of secondary importance and may be due to the involvement of the *recA* gene product in regulation of the SOS responses. The SOS responses include increased synthesis of enzymes involved in excision repair and in other repair pathways.

Mutants at the *uvrD* locus appeared to be as proficient in the repair of *cis* -DDP modified plasmids as wild-type bacteria. Survival of DDP-modified plasmids was not affected even in strain SK3451 which has a truncated UvrD protein due to a Tn5 insertion (data not shown). Cells of strain SK3451, however, were sensitive to *cis* - DDP unlike the *uvrD3* mutation (strain N14-4) which were proficient in repair of these adducts in their chromosomal DNA. This was indicated by the fact that treatment of *uvrD3* mutants with *cis* -DDP did not decrease in their survival (measured as colony forming units) relative to that of wild-type bacteria (unpublished data).

Recent studies have been published on *in vitro* repair assays using purified proteins to reconstitute the UVRABC excinuclease. Results indicate that the *uvrD* gene product, helicase II, enhances the incision reaction by causing turnover of the excision repair complex and further, is necessary for the excision reaction

(Husain et al., 1985b; Caron et al., 1985). The contradictory results observed here in *in vivo* experiments may be due to the diverse phenotypes noted in different *uvrD* mutants (Maples and Kushner, 1982). Efforts to obtain mutants completely devoid of *uvrD* functions have not been successful (S. Kushner, personal communication). Survival of plasmids modified with *cis* -DDP may thus be due to partial or some remaining *uvrD* function. An alternative explanation could be that some of the functions of the *uvrD* gene product in repair of *cis* -DDP damaged plasmids *in vivo* might be performed by other proteins functioning in DNA metabolism.

Treatment with *trans* -DDP was less deleterious to plasmids than treatment with *cis* -DDP. Plasmids containing *trans* -DDP adducts transformed with equivalent frequencies in all mutant strains, but at lower frequencies than in wild-type cells. This data, as well as the fact that induction of SOS functions increased the survival of plasmids damaged with *cis* -DDP but not *trans* -DDP, suggests that *cis* -DDP adducts are repaired in a different fashion than *trans* -DDP adducts. This correlates with the findings of Ciccarelli et al., 1985, who suggest that the differences in the inhibition of SV40 replication, as well as cellular toxicity following treatment with *cis* - or *trans* - DDP is due to differential repair of *cis* - and *trans* -DDP adducts. Possibly these results are due to the greater difficulty in removing *cis* -DDP adducts from DNA than those caused by *trans* -DDP. Monoadducts of *trans* -DDP would react more readily with

intracellular sulfhydryl and amino containing molecules rather than forming bifunctional intrastrand crosslinks as does *cis* -DDP. The latter would cause distortion in DNA and require repair functions for their removal.

In conclusion, adducts produced in plasmid DNA treated with *cis* -DDP were different from and more toxic than those caused by *trans* -DDP. Survival of *cis* -DDP modified DNA was increased by prior induction of SOS responses but this was not the case for *trans* -DDP. *cis* -DDP adducts were repaired mainly by a pathway requiring the specific interaction of the UvrA, UvrB, and UvrC proteins. It was expected then that studies of the incision activity of the UVRABC excinuclease *in vitro* indicated that the specific incision activity was greater for *cis* -DDP modified plasmids than for those modified with *trans* -DDP.

CHAPTER 2

Damage in Plasmid or Chromosomal DNA Caused by *cis* - or *trans* - diamminedichloroplatinum(II) has Different Toxicities in *uvrD* Mutants of *Escherichia coli*

Introduction

In this communication we report how mutations in the *uvrD* gene affect the repair of chromosomal DNA and plasmid DNA damaged with *cis* - diamminedichloropaltinum(II) (*cis* -DDP) or *trans* -DDP. Cell survival was determined when logarithmically growing cells were treated with *cis* - or *trans* -DDP and the survival of plasmids treated with either platinum compound *in vitro* was determined in transformation assays. The phenotypes of the *uvrD* mutants used in this study ranged from resistance similar to that of wild-type, isogenic bacteria, to a five-fold increase in sensitivity to *cis* -DDP. Their different levels of sensitivities to *cis*- DDP and *trans* -DDP suggests that the protein coded by the *uvrD* gene has some properties facilitating repair of DDP-adducts. Transformation frequencies of plasmids damaged with *cis* -DDP in *uvrD* mutants were similar to those of wild-type cells, while the transformation frequencies of

trans -DDP damaged plasmids decreased slightly in *uvrD* mutants. Sensitivity of cells to treatment with *cis* -DDP but not plasmids, suggests that adducts formed in DNA by *cis* -DDP in the chromosome are repaired by a mechanism which is dependent on *uvrD* gene functions, while the repair of these adducts in plasmids has less stringent UvrD protein requirements.

The compound, *cis* -diamminedichloroplatinum (II) (*cis* -DDP), is an effective antitumor agent; while the isomer, *trans* - DDP, is not effective (Rosenberg et al., 1969; Roberts and Thompson, 1979). The interaction of *cis* -DDP with DNA appears to be responsible for its cytotoxic and antitumor properties. Both *cis* - and *trans* -DDP bind to DNA to form a number of monofunctional and bifunctional adducts (Pinto and Lippard, 1985). The major adduct formed when DNA is reacted with *cis* - DDP is an intrastrand crosslink between two adjacent guanines (Eastman, 1986; Fichtinger-Schepman et al., 1985). This may be the biologically active adduct since *trans* -DDP cannot stereochemically bind in this manner (Stone et al., 1976).

Although both *cis* - and *trans* -DDP are reactive with DNA, *cis* -DDP is much more toxic to *E. coli* cells deficient in various DNA repair processes and is a strong mutagen (Beck et al., 1985; Beck and Brubaker, 1973; Beck and Fisch, 1980; Alazard et al, 1982; Fram et al, 1985; Jarosik and Beck, 1984). The *trans* -isomer is lacking in these properties, possibly because DNA damaged with *trans* -DDP is repaired more readily (Ciccarelli et al., 1985). A major pathway for

repair of *cis* - DDP damaged DNA in *E.coli* is excision repair (Popoff et al., 1987). The first step in excision repair is the incision reaction, in which the UvrA, UvrB, and UvrC proteins function in making cuts both 5' and 3' to damage in DNA (Sancar and Rupp, 1983; Yeung et al.,1983). Subsequently, the *uvrD* gene product (helicase II) and DNA polymerase I are requisite for the release of the UVRABC excision nuclease which leads to its turnover (Husain et al., 1985; Caron et al., 1985). This explains the observation that the UvrD protein functions in excision repair *in vivo* and that it stimulates the excision nuclease in cell free extracts (Kuemmerle and Masker, 1980 and 1983).

Previously we studied the repair of DNA damaged *in vitro* with *cis* - or *trans* -DDP by using the reconstituted UVRABC excinuclease. *cis* -DDP damaged plasmids were more readily incised than plasmids treated with *trans* -DDP (Popoff et al., 1987). Damaged end-labeled linear DNA treated with *cis* -DDP was specifically incised both 5' and 3' to adjacent GG's (Beck et al., 1985). Additionally, data from transformation assays on *in vivo* repair indicated that survival of plasmids damaged with *cis* -DDP depended on the function of the *uvrB* gene product (Popoff et al.,1987). Since a mutation in the *uvrD* gene product did not decrease the survival of *cis* -DDP damaged plasmids, these studies were undertaken to observe the affects of three mutations in the *uvrD* gene on the repair of *cis* -DDP adducts.

Materials and Methods

Bacterial strains and plasmids

Relevant genotypes and sources of bacteria used are listed in Table 2-1. Methods for cultivation of bacteria and purification of plasmid pBR322 DNA were as described previously (Beck et al., 1985; Popoff et al., 1987). Transposon Tn5 was maintained in strain SK3451(*uvrD254* ::Tn5) by supplementing LB media with kanamycin (25 μg/ml) and streptomycin (20 μg/ml).

Survival assays

Experiments measuring survival of logarithmically growing cells of *E.coli* treated with DDP (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) were performed as outlined by Beck et al., (1985). Sensitivity of bacteria to UV light was determined by plating 10^2 - 10^4 cells of a log phase culture and irradiating the plate with a 30 Watt G.E. germicidal lamp at a distance of 50 cm for 0 - 45 sec. After irradiation, plates were kept in the dark and incubated overnight at 37°C.

Plasmid pBR322 DNA (50 μg/ml) was treated with 10 μg/ml of DDP in a volume of 200 μl for 20-60 min at 37°C. Reaction conditions, removal of unbound platinum, and the use of atomic absorption spectrophotometry to determine the amount of platinum bound to DNA were according to the procedures of Popoff et al., (1987). Competent cells of *E.coli* were transformed

TABLE 2-1.

BACTERIAL STRAINS

Strain	Other Chromosomal Markers	Source	Reference
AB1157 (*uvr*$^+$,*rec*$^+$)	*thi-1, thr-1, leuB6, proA2, thy-1, hisG4, argE3, lacY1, galK2, ara-14, xyl-5, mtl-1, tsx-33, str-31, supE44*	B.J. Bachmann[a]	
JC8471 (*uvrD252*)	Same as AB1157, also *rpsL31* (formerly *recL152*)	B.J. Bachmann	Horii and Clark, (1973)
SK707 (*uvr*$^+$)	*lacBK1, malA1, mtl-1, xyl-7, argH1, hisG4, ilvD188, metE46, supE44*	S.R. Kushner[b]	
SK3451 (*uvrD254* ::Tn5)	Same as SK707	A. Sancar[c]	Maples and Kushner (1982)
W3623 (*uvr*$^+$)	*trp-56, galT23*	B.J. Bachmann	Ogawa et al., (1968)
N14-4 (*uvrD3*)	Same as W3623, also *rpsL178*	B.J. Bachmann	Ogawa et al., (1968)

[a] *E.coli* Genetic Stock Center, School of Medicine, Yale University
[b] Department of Molecular and Population Genetics, University of Georgia
[c] Department of Biochemistry, University of North Carolina, Chapel Hill

with DDP modified DNA or untreated DNA as a control. Transformation frequencies (number of transformants per viable cell per μg DNA) are the average of three experiments. Relative frequency of transformation is the ratio of the average transformation frequency of DDP treated plasmids to that obtained with untreated plasmid DNA. The average transformation frequency in each strain using untreated plasmid DNA was: 2.0×10^{-3} for AB1157 (*uvr*$^+$), 2.0×10^{-4} for N14-4 (*uvrD3*), 3.0×10^{-4} for JC8471 (*uvrD252*), 1.4×10^{-3} for SK707 (*uvrD*$^+$), and 1.1×10^{-3} for SK3451 (*uvrD254* ::Tn5).

