lation and tends to aggregate upon purification (Griswold et al., 1986). Since the majority of known Sertoli cell secretion products are involved in transport, e.g., ceruloplasmin, transferrin, and androgen binding protein, it is tempting to speculate that SGP-2 could be involved in lipid transport.

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Interaction of *trans*-Diamminedichloroplatinum(II) with DNA: Formation of Monofunctional Adducts and Their Reaction with Glutathione†

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ABSTRACT: Bifunctional reactions with DNA are responsible for the toxic action of the cancer chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP). Thiourea has previously been used to trap transient monofunctional adducts in DNA before they rearrange to the toxic lesions. In these studies, thiourea was used to quantify the monofunctional adducts produced by the ineffective isomer *trans*-DDP. Rather than trapping monofunctional adducts, thiourea labilized them from DNA. At short time periods, 85% of *trans*-DDP bound to double-stranded DNA as monofunctional adducts of deoxyguanosine. Rearrangement to bifunctional adducts in double-stranded DNA was 50% complete in 24 h but was much more rapid in single-stranded DNA with 100% complete rearrangement in 24 h. The ineffectiveness of *trans*-DDP therefore results from a high proportion of monofunctional adducts in DNA that rearrange very slowly to toxic bifunctional adducts. The persistent monofunctional adducts react rapidly with glutathione, which would further reduce their potential toxicity by preventing them from rearranging to more toxic bifunctional adducts.

The cancer chemotherapeutic agent *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] is firmly established as a drug of high potency in the treatment of a variety of tumors. Reaction with DNA appears essential to the toxic action of the drug (Roberts & Thompson, 1979). A complex variety of lesions occur in DNA, including monofunctional adducts, interstrand cross-links, intrastrand cross-links, DNA–protein cross-links and DNA–glutathione cross-links [reviewed in Eastman (1987a)]. The bifunctional reactions are critical to the toxic action as

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); HPLC, high-pressure liquid chromatography.

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monofunctional analogues show no therapeutic activity. In addition, monofunctional adducts can be experimentally quenched with thiourea before they rearrange to bifunctional adducts, and this reduces drug toxicity (Zwelling et al., 1979a) and mutagenicity (Bradley et al., 1982). It still remains to determine the relative significance of each bifunctional lesion to the therapeutic activity of the drug.

The trans isomer (*trans*-DDP) is considered inactive as a therapeutic agent but also reacts extensively with DNA to produce both interstrand cross-links and DNA–protein cross-links. However, it is sterically restricted in the type of intrastrand cross-links it could feasibly produce; that is, it is unable to cross-link neighboring bases in a DNA strand. Such cross-links represent about 90% of the reaction with *cis*-DDP (Eastman, 1987a). This has implicated intrastrand cross-links as important to the activity of *cis*-DDP. The adducts produced by *trans*-DDP remain to be characterized. The experiments reported here address this question.

Materials and Methods

Salmon testes DNA, deoxyribonucleosides, glutathione, and all enzymes were purchased from Sigma Chemical Co., St. Louis, MO. *trans*-DDP was obtained from Alfa Ventron, Danvers, MA. Thiourea was stored in a 1 M solution over AG501-X8 (Bio-Rad, Richmond, CA). Single-stranded DNA was obtained by boiling a 1 mg/mL solution of DNA for 15 min followed by rapid cooling on ice.

In most experiments, 100 μg of DNA was incubated with 1 or 10 μg of *trans*-DDP in 20 mM $NaClO_4$, pH 5.5, for 0–48 h at 37 °C. As required, reactions were terminated by adding either thiourea or glutathione to 10 mM and incubating at 23 °C for various time periods (normally 10 min). The platinated DNA was precipitated with ethanol and dissolved either in 0.1 N HCl for atomic absorption analysis or in 50 mM sodium acetate, 10 mM $MgCl_2$, pH 5.5, and enzymatically digested and chromatographed by HPLC as previously detailed for *cis*-DDP-modified DNA (Eastman, 1986). The column eluent was monitored either by an ultraviolet (A_{254}) detector or by atomic absorption.

