Effect of the Diaminocyclohexane Carrier Ligand on Platinum Adduct Formation, Repair, and Lethality†

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ABSTRACT: Platinum compounds with the diaminocyclohexane (dach) carrier ligand are of particular interest because cell lines that have developed resistance to platinum compounds in general often retain sensitivity to dach–platinum compounds, suggesting that the dach carrier ligand affects the formation, repair, or lethality of platinum–DNA adducts. The effect of the dach ligand on platinum adduct formation was assessed by using the $(HaeIII–HindIII)_{146}$ fragment of pBR322 treated to give equal amounts of dach– or ethylenediamine–platinum adducts. The sites of adduct formation were mapped by digestion with *Escherichia coli* ABC excinuclease. There were no significant effects of the dach carrier ligand on the types or sites of platinum adduct formation. The effect of the dach ligand on platinum adduct repair was determined by using synthetic oligomers designed to have single, specific platinum adducts (G monoadduct; GG, AG, or GNG diadduct) with either the dach or ethylenediamine (en) carrier ligand. These adducts differed significantly in their ability to serve as substrates for ABC excinuclease with GNG ≥ G > AG > GG. The dach carrier ligand had little effect on the recognition of AG and GG adducts by ABC excinuclease, but significantly improved the ability of ABC excinuclease to excise G monoadducts and GNG diadducts. These data suggest that if the carrier ligand has any effect on the repair of platinum adducts, it is more likely to exert that effect on the repair of platinum monoadducts or GNG diadducts rather than on the more abundant AG or GG diadducts. [^{14}C]Thiourea incorporation was used to quantitate the rate of monoadduct to diadduct conversion. The dach carrier ligand was found to slow the rate of monoadduct to diadduct conversion by a factor of 2.4 compared to the en ligand and 1.8 compared to the diammine ligands. Finally, the effect of the dach ligand on platinum adduct lethality was assessed by determining the effect of dach– and en–platinum adducts on the transformation efficiency of pBR322 into a repair-deficient (*recA*$^-$ *uvrA*$^-$) strain of *E. coli*. In the range of 1–14 adducts/pBR322, platinum adducts with the dach carrier ligand were significantly more effective than those with the en ligand at inhibiting transformation. These data suggest that the dach carrier ligand can significantly affect the ability of platinum–DNA adducts to block essential processes such as replication and transcription.

Platinum coordination complexes have shown potent antitumor activity (Rosenberg, 1978) and are used clinically for the treatment of a variety of tumors. It is thought that the cytotoxic effects of platinum complexes are mainly exerted through damage to DNA (Pinto & Lippard, 1985). Cell lines from human ovarian carcinoma 2780, murine leukemia L1210, and murine leukemia P388 have been developed which show selective resistance to platinum drugs on the basis of the carrier ligand attached to the platinum (Burchenal et al., 1978; Eastman & Illenye, 1984; Behrens et al., 1987). For example, the L1210/DDP[1] cell line is resistant to *cis*-diamminedichloroplatinum(II) (*cis*-DDP) and dichloroethylenediamineplatinum(II) [$PtCl_2$(en)] but relatively sensitive to diaminocyclohexanedichloroplatinum(II) [$PtCl_2$(dach)] (Burchenal et al., 1978), while the L1210/DACH line shows the opposite pattern of resistance (Eastman & Illenye, 1984). Thus, the carrier ligand on platinum has a major effect on the ability of these cells to survive platinum treatment. This difference in sensitivity cannot be adequately explained by differential uptake or efflux (Waud, 1987; Richon et al., 1987).

The dach ligand is bulkier and more hydrophobic than the en and diammine carrier ligands, and it alters the N–Pt–N bond angle (Lock & Pilon, 1981). In addition, platinum complexes containing the two dach isomers used in this study (trans-*d* and trans-*l*) have been shown to form adducts with d(GpG) in which one of the axial amino protons of the cyclohexane ring is in close proximity to the O^6 of one of the guanines (the 5′ guanine for the trans-*d* isomer and the 3′ guanine for the trans-*l* isomer) (Inagaki & Kidani, 1986), leading to the possibility of steric hindrance and significant distortion of the DNA. Thus, the dach ligand could potentially affect (1) the type of adduct formed, (2) the rate of adduct formation, (3) the localization of the adducts in the DNA, (4) the rate of adduct repair, and/or (5) the lethality of the adducts.