Results

Effects of cis- or trans-DDP on bacterial survival

cis- DDP was more toxic to wild-type and *uvrD* mutant bacteria than *trans* -DDP (Fig. 2-1). Three different *uvrD* mutants (*uvrD3*, *uvrD252*, *uvrD254* ::Tn5) varied in their ability to survive exposure to DDP, but the mutants exhibited the same order of sensitivity to either platinum isomer and UV light (Table 2-2). Cells bearing the *uvrD3* mutation (strain N14-4) were resistant to killing by *cis* -DDP as were the wild-type cells of the isogenic strain (W3623) and wild-type cells of strain AB1157. These cells underwent one or two divisions after exposure to 30 μg *cis* -DDP / ml and thereafter, further division was inhibited as there was a constant number of colony forming units for 2 hr. Indicators of cell division

Figure 2-1. Survival (colony-forming ability) of *uvrD* and wild-type *E.coli* when logarithmically growing cells were exposed to 30 μg/ml of *cis* -DDP (a) or 60 μg/ml of *trans* -DDP (b) for different periods of time. Strains were: (✱) W3623 (*uvr*$^+$); (●) AB1157 (*uvr*$^+$); (⬣) SK707 (*uvrD*$^+$); (■) N14-4 (*uvrD3*); (◆) JC8471 (*uvrD252*) and (▲) SK3451 (*uvrD254* ::Tn5).

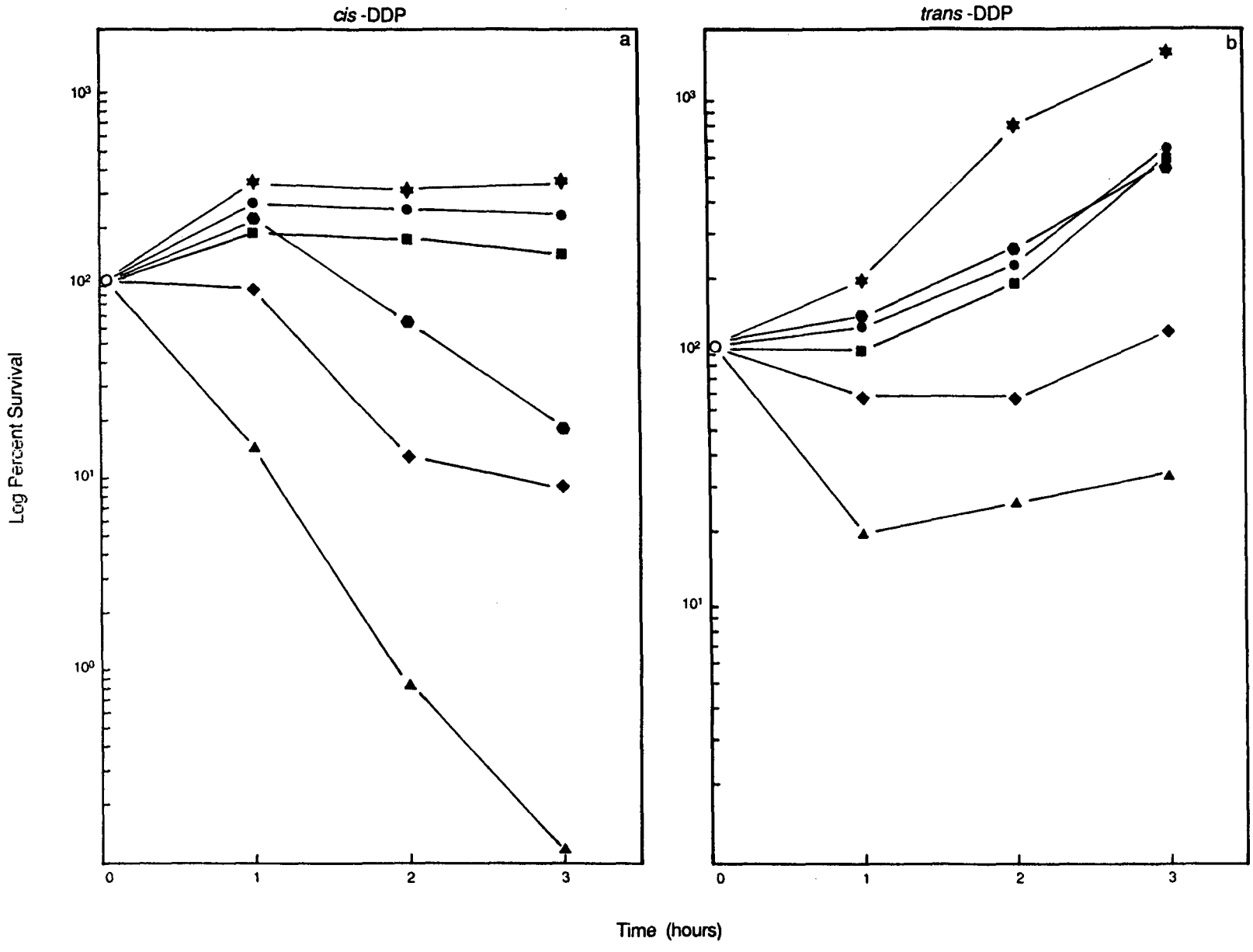

cis -DDP
a
trans -DDP
b
Log Percent Survival
10³
10²
10¹
10⁰
0
1
2
3
Time (hours)

TABLE 2-2.

Effects of platinum compounds and UV light on wild-type bacteria and *uvrD* mutants of *E.coli* [a]

Strain	Time to reduce viability to e^{-1} [a] (min) *cis*-DDP	(sec) UV	Filamentation [b] DDP *cis*	*trans*	Ratio of cell mass [c] minutes of exposure to *cis*-DDP 90	180	*trans*-DDP 90	180
AB1157	>180	25	++	-	0.94	1.0	0.55	0.80
JC8471	90	4	++	+	0.94	1.0	0.55	1.0
SK707	150	13	++	-	0.88	1.0	0.66	1.0
SK3451	30	3	++	+	1.0	1.0	0.70	1.0
W3623	>180	25	++	-	0.93	1.0	0.80	1.0
N14-4	>180	12	++	-	1.0	1.0	0.73	1.0

[a] A culture of logarithmically growing cells upon reaching an absorbance at 550 nm of 0.20, was subdivided and treated with no DDP, 30 μg *cis*-DDP / ml, or 60 μg *trans*-DDP / ml. Bacteria were UV irradiated as described in the methods section. Viability was monitored by plating and $absorbance_{550\ nm}$ measured using a Bausch and Lomb Spectronic 20.

[b] Filamentation was observed microscopically after growth of bacteria in media containing DDP for 3 hr at 37°C: (++) filamentous cells approximately 5-10 times longer than untreated control cells; (+) elongated cells, twice the length of control cells; (-) cells equal in size to the control cells.

[c] Ratio of cell mass was determined by dividing the $absorbance_{550nm}$ of the culture of cells in media containing DDP by the absorbance of the control culture of cells in media without DDP

inhibition were the absence of increases in number of colony forming units and the appearance of filamented cells as observed microscopically (Table 2-2). Filamentous growth of *E.coli* due to inhibition of cell division is one manifestation of the induction of SOS repair in response to DNA damage (Walker, 1984).

Wild-type cells of strain SK707 were sensitive to *cis* -DDP. Colony forming units increased the first hour of treatment with *cis* -DDP as observed in the strains described above. Thereafter, survival decreased to 37% after an additional 90 min exposure to *cis* -DDP. Strains JC8471 (*uvrD252*) and SK3451 (*uvrD254* ::Tn5) were also sensitive to *cis* -DDP, as survival of these cell populations was reduced to 37% upon exposure to *cis* -DDP for 90 or 30 minutes, respectively. This is a five-fold increase in sensitivity for strain SK3451 compared to its parent strain SK707 and a greater than two-fold increase in sensitivity for strain JC8471 relative to its parent AB1157. Cell mass was increasing in all cultures in the presence of either platinum compound, as indicated by turbidity measurements (Table 2-2).

As *trans* -DDP was less toxic than the *cis* - isomer, cells were treated with a greater concentration, 60 µg *trans* -DDP / ml. Wild-type cells and *uvrD3* mutants were most resistant to damage with *trans* -DDP as indicated by their increase in colony forming units after an initial 1 hr lag (Fig 2-1-b). The *uvrD252* and

uvrD254 ::Tn5 mutants were less resistant and exhibited a reduction in the number of colony forming units when populations of cells were treated with the *trans* -compound. Resistant cells did not become filamentous, but the *uvrD252* or *uvr D254* ::Tn5 mutants were slightly elongated after exposure to *trans* -DDP for 3 hr (Table 2-2).

Transformation frequencies of cis- or trans-DDP damaged pBR322 DNA in different uvrD strains of E. coli.

The number of *cis* - or *trans* -DDP adducts in pBR322 plasmid DNA after its reaction with either platinum compound *in vitro* was determined by atomic absorption spectrophotometry (Fig. 2-2). The relative frequencies of transformation for DDP modified plasmids in *uvrD* strains of *E. coli* decrease as a function of the number of platinum adducts per molecule of pBR322 DNA (Fig.2-3). Relative frequency of transformation is defined as the ratio of the average transformation frequency of DDP treated plasmids to that of untreated, control plasmids. Wild-type cells as well as the different *uvrD* mutants exhibited similar relative frequencies of transformation when plasmids contained *cis* -DDP adducts. In all strains, two *cis* - DDP adducts per molecule of plasmid DNA decreased their relative frequencies of transformation to 37%; hence, the *uvrD* mutants were as proficient as wild-type bacteria in the repair of *cis* -DDP adducts in plasmid DNA.

trans -DDP adducts were far less toxic than *cis* -DDP adducts when these

Figure 2-2. The number of *cis* -DDP or *trans* -DDP adducts per molecule of pBR322 DNA formed when plasmid DNA was incubated with 10 μg/ml of either platinum compound for 1 hr at 37° C.