Atomic absorption analysis was performed on a Perkin-Elmer Model 2380 spectrophotometer with a graphite-furnace Model HGA 400 programmer.

Results

trans-DDP Binding to Deoxyribonucleosides. The strategy applied to the analysis of *trans*-DDP adducts in DNA was to platinate DNA in vitro, enzymatically digest it to deoxyribonucleosides, and separate the products by HPLC. It was first necessary to prepare the expected platinated products and assess their HPLC elution characteristics. The preparation of several of these standards was previously reported (Eastman, 1982). Three normal deoxyribonucleosides (thymidine does not react) were incubated at 37 °C for 24 h with *trans*-DDP at equimolar ratios, and the products were separated by HPLC. The elution positions of these mono- and bifunctional standards are shown in Figure 1A. In addition, mixed bifunctional adducts were prepared by first incubating deoxycytidine or deoxyadenosine with *trans*-DDP for 24 h and then adding equimolar deoxyguanosine for a further 24 h to saturate monofunctional adducts. The elution positions are also shown in Figure 1A. It is important to note that the adducts are positively charged and their elution position is dependent upon the buffer concentration in the column eluent, which acts to suppress ionic reactions with the column (Eastman, 1986).

Monofunctional reaction products are expected in DNA, at least at early time periods. Thiourea was used successfully