Several different types of platinum–DNA adducts are possible (Roberts & Thomson, 1979; Royer-Pokora et al., 1981; Fichtinger-Schepman et al., 1982, 1985; Eastman, 1983,

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[1] Abbreviations: dach, *trans-d,l*-1,2-diaminocyclohexane; en, ethylenediamine; dien, diethylenetriamine; $PtCl_2$(dach), *trans-d,l*-1,2-diaminocyclohexanedichloroplatinum(II); $PtCl_2$(en), dichloroethylenediamineplatinum(II); [PtCl(dien)]$^+$, chlorodiethylenetriamineplatinum(II); *cis*-DDP, *cis*-diamminedichloroplatinum(II); L1210/DDP, L1210 cell line with acquired resistance to *cis*-DDP; L1210/DACH, L1210 cell line with acquired resistance to $PtCl_2$(dach); TCA, trichloroacetic acid; r_b, adducts/base pair.

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1986). The most abundant adduct (70–80%) is the GG intrastrand diadduct ([GG]Pt). The other major adducts (5–10% each) are the AG intrastrand diadduct ([AG]Pt), the monoadduct with a single guanine ([G]Pt), and the intrastrand diadduct formed between two guanines separated by a single nucleotide ([GNG]Pt). Interstrand GG diadducts also form, but they represent less than 1% of the total adducts.

The sequence localization of platinum adducts in DNA is distinctly nonrandom. Pinto and Lippard (1985a) have shown that *cis*-DDP reacts preferentially with sites containing multiple G's. However, Hemminki and Thilly (1988) found that the extent of reaction of *cis*-DDP with sites containing multiple G's varied more than 5-fold and that the differences in the extent of reaction did not necessarily correlate with the number of sequential G's. Obviously, the neighboring base sequence also plays a role in determining the site of platination.

Enhanced repair has been implicated as a mechanism of resistance (Eastman & Schulte, 1988; Lai et al., 1988), but the specificity of this enhanced repair for carrier ligand has not been studied. Our objective in these studies was to determine the effects of carrier ligand on (1) the type and location of platinum adducts formed, (2) the recognition of defined platinum–DNA adducts by a purified repair enzyme, and (3) the lethality of platinum–DNA adducts in a repair-deficient strain of *Escherichia coli*. These data have allowed us to propose models for the carrier ligand effects on resistance. Such models should provide a useful basis for the design of future experiments to more fully characterize the mechanism and specificity of resistance in a variety of cell lines.

Experimental Procedures

Unlabeled and ^{3}H-labeled dichloroethylenediamineplatinum(II) [$PtCl_2$(en)], *trans-d,l*-1,2-diaminocyclohexanedichloroplatinum(II) [$PtCl_2$(dach)], and chlorodiethylenetriamineplatinum(II) [[PtCl(dien)]$^+$] were synthesized by Dr. Steve Wyrick as described previously (Wyrick & Chaney, 1988). [^{14}C]Thiourea was obtained from ICN Radiochemicals. After dilution to the appropriate specific activity with unlabeled thiourea, it was stored over AG501X8 resin (Bio-Rad) at 4 °C. *E. coli* strain CSR603 (recA1 uvrA6) was obtained from B. J. Bachmann, *Escherichia coli* Genetic Stock Center, Yale University, New Haven, CT. The cells were routinely grown in Luria broth. pBR322 DNA and frozen competent cells were prepared as described previously (Husain et al., 1985). T4 polynucleotide kinase, T4 DNA ligase, and restriction enzymes were obtained from Bethesda Research Laboratories.