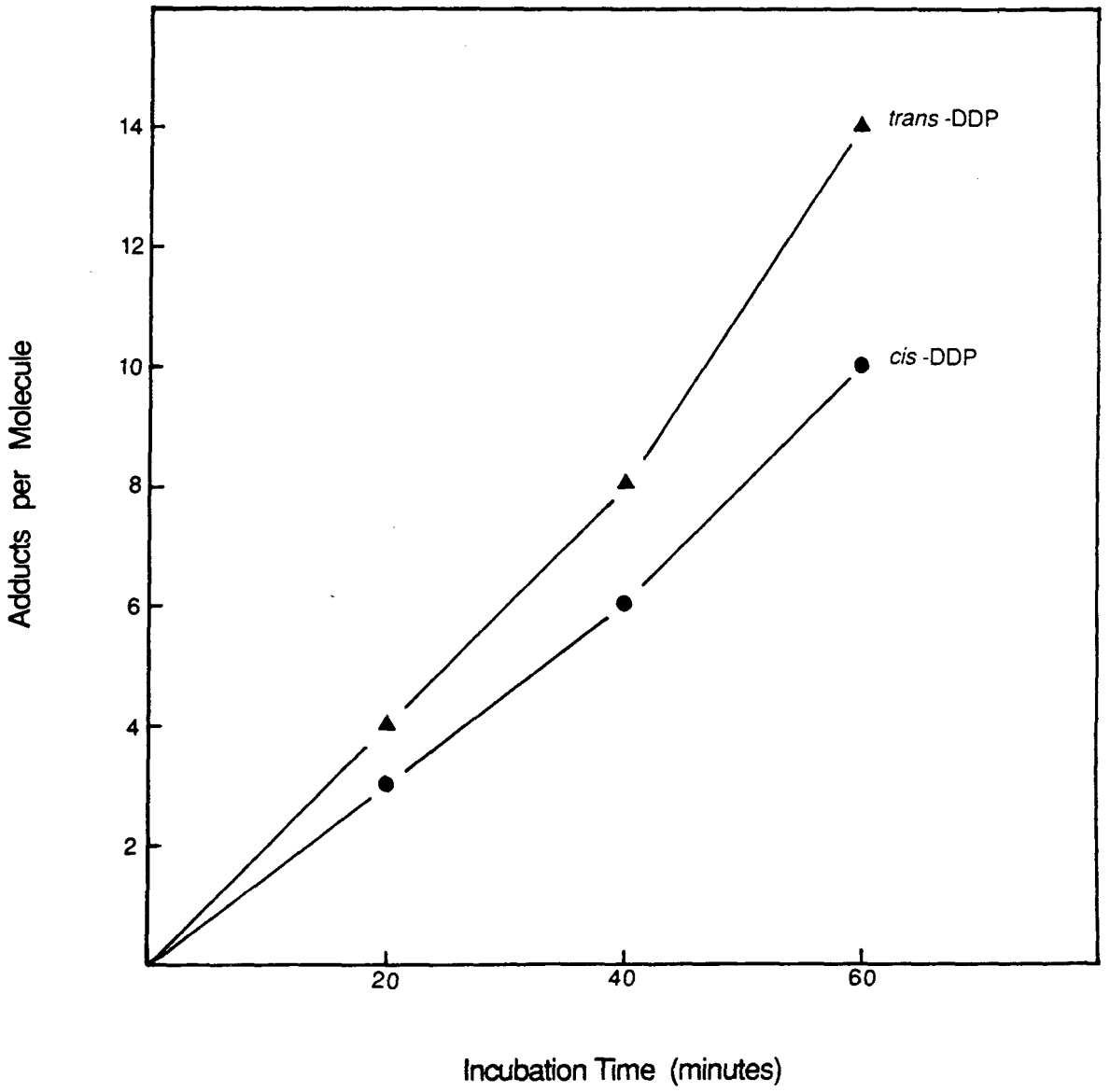
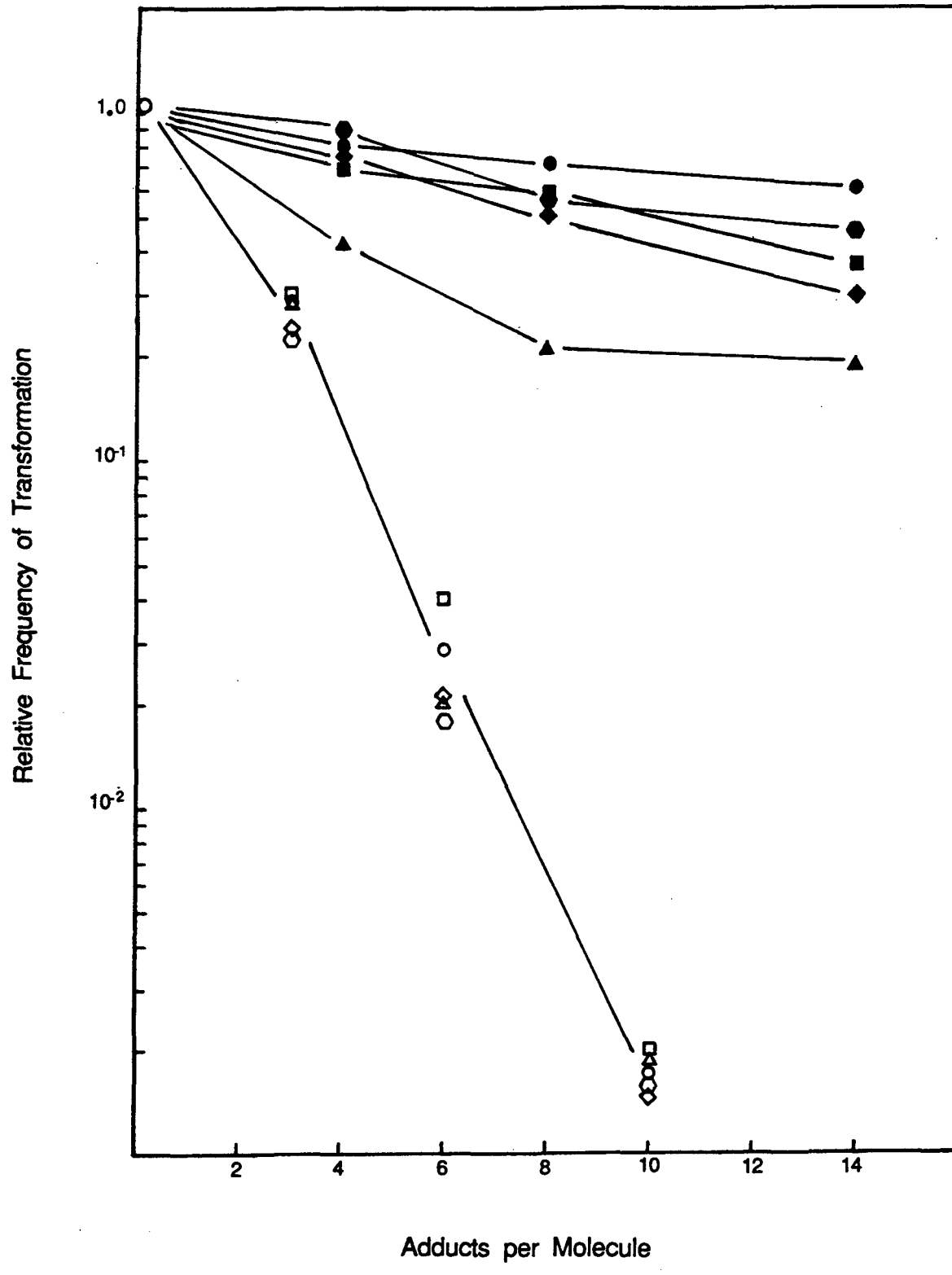

Adducts per Molecule
14
12
10
8
6
4
2
20
40
60
Incubation Time (minutes)
trans -DDP
cis -DDP

Figure 2-3. Relative frequency of transformation of pBR322 DNA damaged with *cis* -DDP (open symbols) or *trans* -DDP (closed symbols) in *E.coli* strains: (✱) W3623 (*uvr*$^+$); (●) AB1157 (*uvr*$^+$); (⬣) SK707 (*uvrD*$^+$); (■) N14-4 (*uvrD3*); (◆) JC8471 (*uvrD252*) and (▲) SK3451 (*uvrD254* ::Tn5).


1.0
10^{-1}
10^{-2}
Relative Frequency of Transformation
2
4
6
8
10
12
14
Adducts per Molecule

modified plasmids were used to transform wild-type or *uvrD* mutant cells (Fig.2-3). Six adducts were required to reduce the relative frequency of transformation of *trans* - DDP damaged plasmids to 37% in the most sensitive *uvrD* mutant (SK3451::Tn5), while 8 adducts reduced it to 70% in the repair proficient strains. The survival of *trans* -DDP treated plasmids in *uvrD* mutants was similar to the mutants ability to survive treatment with *trans* -DDP. This is contrasted with the relative frequencies of transformation of *cis* -DDP damaged plasmids, which were reduced to similar levels in both wild-type and all *uvrD* mutant strains.

Discussion

These results confirm our previous observation that *cis* -DDP is more cytotoxic than *trans* -DDP. We have also shown that excision repair deficient strains of *E.coli* are exceptionally sensitive to *cis* -DDP (Beck et al., 1985). This is due to the formation of intrastrand crosslinks between adjacent guanines. These adducts are specifically incised by the UVRABC excinuclease and thus would not be removed in *uvr* mutants.

The three strains of bacteria mutant at the *uvrD* locus varied in their ability to survive exposure to DDP and had similar cross-resistance to both DDP-isomers and to UV light. The survival of plasmids modified with *trans* -DDP also

resembled the survival of cells treated with *trans* -DDP, in that the transformation frequencies of plasmids decreased most drastically in the most sensitve *uvrD* mutants. The sensitivities of *uvrD* mutants to UV and DDP indicates that the *uvrD* gene product has an important role in DNA repair. The differences in cytotoxicities of the *uvrD* strains suggests that their DNA repair capabilities as mediated by the *uvrD* gene product varied. Since some *uvrD* gene function(s) may be necessary for cell survival, the *uvrD* mutations may not result in cells completely devoid of *uvrD* function. This remaining *uvrD* function may be sufficient for repair of intrastrand crosslinks caused by *cis* -DDP or UV. *uvrD* mutants were found to be proficient in repairing *cis* -DDP damaged plasmids as they were previously shown to be proficient in the repair of UV irradiated plasmids (Roberts and Strike, 1981).