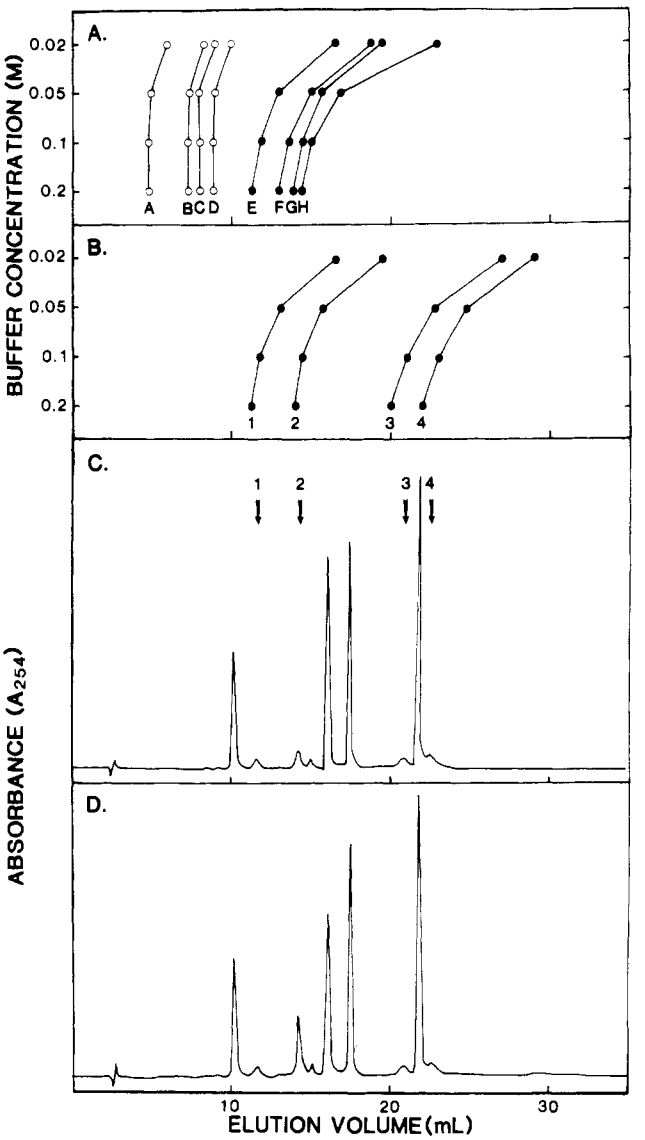

FIGURE 1: (A) HPLC separation of a series of platinated standards. The elution position of each standard is shown for four concentrations of ammonium acetate in the HPLC eluting buffer. The standards are A, dC–Pt; B, N^1-dA–Pt; C, N^7-dA–Pt; D, dG–Pt; E, dC–Pt–dG; F, N^1-dA–Pt–dG; G, dG–Pt–dG; H, N^7-dA–Pt–dG. (B) The elution position of four bifunctional adducts detected in DNA at various concentrations of ammonium acetate. (C) The enzyme digestion products of *trans*-DDP-modified double-stranded DNA in which the reaction with *trans*-DDP was stopped by the addition of thiourea. Elution was with 0.1 M ammonium acetate. The four major peaks are unmodified dC, dG, dT, and dA, respectively. Numbers 1–4 refer to the bifunctional adducts detected. (D) An identical profile as in (C) except no thiourea was added to the reaction.

to trap monofunctional adducts of *cis*-DDP (Eastman, 1983, 1986). This is necessary because during digestion they can react with other nucleosides and chromatograph as bifunctional adducts. Thiourea, 10 mM, was added to the reaction mixtures for various time periods, and the products were separated by HPLC. Two hours were required to convert monofunctional adducts completely to a new peak, although the bifunctional adducts were unaffected. The new peak contained no drug but represented the unmodified deoxynucleoside. Thiourea could not therefore be used to trap the monofunctional adducts but could be used to quantify monofunctional adducts by its ability to displace the drug.

Kinetics of Reaction with DNA. Double-stranded DNA and single-stranded DNA (100 μg) were incubated at 37 °C with 1 or 10 μg of *trans*-DDP. At various times the reactions were

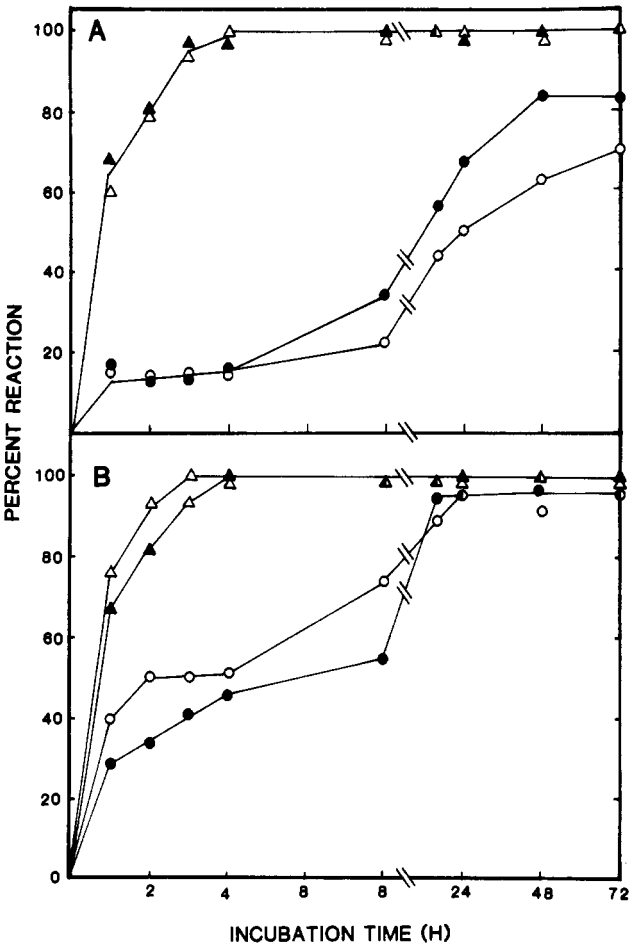

FIGURE 2: Kinetics of reaction of *trans*-DDP with (A) double-stranded DNA and (B) single-stranded DNA at a molar ratio of 0.1 Pt:nucleotide (●, ▲) and 0.01 Pt:nucleotide (○, △). Reactions were stopped with (●, ○) or without (▲, △) thiourea, and Pt associated with DNA was assessed by atomic absorption.