Preparation of Platinated pBR322 Fragment. A 175 base pair fragment [(*Eco*R1–*Hae*III)$_{175}$] isolated from pBR322 was 5′ end labeled with [γ-^{32}P]ATP (ICN) and T4 polynucleotide kinase and digested with *Hin*dIII to remove the label from the *Eco*R1 end. The resulting 146 base pair fragment [(*Hae*III–*Hin*dIII)$_{146}$] was treated with $PtCl_2$(dach) or $PtCl_2$(en) to give 16 adducts/fragment. The reaction conditions used [0.2 μg/mL DNA, 50 μM $PtCl_2$(dach) for 35 min or 50 μM $PtCl_2$(en) for 10 min, 25 mM NaCl, 0.14 mM EDTA, 0.14 mM Tris-HCl, pH 7.4, 37 °C] were chosen on the basis of parallel reactions carried out with the unlabeled 146 base pair fragment and ^{3}H-labeled $PtCl_2$(dach) or $PtCl_2$(en). The reaction was stopped by adjusting the NaCl concentration to 0.5 M and cooling the reaction mixture to 4 °C. Unreacted drug was removed by dialyzing the platinated DNA at 4 °C against TEN 7.4 buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1 mM EDTA) plus 0.5 M NaCl (twice) followed by dialysis against TEN 7.4 buffer alone. Conversion of monoadducts to diadducts was maximized by incubating the platinated DNA at 37 °C for an additional 30 h in TEN 7.4.

Preparation of Site-Specific Platinated Oligomers. (*a*) *DNA Oligonucleotides.* Oligomers were synthesized by phosphotriester chemistry. They were purified on a 20% polyacrylamide/7 M urea DNA sequencing gel. The band corresponding to the correct size was visualized by long-wavelength UV shadowing, cut out, and recovered by electroelution with an IBI Model UEA unidirectional electroelutor.

(*b*) *Platinum-Adducted 12-mers.* Platinum-modified 12-mers were prepared by incubating the platinum drugs at 100 μM with 1 μM 12-mer at 37 °C for 48 h in TEN 7.4 plus 25 mM NaCl. The 12-mers were designed with a single site of platination (either G, GG, AG, or GTG). After 48 h, the excess drug was removed by applying the mixture to a NENSORB column (Du Pont) and washing with 3 mL of 0.1 M Tris-HCl, 10 mM triethylamine, 1 mM Na_2EDTA, pH 7.7, and 3 mL of water. The 12-mer was then eluted with 50% methanol and lyophilized. This mixture of platinated and unplatinated 12-mers was 5′ end labeled, and the platinated 12-mers were purified on a 20% polyacrylamide sequencing gel (the platinated 12-mer runs 1 base slower). Samples of the platinated and unplatinated 12-mers were subjected to the G and A+G sequencing reactions (Maxam & Gilbert, 1977) to verify site-specific platination.

(*c*) *Construction of Defined Substrates for ABC Excinuclease.* To construct the platinum-modified substrates, the platinum-adducted 12-mers were ligated with five other oligomers by using T4 DNA ligase (Bethesda Research Laboratories) as described previously for psoralen-modified DNA substrates (Van Houten et al., 1986). The full-length 43 base pair substrate was then purified on a preparative 12% polyacrylamide sequencing gel.

Digestion with ABC Excinuclease. The UvrA, UvrB, and UvrC subunits of ABC excinuclease were purified by the method of Thomas et al. (1985). The digestion was carried out essentially as previously described (Sancar et al., 1985). Briefly, the DNA was incubated with UvrA, UvrB, and UvrC at 37 °C in a buffer containing 50 mM KCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM ATP, and 100 μg/mL bovine serum albumin. At appropriate times, an aliquot (50 μL) of this mixture was taken and the reaction stopped by adding 250 μL of 300 mM sodium acetate and 10 mM EDTA at 4 °C. The DNA was immediately precipitated with ethanol in the presence of 10 μg of oyster glycogen as a carrier, lyophilized, and resuspended in formamide dye for analysis on a sequencing gel. The quantities of DNA and the Uvr subunits used in each assay are described in the figure legends.

Quantitation of ABC Excision Efficiency for Oligonucleotide Substrates. The ABC excinuclease treated oligonucleotide was fractionated on a 20% polyacrylamide sequencing gel. The extent of excision was quantitated by densitometric scanning of the autoradiograms with a Hoeffer Model GS300 scanning densitometer linked to an Apple IIe computer with the Appligration program (Dynamic Solutions Corp., Pasadena, CA). This program gave integrated values as a percent of total area for the peaks detected on the autoradiogram.