Possibly the incision step of excision repair as mediated by the UvrA, UvrB, and UvrC proteins is sufficient for alleviating the lethal effects of intrastrand crosslinks in plasmid molecules, but not chromosomal DNA. Husain et al., (1985) have previously demonstrated that the removal of *cis* -1,2-diaminocyclohexyldichloroplatinum(II) adducts in plasmid pBR322 by the UVRABC excinuclease, restores the biological activity of damaged plasmids in transformation assays. In *uvrD* mutants the incision step occurs normally, but the excision step is impaired (Kummerle and Masker, 1980; van Sluis et al.,1974). The incision step may be functional in allowing removal of *cis* -DDP adducts in

transforming plasmid DNA and the excision step may not have stringent requirements for the *uvrD* gene function. Replication of plasmid DNA in competent cells may be sufficient to complete the excision step.

A second alternative may be that *cis* -DDP adducts formed in plasmid DNA *in vitro* differ from the adducts formed in chromosomal DNA *in vivo*. Recently Villani et al., (1987) have demonstrated that *cis* -DDP adducts in plasmids differed from those in the bacterial chromosome. This is not surprising since the conditions *in vitro* would necessarily be different than the intracellular environment. *cis* -DDP adducts unique to chromosomal DNA may be repaired by a mechanism requiring greater participation of the UvrD protein. A final alternative could be that the UvrD protein may have a role in recombination repair, a repair pathway that would not be likely to be operative in the repair of plasmids. It has been reported that some mutants in the *uvrD*, *recL*, and *uvrE* genes (alleles of the same cistron) have increased levels of genetic recombination (Zieg et al., 1978).

In summary, *cis* -DDP causes decreases in cells survival, but not plasmid survival in *uvrD* mutants. Ability of *uvrD* mutants to repair adducts caused by *cis* - DDP but not *trans* -DDP in plasmid DNA is another difference in repair of damage caused by these two compounds (Popoff et al., 1987). Plasmids damaged with *cis* -DDP are primarily repaired by excision repair. Damage caused by *cis* -DDP is far more lethal than that of *trans* -DDP as two *cis* -DDP adducts in plasmid DNA constitute a lethal hit; thus turnover of the excinuclease

might not enhance repair. Differences in repair requirements (a functional *uvrD* gene) of *cis* -DDP adducts in chromosome and plasmid DNA which have been observed here may also be explained by the different spectrum of adducts formed in the reaction of DNA with *cis* -DDP under *in vitro* and *vivo* conditions.

CHAPTER 3

Mutations Induced in the Tetracycline-Resistance Gene of Plasmid pXf3 DNA by *cis* - or *trans* -diamminedichloroplatinum(II)

Introduction

In this study we examine the types of mutations induced by *cis* - or *trans* -DDP using a mutagenesis assay developed by Livneh (1983). A 276 bp Bam HI - Sal I fragment of the tetracycline-resistance gene of plasmid pXf3 (3.1kb) was isolated and treated with *cis* - or *trans* -DDP *in vitro* . The plasmid was then reconstructed by ligating the treated fragment to the untreated large fragment of the plasmid. The reconstructed plasmids were used to transform competent cells of *E.coli* of different DNA repair capacities and mutation frequencies to tetracycline sensitivity were determined. The mutation frequencies were greatest when the Bam HI - Sal I fragment had been damaged with *cis* -DDP. Mutations induced with *cis* -DDP in this system were independent of excision repair, as some mutant plasmids (*tet* ^{S}) were obtained in *uvrB* mutants. SOS repair enhanced *cis* -DDP mutagenesis since the mutation frequencies of plasmids

were greater when competent cells were preinduced for SOS repair functions. Determination of the base pair sequence of the Bam HI - Sal I fragment in *tet*^{S} plasmids indicated that the major type of mutation induced with *cis* -DDP was an A:T to T:A transversion at AG adducts. A:T to T:A transversions were also induced at a GTG sequence by both *cis* - and *trans* -DDP. These experiments are the first to provide direct sequence analysis of mutations induced by platinum compounds and will hopefully extend information available on the nucleotide lesion responsible for their mutagenic properties.

Platinum compounds, particularily *cis*- DDP have been used successfully in chemotherapy (Rosenberg et al., 1969; Roberts and Thompson, 1979; Pinto and Lippard, 1985a). The antitumor efficacy of *cis*- DDP appears to correlate with its interaction with DNA, whereby a pair of *cis*- labile chloride ligands undergo hydrolysis to enable the platinum compound to bind to DNA. Both platinum compounds have been shown to bind to DNA and to cause different types of monofunctional and bifunctional adducts (Pinto and Lippard, 1985a). In conjunction with its reactivity with DNA, *cis*- DDP has been found to be damaging to chromosomal and plasmid DNA, toxic to DNA repair deficient mutants of *E. coli* and mutagenic (Beck and Brubaker, 1973; Beck et al., 1985; Alazard et al., 1982; Beck and Fisch, 1980; Fram et al., 1986; Popoff et al., 1987). *trans*- DDP, although reactive with DNA, is less toxic and only slightly mutagenic (Popoff et al., 1987; Mattern et al., 1982).

Little information is known on the type of nucleotide lesions responsible for the mutagenic properties of *cis* -DDP, thus we chose to determine base changes caused by *cis*- DDP using a directed mutagenesis assay of a fragment within the *tet* gene (Livneh, 1983). Previously, Brouwer et al., (1981) found that *cis* -DDP induced nonsense mutations in the *lacI* gene of *E. coli* primarily at GAG and GCG nucleotide sequences. No *cis* -DDP induced mutations were obtained in the *lacI* gene of *uvrB* mutants, but mutations were obtained in *recA* mutants. In contrast, Fram et al., (1986) demonstrated that *cis* -DDP induced mutations were dependent on SOS repair as no mutants were obtained in *umuC* mutants and that mutagenesis was independent of excision repair as mutants were obtained in *uvrA* mutant cells. An additional objective of this study was to examine further, the involvement of SOS and excision repair processes in mutagenesis by DDP.

Materials and Methods

Bacterial strains , plasmids and media

E.coli strains used in these experiments were AB1157 (*uvr*$^+$, *rec*$^+$) and AB1885 (*uvrB5*), which were obtained from Barbara Bachmann (*E.coli* Genetic Stock Center, Yale University). Plasmid pXf3 was obtained from Z. Livneh (Stanford University). Purification of plasmid DNA utilized the procedures

described by Maniatis et al., (1982). Bacteria were routinely grown in Luria Broth or Luria Agar (Maniatis et al., 1982).

Chemicals and enzymes

Platinum compounds were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Restriction enzymes, bacterial alkaline phosphatase, T4 DNA ligase , polynucleotide kinase, Klenow fragment, and chemicals used for electrophoresis were purchased from Bethesda Research Labs (BRL). All enzyme reactions were performed as described by the supplier. DNA fragments were 5'-end-labeled with [γ-^{32}P]ATP (>7000 Ci/mmol) or 3'-end-labeled with [α-^{32}P]GTP (>3000 Ci/mmol) which were purchased from NEN.

Isolation and Modification of DNA fragments

Plasmid pXf3 DNA was digested with restriction endonucleases Bam HI and Sal I. The fragments generated in this digest (large fragment, 2825 bp and small fragment, 276 bp) were resolved on 5% polyacrylamide gels (Maniatis et al., 1982). The large and small fragments were isolated and purified after electroelution of gel pieces, using a unidirectional electroeluter (International Biotechnologies Inc.).

The large, 2825 bp Bam HI - Sal I DNA fragment which was isolated, was dephosphorylated using bacterial alkaline phosphatase and the fragment was purified by two phenol extractions, an ether extraction, and precipitation with

ethanol. Fragments were dried and resuspended in TEN 7.4 (10 mM tris-HCl, 1mM EDTA, 10 mM NaCl, pH 7.4).

The small Bam HI - Sal I fragments (0.50 μg) were suspended in TEN 7.4 and treated with 10 μg/ml of *cis* -DDP or *trans* -DDP in a reaction volume of 50 μl at 37°C for 1 hr. Reactions of DDP with DNA were stopped by addition of 0.1 volume of 5 M NaCl. Unbound platinum was removed by spot dialysis and the amount of platinum bound to DNA fragments was determined by atomic absorption spectrophotometry (Beck et al., 1985).

Ligation

The ligation mixture (75 μl) consisted of dephosphorylated large fragments (0.20 μg) and small fragments (0.40 μg) which had been treated with *cis* -DDP, *trans* -DDP, or untreated; large and small fragments were digested with T4 DNA ligase overnight in a 17°C water bath.

Transformation of bacterial cells

The methods used for making *E. coli* cells competent and the induction of SOS functions prior to making cells competent have been described previously (Popoff et al.,1987). Ligated DNA (40 ng) was added to 300 μl of competent cells which were either not induced or induced for SOS repair functions. This mixture was incubated on ice for 1 hr and "heat shocked" for 2 min at 42°C. Cells and DNA were added to 5 ml of LB broth; 1 ml aliquots were immediately taken from each mixture and added to 5 separate sterile test-tubes and these were

incubated at 37°C for 2 hr in a shaking-water bath. Bacteria from each tube were then plated on Luria Agar supplemented with ampicillin (50 µg/ml) to select for transformants.

Isolation of "mutants"

Transformants (*amp* ^{r} colonies) were screened for sensitivity to tetracycline (*tet* ^{s}) by picking well isolated transformants with tooth picks and replating these isolates on plates of Luria Agar containing ampicillin (50 µg/ml) or tetracycline (20 µg/ml). Isolates which were ampicillin resistant and tetracycline sensitive (*amp* ^{r}, *tet* ^{s}) were grown in 5 ml LB with ampicillin (50 µg/ml) and plasmid DNA was purified using standard procedures (Maniatis et al., 1982). Plasmids were analyzed by agarose gel electrophoresis. Mutant plasmids (*amp* ^{r}, *tet* ^{s}) which were of the same size as wild-type plasmids (*amp* ^{r}, *tet* ^{r}), were analyzed further by restriction endonuclease digestion with Bam HI and Sal I to determine if the recognition sequences for these enzymes were retained. Mutant plasmids (*amp*^{r}, *tet* ^{s}) which were of equivalent size to wild-type plasmids and retained their Bam HI and Sal I restriction endonuclease sites were purified using CsCl gradient centrifugation. The 276 bp Bam HI - Sal I fragments were purified and the sequence of bases in it determined according to the methods of Maxam and Gilbert (1980). The reaction products were subjected to electrophoresis on 6% buffer gradient or 20% denaturing polyacrylamide gels (Biggin et al., 1983; Maxam and Gilbert, 1980).