stopped by ethanol precipitation of the DNA. In parallel tubes, reactions were stopped by addition of thiourea to 10 mM for 10 min, and then ethanol precipitation. The DNA was redissolved, and Pt content was analyzed by atomic absorption spectrometry. The reaction of *trans*-DDP with DNA was maximum by 4 h (Figure 2). However, thiourea displaced 85% of the drug from double-stranded DNA at early time points. The rate of rearrangement of monofunctional to bifunctional adducts was very slow, with 24 h required for about 50% rearrangement. This rearrangement was slightly faster at the higher level of platination. In experiments not shown 5–60-min incubations with thiourea gave the same results, showing complete displacement of monofunctional adducts but no displacement of bifunctional adducts.

In single-stranded DNA 30–50% of the adducts were bifunctional at early time points and the remainder became bifunctional by 24 h (Figure 2B).

An alternate way to measure the rate of reaction of *trans*-DDP with DNA is to assess the disappearance of unmodified deoxyribonucleosides over the course of the reaction. This provides additional information as to the nucleosides involved in binding. DNA (100 μg) was incubated with 10 μg of *trans*-DDP for various times, the reaction was stopped by addition of thiourea as required, the DNA was precipitated with ethanol and enzymatically digested, and the products were separated by HPLC. The profiles obtained after a 24-h reaction with *trans*-DDP are shown in Figure 1C,D. The four major peaks represent unmodified dC, dG, dT, and dA. The

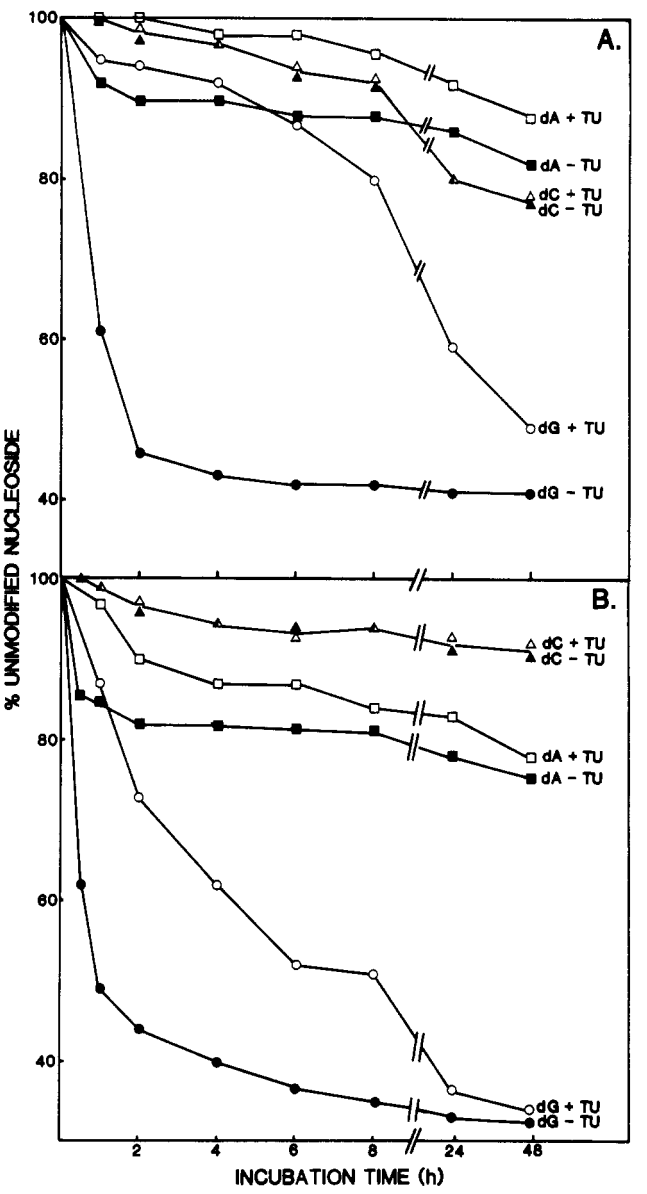

FIGURE 3: Kinetics of reaction of *trans*-DDP with (A) double-stranded DNA and (B) single-stranded DNA at a molar ratio of 0.1 Pt:nucleotide. Reactions were stopped with (open symbols) or without (closed symbols) thiourea, and the DNA was digested and analyzed by HPLC. The peak height of remaining, unmodified deoxyribonucleoside was compared to the peak height in unplatinated DNA.

only difference in the relative heights of these peaks is that for dG. In the absence of thiourea less dG was detected because it reacted with monofunctional adducts, causing a concomitant increase in adduct 2 (discussed below).

Thymidine was used as an internal reference because it does not react with *trans*-DDP. The heights of the other three deoxyribonucleoside peaks were compared to the thymidine peak and then expressed as a percentage of each nucleoside remaining. By this calculation, the dG peak was reduced by 60% during 4 h and then remained constant for the remainder of the experiment (Figure 3). Displacement of monofunctional adducts by thiourea had a marked effect on the dG peak. In double-stranded DNA (Figure 3A) the dG peak was reduced slightly at 1 h but became involved in bifunctional reactions with the same slow kinetics as those seen in the experiments described above. In single-stranded DNA (Figure 3B) the dG peak was reduced much faster as it became involved in bifunctional reactions.

These experiments also monitored changes in dC and dA. Up to 4 h of incubation of *trans*-DDP with double-stranded

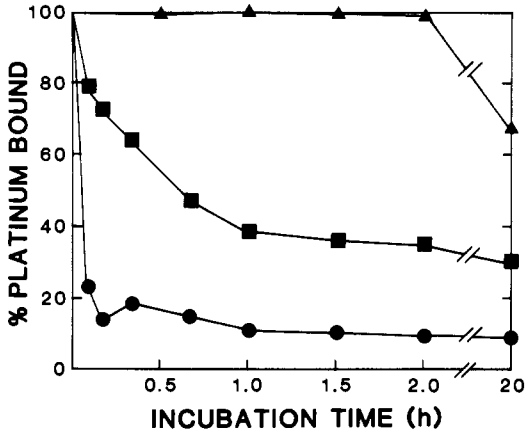

FIGURE 4: Reaction of glutathione and thiourea with *trans*-DDP adducts in DNA. DNA was reacted with *trans*-DDP for 4 h, and then 10 mM glutathione (▲) or 10 mM thiourea (●) was added and incubation continued for the indicated time. The amount of Pt remaining bound to DNA was assayed by atomic absorption. To one sample (■) 10 mM glutathione was added for 10 min; then 10 mM thiourea was added, and incubation continued for the indicated time.

DNA, little if any dA or dC complexed to the drug after thiourea treatment. This demonstrates that practically all monofunctional reaction is with dG. Only at 24–48 h was the dC peak reduced by 20% and the dA peak by 10%, demonstrating their subsequent incorporation into bifunctional adducts. The reaction with single-stranded DNA was different in that dA was notably more involved in bifunctional adducts than dC.

Interaction of Glutathione with Platinated DNA. Experiments were performed to compare the ability of thiourea to reverse monofunctional adducts with a potentially similar phenomenon that might occur with the natural cellular thiol glutathione. A monofunctional standard, dG–Pt, was incubated with 10 mM glutathione for 2 h, the same conditions used to obtain complete reversal with thiourea. Analysis by HPLC showed that only about 50% of the adduct reacted with glutathione. This gave two products of approximately equal quantity, dG–Pt–glutathione and unmodified dG. At longer time periods the reversal went to completion.