Results

Transformation (amp^r) and mutation (tet^s) frequencies of reconstructed pXf3 plasmids.

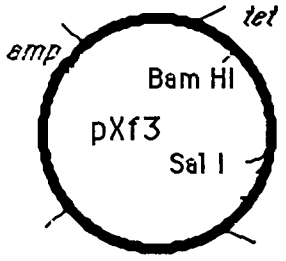
The protocol used for DDP treatment of the 276 bp Bam HI - Sal I fragment of plasmid pXf3 and for plasmid reconstruction is shown in Fig. 3-1. When the purified fragment was treated *in vitro* with *cis* - or *trans* -DDP for 1 hr, approximately 3 and 5 DDP adducts were formed, respectively, as determined by atomic absorption spectrophotometry.

Ligated plasmids containing untreated small fragments, or small fragments treated with *cis* - or *trans* -DDP were used to transform competent cells of *E. coli* of different DNA repair capacities. The DNA repair proficient strain AB1157 and excision repair deficient strain AB1885 (*uvrB5*) were used and in some instances the repair proficient cells were induced for SOS repair functions. The transformation frequencies of each strain and treatment group are the average number of transformants per μg of ligated plasmid (Table 3-1). Transformation frequencies of ligated plasmids bearing the *cis* -DDP treated 276 bp Bam HI - Sal I fragments were lower than when DNA fragments of ligated plasmids were treated with *trans* -DDP or untreated.

Approximately eleven thousand transformants (*amp*^{r} colonies) were screened for sensitivity to tetracycline. Mutants are the number of *amp*^{r}, *tet*^{s}

Figure 3-1. *In vitro* mutagenesis strategy. The 276 bp Bam HI - Sal I fragment of plasmid pXf3 is damaged with diamminedichloroplatinum(II) and the plasmid was reconstructed by ligating the damaged fragment with the large fragment. These ligated molecules were used to transform competent cells of *E.coli* to resistance to ampicillin. Mutant plasmids (*amp* ^{r}, *tet* ^{s}) were obtained by screening ampicillin resistant transformants for sensitivity to tetracycline.

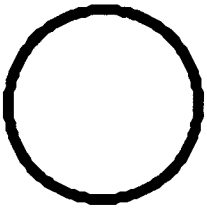
IN VITRO MUTAGENESIS



Bam HI
Sal I

2825 bp (dephosphorylate)
+
276 bp (treat with cis or trans-DDP)

T4 DNA Ligase



Ligated DNA used to transform competent cells of E.coli

1) Select for ampicillin resistant transformants.
2) Screen transformants for sensitivity to tetracycline.
3) Select amp-resistant, tet-sensitive mutant plasmids.
4) Sequence the 276 bp, Bam HI - Sal I fragment of mutants.

TABLE 3-1.

Transformation frequencies of ligated plasmids in strains of *Escherichia coli.*[a]

	Strain			
DNA Fragment Treatment	AB1157 (*uvr*$^+$)	AB1157 (SOS induced) (uv)	AB1157 (SOS induced) (DDP)	AB1885(*uvrB5*)
none	2.45×10^5	1.59×10^5	7.75×10^3	3.14×10^5
cis-DDP	1.39×10^4	1.19×10^4	8.25×10^2	1.70×10^4
trans-DDP	1.58×10^5	7.30×10^4	2.35×10^4	2.80×10^5

[a] Transformation frequencies are the average number of ampicillin resistant transformants per μg ligated DNA.

colonies obtained per number of *amp* ^{r} transformants screened and are referred to as class I mutants (Table 3-2). The mutation frequencies of class I mutants were greatest when the small Bam HI - Sal I 276 bp DNA fragments of ligated plasmids were treated with *cis* -DDP. These plasmids exhibited mutation frequencies 4-fold and 3-fold higher than ligated plasmids from the control or *trans* -DDP treated group in wild-type cells, respectively. In *uvrB* cells, the mutation frequencies of plasmids bearing *cis* -DDP damage were approximately 18-fold greater than the mutation frequencies of plasmids from either the control or *trans* -DDP treated groups. When cells were induced for SOS repair processes, the mutation frequencies of plasmids damaged with *cis* -DDP were increased. Class I mutation frequencies of *cis* -DDP damaged molecules were 10-fold higher than undamaged plasmids when wild-type cells were preinduced with UV light and even greater differences were observed when cells were induced for SOS functions with *cis* -DDP, as no class I mutants were observed for the untreated control group.

Class I mutant plasmids (*amp* ^{r}, *tet* ^{s}) were characterized further by determining if these plasmids were equivalent in size to wild-type plasmids (*amp*^{r}, *tet* ^{s}) and also retained their recognition sites for both restriction endonucleases Bam HI and Sal I. Tetracycline sensitive plasmids with both of these characteristics represent a subset of the class I mutants and are defined as class II mutants (Table 3-3). The number of mutant plasmids bearing the *tet* ^{s}

TABLE 3-2.

Class I mutants of plasmid pXf3 which were damaged within their 276 bp Bam HI - Sal I fragment with *cis* - or *trans* -DDP *in vitro* .

Strain	DNA fragment treatment	# of *amp*^{R} transformants screened	*amp*^{R}, *tet*^{S} phenotype	mutation[a] frequency
AB1157	none	1786	15	8.4×10^{-3}
	cis -DDP	1752	56	3.2×10^{-2}
	trans -DDP	1434	15	1.1×10^{-2}
AB1157 (SOS induced)				
(UV)	none	754	1	1.3×10^{-3}
	cis -DDP	830	11	1.3×10^{-2}
	trans -DDP	642	11	1.7×10^{-2}
(*cis* -DDP)	none	208	0	------
	cis -DDP	19	7	3.7×10^{-1}
	trans -DDP	52	1	1.9×10^{-2}
AB1885	none	1410	2	1.4×10^{-3}
	cis -DDP	930	23	2.5×10^{-2}
	trans -DDP	1534	2	1.3×10^{-3}

[a] Mutation frequencies of class I mutants are the number of mutant plasmids (*amp*^{R},*tet*^{S}) per number of ampicillin resistant transformants screened.

TABLE 3-3.

Class II mutants of plasmid pXf3.

Strain	276 bp fragment treatment	# of full size plasmids retaining Bam HI - Sal recognition sequences	mutation frequency[a]
AB1157	none	2	1.1×10^{-3}
	cis -DDP	21	1.2×10^{-2}
	trans -DDP	5	3.5×10^{-3}
AB1157 (SOS induced)			
(UV)	none	0	-----
	cis -DDP	6	7.2×10^{-3}
	trans -DDP	0	-----
(*cis* -DDP)	none	-	-----
	cis -DDP	3	1.6×10^{-1}
	trans -DDP	0	-----
AB1885	none	0	-----
	cis -DDP	7	7.3×10^{-3}
	trans -DDP	0	-----

[a] Mutation frequencies of class II mutants which are a subset of mutant plasmids (*amp* ^{r}, *tet* ^{s}) from class I. This subset was defined as those of equivalent molecular weight to wild-type plasmids (*amp*^{r}, *tet* ^{s}) and retained their recognition sequence for the restriction endonucleases Bam HI and Sal I. Mutation frequencies are the number of plasmids per number of ampicillin resistant transformants screened.

phenotype from class I which could be caused by anomalies of the ligation reaction or other manipulations, would be eliminated by this second analysis and excluded from the pool of mutants to be sequenced. In many instances it was evident that *tet* ^{S} was due to dimerization of DNA molecules, reannealing of the large DNA fragments, or loss of a restriction endonuclease site. This was revealed by analyzing plasmid molecules or plasmids digested with Bam HI and Sal I, on gels.

Mutation frequencies of class II mutants were highest when DNA fragments were damaged with *cis* -DDP (Table 3-3). The mutation frequency of plasmids with *cis* -DDP damaged 276 bp Bam HI - Sal I fragments were 12-fold higher than the mutation frequency of untreated molecules and 4-fold greater than when the 276 bp fragments were treated with *trans* -DDP, when either of these plasmids were used to transform wild-type cells. There were no class II mutants obtained when the Bam HI - Sal I fragment of the ligated plasmids were untreated or treated with *trans* -DDP and used to transform wild-type cells induced for SOS repair or in *uvrB* mutants, thus direct comparisons could not be made.

DNA Sequence Analysis of DDP - Induced Mutations

In eight of the twelve class II mutants which were independent isolates and sequenced within their Bam HI - Sal I fragments, base pair substitutions occured in the form of A:T to T:A transversions (Table 3-4). Four of the twelve class II mutant plasmids which were sequenced within this region did not have any base

TABLE 3-4.

DDP induced mutations in the Bam HI - Sal I 276 bp fragment of the tetracycline gene of plasmid pXf3.

Host Cell	SOS induction to host cell	DNA fragment treatment	Isolate #	Site of mutation	Type of mutation
AB1157	UV	*cis* -DDP	108-39	482	A:T to T:A
AB1157	none	*cis* -DDP	402-14	482	A:T to T:A
AB1157	none	*cis* -DDP	402-40	482	A:T to T:A
AB1157	none	*cis* -DDP	409-23	482	A:T to T:A
AB1157	UV	*cis* -DDP	127-50	606	A:T to T:A
AB1885	none	*cis* -DDP	406-36	531	A:T to T:A
AB1885	none	*cis* -DDP	406-14	531	A:T to T:A
AB1157	none	*trans* -DDP	616-51	531	A:T to T:A
AB1157	UV	*cis* -DDP	152-33	outside	?
AB1885	none	*cis* -DDP	155-25	outside	?
AB1157	*cis* -DDP	*cis* -DDP	405-31	outside	?
AB1157	*cis* -DDP	*cis* -DDP	404-32	outside	?

pair changes, thus it is presumed that the mutation within the tetracycline gene occured outside of the targeted 276 bp region. The wild-type base pair sequence of this fragment of the tetracycline gene was in agreement with Livneh (1983) and Peden (1983) in that a G:C base pair at position 526 which was missing from the first base sequence analysis which was published by Sutcliffe (1979) was detected. The numbering of bases will follow those adopted by Sutcliffe (1979) except for the addition of one nucleotide after position 526.

All of the A:T to T:A transversions occurred at three different sites within the Bam HI - Sal I region of the *tet* gene (Fig. 3-2). At position 482, four isolates from the *cis* -DDP treatment group exhibited base pair substitutions at AG sequences, three of the mutant plasmids were from wild-type host cells and one of the four mutants was obtained when the transformed repair proficient cells were induced for SOS repair functions with UV. Three of the isolates had mutations at site 531 at GTG sequences. Two were from *tet* ^{S} plasmids isolated from *uvrB* cells in which the small Bam HI - Sal I fragment of the plasmid was damaged with *cis* -DDP. One of the mutant plasmids was from an isolate in which the Bam HI - Sal I region of the *tet* gene was damaged with *trans* -DDP and was in a wild-type host strain. The surrounding sequence of bases at site 531 are extremely G:C rich, so it is not surprising that mutations could be induced with either platinum compound at this site. A third site was also at an AG sequence at position 606, whereby the mutant plasmid was isolated when the

Figure 3-2. Location and surrounding DNA sequence of DDP induced mutations within the Bam HI - Sal I fragment of the tetracycline gene of plasmid pXf3. In all instances the wild-type sequence is shown. The sites of mutations are in bold-faced letters and at these sites, base pair substitutions occurred in the form of A:T to T:A transversions.

Position: 482

```
              470                 480                 490                 500
5' GGGGAAGATC GGGCTCGCCA CTTCGGGCTC ATGAGCGCTT
  CCCCTTCTAG CCCGAGCGGT GAAGCCCGAG TACTCGCGAA
```

Change in Amino Acid:
at position 482: TTC = phenylalanine; mutation changes this to ATC = isoleucine

---

Position: 531

```
                                  530                 540                 550
5' GGGTATGGTG GCAGGCCCCG TGGCCGGGGG ACTGTTGGGCG
   CCCATACCAC CGTCCGGGGC ACCGGCCCCC TGACAACCCGC
```

Change in Amino Acid:
at position 530: GTG =valine; mutation changes this to GAG = glutamic acid

---

Position: 606

```
              590                 600                 610                 620
5' GCGGCGGTGC TCAACGGCCT CAACCTACTA CTGGGCTGCT
  CGCCGCCACG AGTTGCCGGA GTTGGATGAT GACCCGACGA
```

Change in Amino Acid:
at position 606: CTA = leucine; mutation changes this to CAA = glutamine

---

small Bam HI - Sal I fragment of the ligated plasmid was treated with *cis*- DDP and used to transform repair proficient cells induced for SOS repair functions with UV light.

In all instances, the A:T to T:A transversions induced with the platinum compounds at the three different sites within the tetracycline gene of plasmid pXf3 altered the nucleotide sequence such that there would be a change in the amino acid (Fig. 3-2). The detection of these changes are derived by comparing the projected amino acid sequence of the tetracycline gene reported previously (Peden, 1983), with how the specific base change would alter the codon. The mutation at position 482 would change phenylalanine (TTC) to isoleucine (ATC). The base pair substitution at position 531 could change the wild-type sequence from GTG, which codes for valine, to GAG, which now would serve as the codon for the amino acid glutamic acid. The A:T to T:A transversion at 606 would change CTC, the codon for leucine, to CAA, which would code for glutamine.

Discussion

In the mutagenesis assays described here, the primary type of mutation induced with DDP were base pair substitutions in the form of A:T to T:A transversions. The mutation frequencies of tetracycline sensitive plasmids were greatest when DNA molecules were damaged with *cis* -DDP. *trans* -DDP was a

much weaker mutagen, which is in agreement with previous observations (Beck and Fisch, 1980; Fram et al., 1986; Mattern et al., 1982). Mutant plasmids (*amp* ^{r}, *tet* ^{s}) were obtained in both wild-type and *uvrB* mutant cells and the induction of SOS functions increased the mutation frequencies of *cis* -DDP induced mutations further. Formerly, it had been reported that no mutations were induced with *cis* -DDP in the *lacI* gene of *uvrB* mutants (Brouwer et al., 1982). It may be that these investigators were not obtaining any mutants because of lethality as some *cis* -DDP induced mutations were obtained in excision repair deficient mutants by others (Fram et al., 1986; Mattern et al., 1982), although the frequencies were lower than in wild-type cells. Additionally, both of the latter two groups found that SOS repair was involved in *cis* -DDP induced mutagenesis, which also supports data presented here.

The type of adduct involved in the formation of A:T to T:A transversions at the three sites within the region of the tetracycline gene examined, could be inferred by examination of nucleotides at the site of mutation and bases adjacent to that site. Mutations at position 482 and 606 may have been caused by an intrastrand crosslink between an AG, whereby the A would lie 5' to the G. This AG adduct, in addition to a GG adduct, have been the major adducts detected biochemically when DNA is reacted with *cis* -DDP *in vitro* ; these adducts accounted for 25% and 65% of the total platinum bound, respectively (Fichtinger-Schepman et al.,1985; Eastman, 1986). A GXG adduct could be the mutagenic lesion at site

531 and this type of adduct could be feasibly formed with either *cis* - or *trans* -DDP. In the case of site 531 the adduct would be GTG, with DDP potentially bound to two guanine molecules separated by thymine.

The formation of GXG adducts have been revealed by both genetic analysis (Brouwer et al., 1981) and biochemical analysis (Eastman, 1986; Fichtinger-Schepman et al., 1985). The biochemical evidence indicates that GXG adducts are a minor proportion of the lesions formed with *cis* -DDP (6% according to Eastman, 1986), however it was the major adduct detected with *trans* -DDP from *in vitro* DNA replication studies (Pinto and Lippard, 1985b). Brouwer et al., (1981) have observed that GAG and GCG sequences were involved in inducing nonsense mutations with *cis* - DDP in the *lacI* gene of *E.coli.* Both of these sequences were involved in the formation of base pair substitutions. Seventy-four percent of these mutations were G:C to T:A transversions and the majority of the transversions were at GAG sequences (72% of the 74%). It may be that the AG sequence of their reported GAG sequence is the actual adduct involved in mutagenesis since this adduct makes up a greater proportion of the adducts formed with *cis* -DDP than do GXG adducts.

There may be other *cis* -DDP adducts involved in mutagenesis that have not been revealed by this study or previously (Brouwer et al., 1981). Since GG adducts are the predominant adducts formed with *cis* -DDP, it would be expected that they would be involved in mutagenesis. It may be that these adducts are the

most readily repaired and if they are not repaired, then they are lethal and consequently are never fixed as mutations by the cell. It has been demonstrated that the GG adduct formed with *cis* -DDP is the major adduct recognized by the UVRABC excision repair complex of *E.coli* (Beck et al., 1985). GXG adducts are not the primary adducts detected biochemically and are probably not lethal since both *cis* - and *trans* -DDP can form them and mutations were induced by both platinum isomers at such sites and occurred in *uvrB* cells, also. The AG adducts alone, or as part of GAG sequences, appear to be a prime candidate for mutagenesis with *cis* -DDP.

There are possible mechanisms for mutagenesis with DNA damaging agents such as DDP. One or more bases may be damaged to disrupt base pairing and DNA replication could be blocked. The induction of the SOS system would be required to replicate past such lesions, this would allow for a bypass of these mispaired sites and mutations may be generated opposite these lesions (Miller, 1983). The primary types of mutations induced with the SOS system are G:C to T:A and A:T to T:A transversions and these have been the major types of mutations observed with *cis* -DDP (Brouwer et al., 1981; this study). Further, it has been demonstrated that *cis* - and *trans* -DDP can destabilize base pairing to cause local regions of denaturation which have been detected by using S-1 nuclease (Scovell and Capponni, 1982) or anti-nucleoside antibodies (Sundquist, 1986), thus DNA damage with DDP could feasibly be fixed as

mutations by SOS processing.

The data presented here suggests that SOS mutagenesis has a role in mutations induced with *cis* -DDP. Mutation frequencies of plasmids were highest when cells were preinduced for SOS repair functions and four of the twelve mutants sequenced did not have nucleotide changes within the 276 bp targeted region, thus indicating that SOS mutagenesis was involved in fixing mutations at another region within the *tet* gene. Such non-targeted mutations have been detected when mutagenesis assays of this nature were used to detect mutations with other DNA damaging agents. Nearly 40% of the mutations induced with UV light as analyzed by Livneh (1983) were untargeted and approximately 25% of the mutations detected by Saffran and Cantor (1984) for DNA damaged with psoralen were outside of the targeted region.

In summary, mutations in the form of A:T to T:A transversions were induced with *cis* -DDP primarily at AG sequences. GXG sequences appeared to be involved in the formation of mutations with both *cis* - and *trans* - DDP in a very G:C rich region of the targeted area. The SOS repair system stimulated the mutation frequencies of plasmids which were damaged with *cis* -DDP within their 276 bp Bam HI - Sal I region of the *tet* gene. It appears that SOS functions have a role in targeted and non-targeted *cis* -DDP mutagenesis. Mutations induced with *cis* -DDP were independent of excision repair since some mutant plasmids were obtained in *uvrB* mutant cells.

CONCLUSIONS

A. Repair of adducts in DNA caused by DDP

Both *cis* - and *trans* - DDP bind to DNA with similar kinetics as measured by atomic absoprtion spectrophotometry to determine platinum bound to DNA. Since *trans* -DDP causes adducts which do not have exceptional effects on biological systems, we have used it as an indicator for the adducts responsible for the properties associated with *cis* -DDP. It is expected then, that the adduct(s) effective in biological systems is (are) unique to *cis* -DDP or else formed at much lower frequencies by *trans* -DDP.

For studies of repair of adducts caused by DDP, plasmid DNA was treated *in vitro* and used to transform competent bacteria. The *cis* - compound was exceedingly toxic as two adducts in plasmid DNA were required for a lethal hit when transforming wild-type bacteria. Four times as much bound platinum was required for *trans* -DDP modification to reduce survival to 37% that of control, untreated plasmids. Proficiency of competent cells in excision repair enhance survival of plasmids modified with *cis* -DDP, but not *trans* -DDP. Although a functional *uvrB* gene product was essential for plasmid survival after its damage with *cis* -DDP, a functional *uvrD* gene product was not. A functional *recA* gene

was of secondary importance in affecting plasmid survival after its modification with *cis* -DDP. Repair of adducts caused by *trans* -DDP was different from that of *cis* -DDP as indicated by the fact that mutants deficient in either recombination or excision repair gave similar kinetics for plasmid survival when damaged by *trans* -DDP. These differences may be attributed to differential repair mechanisms. Mutants at the *uvrD* locus which were sensitive to DDP in comparison to the isogenic wild-type strains had similar repair capabilities for *trans* -DDP adducts in plasmid DNA modified *in vitro.*

In contrast, plasmid DNA treated with *cis* -DDP survived as well in all *uvrD* isolates as in their parental wild-type strain. Since these mutants were sensitive to *cis* -DDP treatment, the composition of adducts caused by *cis* -DDP when DNA is treated *in vitro* most likeley was different than those caused by *in vivo* treatment. In addition, the repair of these adducts in plasmid and chromosomal DNA may have different requirements for *uvrD* gene functions which may operate in recombination and mismatch repair, as well as excision reapir.