The reaction of glutathione with *trans*-DDP adducts in DNA was investigated. DNA was incubated with *trans*-DDP for 4 h; 10 mM glutathione was added and incubation continued for up to 20 h. The DNA was precipitated with ethanol, and the bound Pt was assessed by atomic absorption spectrometry (Figure 4). No reversal of platination occurred during the initial 2 h, but about 30% was reversed by 20 h. This is contrasted to the very rapid displacement by thiourea of 85% of the Pt. Little further reversal occurred up to 20 h.

It was necessary to determine whether glutathione had reacted with the monofunctional adducts in DNA. This was achieved by incubating platinated DNA with 10 mM glutathione for 10 min and then quantitating the remaining monofunctional adducts by incubation with thiourea. Instead of a rapid reversal of platination, the thiourea took more than an hour to reduce the platination to a plateau value. This demonstrated that glutathione rapidly saturated monofunctional adducts and that thiourea could only slowly displace the drug from a glutathione–Pt–DNA cross-link.

Detection of Bifunctional Adducts in DNA. At high levels of DNA platination (1 Pt:10 nucleosides) new A_{254}-absorbing peaks were seen in the HPLC profiles (Figure 1C,D). These were numbered 1–4 and represent bifunctional adducts. At certain concentrations of elution buffer these adducts coeluted with unmodified nucleosides, but by adjustment of the buffer concentration they could be detected. The elution position of each adduct at various buffer concentrations is shown in Figure 1B.

Adducts 1 and 2 cochromatographed with the standards dC–Pt–dG and dG–Pt–dG, but adducts 3 and 4 did not cochromatograph with any of the standards. In single-stranded DNA adduct 1 was markedly less and another minor adduct occurred that cochromatographed with a standard for dA–Pt–dG (not shown). This would agree with the relative disappearance of unmodified nucleosides discussed above. The structures of these adducts will be the subject of a subsequent communication.

Discussion

In establishing a system to analyze *trans*-DDP adducts in DNA, thiourea was used in an attempt to saturate monofunctional adducts. This approach was used successfully for *cis*-DDP adducts (Eastman, 1986). However, rather than trapping monofunctional adducts, thiourea displaced the drug. In retrospect, this was not surprising. The strength of any ligand is markedly influenced by the ligand in a trans orientation. Hence, a sulfur (thiourea) ligand is much stronger than a nitrogen (guanine). This weakens the nitrogen bond which makes it susceptible to further substitution. The experiments reported here demonstrate that thiourea readily trans labilizes *trans*-DDP from both deoxyribonucleosides and from DNA. The reaction of thiourea with monofunctional adducts was much faster in DNA than in nucleosides, but the subsequent rate of labilization was fast in both cases as no intermediate thiourea complexes were detected.

Glutathione occurs in cells at concentrations up to about 10 mM. This concentration of glutathione rapidly saturated monofunctional adducts. However, it caused little reversal of the adducts. Glutathione has also been shown to cross-link to DNA through *cis*-DDP (Eastman, 1987b), but its efficiency was much less than that for *trans*-DDP shown here.

The significance of the reaction of glutathione with *trans*-DDP adducts would be dependent upon the frequency of monofunctional adducts in DNA. These experiments demonstrated that 85% of adducts in double-stranded DNA were monofunctional and that rearrangement to bifunctional adducts was very slow. Similar observations have recently been reported with [^{14}C]deoxyguanosine used to trap the monofunctional adducts, but the reaction required up to 100 h to reach maximum (Butour & Johnson, 1986). The rearrangement to bifunctional adducts occurred considerably faster in single-stranded DNA, where the increased freedom of movement facilitated formation of bifunctional adducts. The presence of high levels of monofunctional adducts in cells, as well as a slow rearrangement, is suggested by the ability of extracellular thiourea to reverse the toxicity of *trans*-DDP (Zwelling et al., 1979a).

The structure of the bifunctional adducts has not yet been definitely proven, but deoxyguanosine is the preferred site of reaction. The initial bifunctional adduct formed is a cross-link between two deoxyguanosines as determined both by cochromatography with a standard and by the fact that, at an early time point, no other deoxyribonucleosides have reacted with the drug. A striking difference exists between double- and single-stranded DNAs after prolonged incubation in that much more deoxycytidine is involved in bifunctional adducts in double-stranded DNA while more deoxyadenosine is present in the adducts in single-stranded DNA.