The fact that *uvrD* mutants were as capable as wild-type bacteria in the repair of plasmid DNA treated with *cis* -DDP was not expected, particularily since the three different mutants were sensitive to *cis* -DDP at the chromosomal level. Survival of *uvrD* cells was variable, however. This phenotypic variation of the different *uvrD* isolates is most probably due to the fact that they are not null mutants with respect to *uvrD* gene function, but have varying degrees of the *uvrD*

gene functions remaining. This is also indicated by the fact that attempts to obtain mutants completely devoid of *uvrD* activity have not been successful, thus it appears that the UvrD protein is necessary for survival. The ability of these mutants to repair *cis* -DDP adducts in plasmid DNA may be due to the remaining *uvrD* activity.

B. Mutagenicity of DDP

cis -DDP was more mutagenic than *trans* -DDP as indicated by the mutation frequencies of mutant plasmids in the *in vitro* mutagenesis assays reported here. Further, mutation requencies to tetracycline sensitivity for plasmids damaged with *cis*- DDP were highest when these plasmids were used to transform wild-type cells which had been induced for SOS functions. This was not the case when plasmids contained *trans* -DDP adducts and suggests that mutagenesis depends on SOS repair processes for fixation of mutations at *cis* -DDP adducts, but not *trans* -DDP.

When independent mutant isolates were sequenced within the 276 bp Bam HI - Sal I region of these plasmids, base pair substitutions occurred in the form of A:T to T:A transversions at AG adducts produced by *cis* -DDP. This type of mutation was found at two different sites at such adducts within the fragment and comprised 40% of the mutations which were analyzed by DNA sequencing. Both

cis - and *trans* -DDP produced A:T toT:A transversions at a GTG adduct in a very G:C rich region of the fragment. From this data and observations of repair of DDP adducts it may be that adducts are fixed as mutations because they are not repaired. Some adducts, such as GG adducts would be expected to be involved in *cis* -DDP mutagenesis since they have been the major adducts identified biochemically. It may be that these adducts are more readily repaired or if not repaired are lethal and thus mutations are not introduced at such sites.

C. Future Directions

Since DDP adducts formed *in vivo* would potentially be different than those formed *in vitro*, it would be of interest to extend the results reported here with experiments using DNA molecules damaged *in vivo.* This could be accomplished by treating bacterial cells which hosted plasmids with platinum compounds *in vivo* , at a time interval following plasmid amplification to ensure for the presence of large population of plasmid molecules. Plasmids could be isolated, purified, and the DNA could be used in transformation assays as described in Chapter 1. This would provide additional information on which DNA repair processes alleviated DNA damage with DDP and if these results differed from those obtained when DNA was damaged *in vitro.* Additionally, the number of platinum adducts per DNA molecule could be quantitated by using

atomic absorption spectrophotometry.

To dilineate further which DDP adduct is responsible for inducing mutations with *cis* -DDP, it would be most useful to have a DNA molecule such as a small plasmid or phage that had a defined DDP adduct introduced in it. Mutations could be scored for by a direct change in phenotype, given the premise that there was a readily selectable system. A region of the DNA which contained the defined adduct could be purified from the mutant plasmid or phage and the nucleotide change could be discerned by DNA sequencing methods. It would be expected that different adducts may introduce different mutations in DNA and also test to see if certain adducts were lethal, preferentially repaired, or fixed as mutations. This would extend as well as substantiate the information reported here.

A final question to address would be to examine the role of SOS repair by another method. This would be accomplished by making use of *umuC* or *umuD* mutants of *E.coli*. The *umuC* and *umuD* gene products have been reported to be necessary for mutagenesis. These mutant bacteria could be transformed with the reconstructed damaged plasmids as described in Chapter 3 and screened for the presence of mutant, *tet*^{S} plasmids. If no or significantly fewer mutant plasmids were obtained from these bacteria, then this would confirm the data presented here that SOS processing clearly has a role in *cis* -DDP mutagenesis.

Information presented here as well as questions yet to be answered, clearly

provide important concepts which will assist in understanding which DNA repair pathways function to prevent mutation and cancer. Additionally, providing information on the biologically important adduct(s) of a potent antitumor drug. Results will be useful in understanding the mechanism of the antitumor activities of *cis* -DDP.

LITERATURE CITED

Alazard, R., M. Germanier and N.P. Johnson (1982) Mechanism of toxicity of platinum(II) compounds in repair-deficient strains of *Escherichia coli* , Mutation Res., 93, 327-337.

Allan, S.G., J.F. Smyth, F.G. Hag, R.C.F. Leonard and C.R. Wolf (1986) Protective effect of sodium-2-mercaptoethanesulfonate on the gastrointestinal toxicity and lethality of *cis* -dimminedichloroplatinum, Cancer Res., 46, 3569-3573.

Beck, D.J. and R.R. Brubaker (1973) Effect of *cis* -platinum(II)diamminedichloride on wild-type and deoxyribonucleic acid repair-deficient mutants of *Escherichia coli*, J. Bacteriol., 116, 1247-1252.

Beck, D.J. and R.R. Brubaker (1975) Mutagenic properties of *cis* -platinum(II)diamminedichloride in *Escherichia coli* , Mutation Res., 27, 181-189.

Beck, D.J. and J.E. Fisch (1980) Mutagenicity of platinum coordination complexes in *Salmonella typhimurium* , Mutation Res., 77, 45-54.

Beck, D.J., S. Popoff, A. Sancar and W.D. Rupp (1985) Reactions of the UVRABC excision nuclease with DNA damaged by diamminedichloroplatinum(II), Nucleic Acids Res., 13, 7395-7412.

Bergerat, J.P., B. Drewinko, P. Corry, B. Barlogie and D.H. Ho (1981) Synergistic lethal effect of *cis* -dichlorodiammineplatinum and 1-ß-D-arabinofuranosylcytosine, Cancer Res., 41, 25-30.

Biggin, M.D., T.J. Gibson and G.F. Hong (1983) Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination, Proc. Natl. Acad. Sci. (U.S.A.), 80, 3963-2965.

Brodberg, R.K., R.F.Lyman and R.C. Woodruff (1983) The induction of chromosome aberrations by *cis* -platinum(II)-diamminodichloride in *Drosophila melanogaster*, Environ. Mutagen., 5, 285-297.

Brouwer, J., P. van de Putte, A.M.J. Fichtinger-Schepman and J. Reedijk (1981) Base pair substitution hotspots in GAG and GCG nucleotide sequences in *Escherichia coli* K-12 induced by *cis* -diamminedichloroplatinum(II), Proc. Natl. Acad. Sci. (U.S.A), 78, 7010-7014.

Caron, P.R., S.R. Kushner and L. Grossman (1985) Involvement of helicase II (*uvrD* gene product) and DNA polymerase I in excision mediated by the uvrABC protein complex, Proc. Natl. Acad. Sci. (U.S.A.), 82, 4925-4929.

Cicarelli, R.B., M.J. Solomon, A. Varshavsky and S.J. Lippard (1985) *In vivo* effects of *cis* - and *trans* - diamminedichloroplatinum(II) on SV40 chromosomes: differential repair, DNA-protein crosslinking, and inhibition of replication, Biochemistry, 24, 7533-7540.

Comis, R.L. (1980) Cisplatin nephrotoxicity: the effect of dose schedule and hydration scheme, In: A.W. Prestayko, S.T. Crooke and S.K. Carter (eds.), Cis-platin - current status and new developments, Academic Press, Inc., New York, New York.

Drobnik, J., A. Blahuskova, S. Vasilukova and A. Krekulova (1975) Inactivation of bacteriophages with *cis* -platinum(II)diamminedichloride, Chem. -Biol. Interact., 11, 365-375

Eagan, R.T., S. Frytak, E.T. Creagan, R.L. Richardson, D.T. Coles and J.R. Jett (1986) Differing response rates and survival between squamous and non-squamous non-small cell lung cnacer, Am. J. Clin. Oncol., 9, 249-254.

Eastman, A. (1983) Characterization of the adducts produced in DNA by *cis* -diamminedichloroplatinum(II) and *cis* -dichloro(ethylene-diamine) platinum(II), Biochemistry, 22, 3927-3933.

Eastman, A. (1986) Reevaluation of interaction of *cis* - dichloro(ethylenediamine)platinum(II) with DNA, Biochemistry, 25,3912-3915.

Einhorn, L.H. and J.D. Donohue (1977) *cis* -diamminedichloroplatinum, vinblastine and bleomycin combination chemotherapy in disseminated testicular cancer, Ann. Intern. Med., 87, 293-298.

Fichtinger-Schepman, A.M.J., J.L. van der Veer, J.H.S. den Hartog, P.H.M. Lohman and J. Reedijk (1985) Adducts of the antitumor drug *cis* - diamminedichloroplatinum(II) with DNA: Formation, identification, and quantitation, Biochemistry, 24, 707-713.

Fram, R.J., P.S. Cusick, J.M. Wilson and M.G. Marinus (1985) Mismatch repair of *cis*- diamminedichloroplatinum - induced DNA damage, Mol. Pharmacol., 28, 51-55.

Fram, R.J., P.S. Cusick and M. G. Marinus (1986) Studies on mutagenesis and repair induced by platinum analogs, Mutation Res., 173, 13-18.

Fraval, H.N.A., C.J. Rawlings and J.J. Roberts (1978) Increased sensitivity of UV-repair-deficient human cells to DNA bound platinum products which unlike thymine dimers are not recognized by an endonuclease extracted from *Micrococcus luteus*, Mutation Res., 51, 121-129

Hori, Z.I. and A.J. Clark (1973) Genetic analysis of the *recF* pathway to genetic recombination in *Escherichia coli* : isolation and characterization of mutants. J. Mol. Biol., 80, 327-344

Husain, I., S. G. Chaney and A. Sancar (1985a) Repair of *cis* -platinum DNA adducts by ABC excinuclease *in vivo* and *in vitro*, J. Bacteriol., 163, 817-823.