The observations reported here provide a reason for the ineffectiveness of *trans*-DDP. For the most part *trans*-DDP

reacts with DNA like a monofunctional analogue. However, the free ligand would be available to react with other molecules within the cell. This explains the relatively high levels of DNA–protein cross-links produced by this drug (Zwelling et al., 1979b). It is possible that some of these cross-links might labilize the drug from DNA. This may explain the report that *trans*-DDP-induced DNA–protein cross-links are removed in an excision repair deficient cell line (Gantt et al., 1984). Rather than postulate a new repair pathway, it is possible that the protein cross-links could have spontaneously dissociated from DNA. It is probable, however, that the major reaction of the monofunctional adducts in cells is with glutathione. That this is a significant reaction is supported by the observation that reducing glutathione levels in cells markedly enhances toxicity to *trans*-DDP but has little if any effect on *cis*-DDP toxicity (Andrews et al., 1985).

It has recently been proposed that the limited toxicity of *trans*-DDP is attributable to preferential repair of the DNA adducts as compared to repair of *cis*-DDP adducts (Ciccarelli et al., 1985). The majority of adducts were repaired in less than 6 h. The present report would suggest that the adduct rapidly repaired is a glutathione–Pt–DNA cross-link. However, such a repair process may contribute little to detoxifying the drug if the adducts are already detoxified by reaction with glutathione. *trans*-DDP-induced interstrand cross-links are also apparently repaired more rapidly than their *cis*-DDP counterparts (Plooy et al., 1984); however, the difference in repair rates is much less dramatic than the repair of total adducts in that the repair of interstrand cross-links took 24 h.

In summary, the ineffectiveness of *trans*-DDP is related to the high proportion of monofunctional adducts in DNA that rearrange very slowly to toxic bifunctional adducts. Persistent monofunctional adducts react rapidly with glutathione and become potentially less toxic. In addition, there is the possibility that some reactions may result in trans labilization of the drug from DNA, thereby rendering it innocuous.

Registry No. *trans*-DDP, 14913-33-8; glutathione, 70-18-8.

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Sequence Context Effects on 8-Methoxypsoralen Photobinding to Defined DNA Fragments†

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ABSTRACT: The photoreaction of 8-methoxypsoralen (8-MOP) with DNA fragments of defined sequence was studied. We took advantage of the blockage by bulky adducts of the 3′–5′-exonuclease activity associated with the T4 DNA polymerase. The action of the exonuclease is stopped by biadducts as well as by monoadducts. The termination products were analyzed on sequencing gels. A strong sequence specificity was observed in the DNA photobinding of 8-MOP. The exonuclease terminates its digestion near thymine residues, mainly at potentially cross-linkable sites. There is an increasing reactivity of thymine residues in the order T < TT ≪ TTT in a GC environment. For thymine residues in cross-linkable sites, the reactivity follows the order AT ≪ TA ~ TAT ≪ ATA < ATAT < ATATAA. Repeated A-T sequences are hot spots for the photochemical reaction of 8-MOP with DNA. Both monoadducts and interstrand cross-links are formed preferentially in 5′-TpA sites. Our results highlight the role of the sequence and consequently of the conformation around a potential site in the photobinding of 8-MOP to DNA.

Psoralens are a class of heterocyclic compounds (furocoumarins) which are used as photosensitizing agents in the treatment of various skin diseases (referred to as PUVA therapy). Psoralen plus UVA treatment has lethal and mutagenic effects on bacteria, yeast, and mammalian cells [for reviews, see Ben-Hur and Song (1984) and Averbeck (1985)]. It is also known to be carcinogenic in mice [for a review, see Ben-Hur and Song (1984)]. Nucleic acids are targets for the

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