Husain, I., B. Van Houten, D.C. Thomas, M. Abdel-Monem and A. Sancar (1985b) Effect of DNA polymerase I and DNA helicase II on the turnover rate of UvrABC excision nuclease, Proc. Natl. Acad. Sci. (U.S.A.), 82, 6774-6778.

Jarosik, G.P. and D.J Beck (1984) The effects of *cis* -platinum(II)diamminodichloride, UV light and *N* -methyl-*N'*-nitro-*N* -nitrosoguanidine on *Escherichia coli* : plasmid mediated resistance to mutagens, Chem. Biol. Interactions, 51 247-259.

Johnson, N.P., J.D. Hoeschele, R.D. Rahn, J.P. O'Neil and A.W. Hsie (1980) Mutagenicity, cytotoxicity and DNA binding of platinum(II) chloroamine in Chinese Hamster Ovary Cells, Cancer Res., 40, 1463-1468.

Kuemmerle, N.B. and W.E. Masker (1980) Effect of the *uvrD* mutation on excision repair, J. Bacteriol., 142, 535-546.

Kuemmerle, N.B. and W.E. Masker (1983) An *in vitro* complementation assay for the *Escherichia coli uvrD* gene product, Nucleic Acids Res., 11, 2193-2204.

Livneh, Z. (1983) Directed mutagenesis method for analysis of mutagen specificity: Application to ultraviolet-induced mutagenesis, Proc. Natl. Acad. Sci. (U.S.A.), 80, 237-241.

Maniatis, T., E.F. Fritsch and J. Sambrook (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Maples V.F. and S.R. Kushner (1982) DNA repair in *Escherichia coli* : Identification of the *uvrD* gene product, Proc. Natl. Acad. Sci. (U.S.A.), 79, 5616-5620.

Markham, B.E. and R.R. Brubaker (1980) Influence of chromosome integrity on *Escherichia coli* cell divisions, J. Bacteriol., 143, 455-462.

Marusyk, R. and A. Sargeant (1980) A simple method for dialysis of small-volume samples, Analytical Biochem., 105, 403-404.

Mattern, I. E., L. Cocchiarella, C.G. van Kralinger and P.H.M. Lohman (1982) Prophage induction and mutagenicity of a series of anti-tumor platinum(II) and platinum(IV) co-ordination complexes, Mutation Res., 95, 79-93.

Maxam, A.M. and W. Gilbert (1980) Sequencing end-labelled DNA with base-specific chemical cleavages, in Grossman, L. and K. Moldave (Eds.), Methods in Enzymology, vol. 65, Academic Press, New York, pp. 497-559.

Miller, J.H. (1983) Mutational specificity in bacteria, Ann. Rev. Genet., 17, 215-238.

Muggia, F.M., M.K. Wolpert-DeFilippes, P.Ribaud and G. Mathe (1980) Clinical results with cisplatin analogs, In: A.W. Prestayko, S.T. Crooke and S.K. Carter (eds.), Cis-platin - current status and new developments, Academic Press, Inc., New York, New York.

O'Connel, M.J. , C.G. Moertel, L.K. Dools, R.G. Hahn and J. Rubin (1986) Clinical trials of cisplatin and intensive course 5-fluorouracil for the treatment of advanced colorectal cancer, Am. J. Clin. Oncol., 9, 192-195.

Ogawa, H. K. Shimada and J. Tomizawa (1968) Studies on radiation-sensitive mutants of *E.coli* I. Mutants defective in the repair synthesis, Molec. Gen. Genetics, 101, 227-244.

Pascoe, J.M. and J.J. Roberts (1972) Cross-linking of complementary strands of DNA in mammalian cells by antitumor compounds, Nature, 235, 282-284.

Pinto, A.L. and S.J. Lippard (1985a) Binding of the antitumor drug *cis*-diamminedichloroplatinum(II) (*cis* -platin) to DNA, Biochimica et Biophysica Acta, 780, 167-180.

Pinto, A.L. and S.J. Lippard (1985b) Sequence dependent termination of *in vitro* DNA synthesis by *cis* - and *trans* -diamminedichloroplatinum(II), Proc. Natl. Acad. Sci. (U.S.A.), 82, 4616-4619.

Popoff, S.C., D.J. Beck and W.D. Rupp (1987) Repair of plasmid DNA damaged *in vitro* with *cis*- or *trans* -diamminedichloroplatinum (II) in *Escherichia coli*, Mutation Res., 183, 129-137.

Roberts, J.J. and H.N.A. Fraval (1980) Repair of Cisplatin induced DNA damage and cell sensitivity, In: A.W. Prestayko, S.T. Crooke and S.K. Carter (eds.), Cis-platin - current status and new developments, Academic Press, Inc., New York, New York.

Roberts, J.J. and A.J. Thompson (1979) The mechanism of action of antitumor platinum compounds, in Progress in Nucleic Acids Research and Molecular Biology, Davidson, I.W. and W.E Cohn, Eds., vol. 22, 71-133.

Roberts, R.J. and P. Strike (1981) Efficiency of *Escherichia coli* repair processes on UV-damaged transforming plasmid DNA, Plasmid, 5, 213-220.

Rodriguez, R.L. and R.C. Tait (1983) Recominant DNA Techniques: An Introduction, Addison-Wesley Publishing Company, Reading, Massuchusetts.

Rosenberg, B. (1980) Cisplatin: Its history and possible mechanisms of action, In: A.W. Prestayko, S.T. Crooke and S.K. Carter (eds.), Cis-platin - current status and new developments, Academic Press, Inc., New York, New York.

Rosenberg, B., L. Van Camp, J.E. Trosko and V.H. Mansour (1969) Platinum compounds: a new class of potent antitumor agents, Nature, 222, 385-386.

Saffran, W. and C.R. Cantor (1984) Mutagenic SOS repair of site-specific psoralen damage in plasmid pBR322, J. Mol. Biol., 178, 595-609.

Sancar, A. and D.W. Rupp (1983) A novel repair enzyme: UVRABC excision nuclease of *Escherichia coli* cuts a DNA strand on both sides of the damaged region, Cell, 33, 249-260.

Scovell, W.M. and V.J. Capponi (1982) *cis* -diamminedichloroplatinum(II) modified DNA stimulates far greater levels of S-1 nuclease sensitive regions than does the modification produced by the *trans* -isomer, Biochem. Biophys. Res. Commun., 107, 138-143.

Scovell, W.M. and V.J. Capponi (1984) S-1 nuclease sensitivity to *cis* - and *trans* - diamminedichloroplatinum(II) modified DNA's. Influence of (G + C) content and nucleotide sequence, Biochem. Biophys. Res. Commun., 124, 367-374.

Scovell, W.M. and F. Collart (1985) Unwinding of supercoiled DNA by *cis* - and *trans* -diamminedichloroplatinum(II): influence of the torsional strain on DNA unwinding, Nucleic Acids Res., 13, 2882-2895.

Sherman, S.E., D. Gibsom, A.H.J. Wang and S.J. Lippard (1985) X-ray structure of the major adduct of the anticancer drug *cis* -platin with DNA: cis-[Pt(NH_3)$_2${d(pGpG)}], Science, 230, 412-417.

Smeyers-Verbeke, J., M.R. Detaevernier, L. Denis and D.L. Massart (1981) The determination of platinum in biological fluid by means of graphite furnace atomic absorption spectrophotometry, Clinica Chimica Acta, 113, 392-333.

Stewart, D.J., T.A. Maroun, G. Laframboise and J. Gerin-Lojore (1986) Cisplatin plus cytosine arabinoside in the treament of squamous cell carcinoma of the head and neck, Am. J. Clin. Oncol., 9, 200-203.

Stone, P.J., A.D. Kelman, F.M. Sinex, M.M. Bhargara and H.O. Halvorson (1976) Resolution of α, ß and γ DNA of *Saccharomyces cerevisiae* with antitumor drug *cis* -Pt (NH_3)$_2Cl_2$. Evidence for preferential drug binding by GpG sequences of DNA, J. Mol. Biol., 104, 792-801.

Sundquist, W.I. and S.J. Lippard (1986) Binding of *cis* - and *trans*- diamminedichloroplatinum(II) to deoxyribonucleic acid exposes nucleosides as measured immunochemically with anti-nucleoside antibodies, Biochemistry, 25, 1520-1524.

Sutcliffe, J.G. (1979) Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322, Cold Spring Harbor Symp. Quant. Biol., 43, 77-90.

Taylor, R.T., J.H. Carver, M.L. Hanna and D.C. Wanders (1979) Platinum induced mutations to 8-azoguanine resistance in Chinese hamster ovary cells, Mutation Res., 67, 65-80.

van Sluis, C.A., I.E. Mattern and M.C. Paterson (1974) Properties of *uvrE* mutants of *Escherichia coli* K12. Effects of UV irradiation on DNA metabolism, Mutation Res., 25, 273-279.

Villani, G., C. Lherisson, M. Defais and N.P. Johnson (1987) Further characterization of an *E.coli* strain resistant to the toxic and mutagenic action of *cis* -diamminedichloroplatinum(II), Mutation Res., 183, 21-29.

Walker G.C. (1984) Mutagenesis and inducible responses to deoxyribonucleic acid damage in Escherichia coli, Microbiol. Rev., 48, 60-93.

Woodruff, R.C., R. Valencia, R.C. Lyman, B.A. Earle and J.T. Boyce (1980) The mutagenic effect of platinum compounds in *Drosophila melanogaster*, Environ. Mutagen., 2, 133-138.

Yeung, A.T., W.B. Mattes, E.Y. Oh and L. Grossman (1983) Enzymatic properties of purified *Escherichia coli* uvrABC proteins, Proc. Natl. Acad. Sci. (U.S.A.), 80, 6157-6161.

Zieg, J., V.F. Maples and S.R. Kushner (1978) Recombination levels of *Escherichia coli* K-12 mutants deficient in various replication, recombination, or repair genes, J.Bacteriol., 134, 958-966.

Zwelling, L.A., M.O. Bradley, N.H. Sharkey, T. Anderson and K.W. Kohn (1979) Mutagenicity, cytotoxicity, and DNA crosslinking in V79 Chinese Hamster Cells treated with *cis* - and *trans* -Pt(II)diamminedichlride, Mutation Res., 67, 271-280.