Monoadduct to Diadduct Conversion. A modification of the method of Malinge and Leng (1988) was used to measure the rates of monoadduct to diadduct conversion. The original protocol required ethidium bromide and acridine for the formation of monoadducts. Since different types of monoadduct to diadduct conversions appear to occur at different

rates (Eastman, 1986; Johnson et al., 1985) and ethidium bromide is known to alter the sequence specificity of platinum adduct formation (Bowler & Lippard, 1986; Malinge et al., 1987), it was thought that the use of ethidium bromide to form the monoadducts might influence the rate of monoadduct to diadduct conversion. In the modified protocol 0.5 mg/mL salmon sperm DNA was incubated with either 122 μg/mL $PtCl_2$(dach), 80 μg/mL $PtCl_2$(en), or 200 μg/mL *cis*-DDP in 25 mM NaCl, 0.2 mM EDTA, and 2 mM Tris-HCl, pH 7.4, for 30 min at 37 °C. These drug concentrations were chosen to produce the same level of platinum–DNA adducts. The reaction was stopped by increasing the NaCl concentration to 0.5 M, cooling to 4 °C, and dialyzing versus two changes of high-salt TEN 7.4 (TEN 7.4 plus 0.5 M NaCl) at 4 °C. The samples were then precipitated with 0.8 volume of 2-propanol, resuspended at 1 mg/mL in high-salt TEN 7.4, and dialyzed versus two changes of 10 mM $NaClO_4$ for a total of 2 h at 4 °C.

After aliquots were removed for determination of platinum content (Zeeman Model 3030 atomic absorption spectrometer) and DNA concentration, the samples were divided into 200-μL aliquots and either left at 4 °C (controls) or incubated at 37 °C. The amount of monoadduct present at each time point was determined by adding 20 μL of 110 mM [^{14}C]thiourea (specific activity 5.23 μCi/μmol) and incubating for 10 min at 37 °C. The reaction was stopped by adding 2 mL of cold 5% TCA and cooling to 4 °C. To reduce background, the samples were centrifuged, the supernatant was removed by aspiration, and the pellet was redissolved in 0.2 mL of 0.1 N NaOH. After reprecipitation of the DNA with cold 5% TCA, the samples were analyzed by standard filtration techniques (Malinge & Leng, 1988). Background was determined by using unplatinated salmon sperm DNA.

Transformation Assay. pBR322 DNA was platinated with $PtCl_2$(dach) or $PtCl_2$(en) to give 1–14 adducts/pBR322 essentially as described above for the 146 base pair fragment. The number of adducts was quantitated on the basis of radioactivity incorporated and checked by atomic absorption for the two samples with the most adducts per pBR322. The transformation assay was carried out as described previously (Husain et al., 1985). The pBR322 transformants were selected on Luria agar containing 20 μg of tetracycline/mL.

Results

Effect of Carrier Ligand on the Site of Platination. Since ABC excinuclease cleaves DNA 7 base pairs 5′ to the site of bulky DNA adducts such as platinum adducts (Sancar & Rupp, 1983; Sancar & Sancar, 1988), it can be used to precisely locate such adducts in terminally labeled DNA fragments of known sequence. Assuming that the recognition frequencies were known for different types of adducts, such cleavage reactions could also be used to quantitate the amount of each adduct present. In these experiments a 146 base pair fragment, 5′ end labeled on one strand, was prepared from pBR322 and platinated with either $PtCl_2$(dach) or $PtCl_2$(en). The site of platination was determined by digesting the platinated DNA with ABC excinuclease and running the digested DNA on a sequencing gel. Control experiments showed no detectable differences in the sites of platination in DNA fragments containing between 2 and 16 adducts/146 base pair fragment (The number of adducts was estimated from parallel reactions carried out with unlabeled 146 base pair fragment and ^{3}H-labeled platinum compounds of known specific activity.) Thus, the sites of platination were compared for $PtCl_2$(dach)- and $PtCl_2$(en)-treated DNA containing 16 platinum adducts/146 base pair fragment because of better

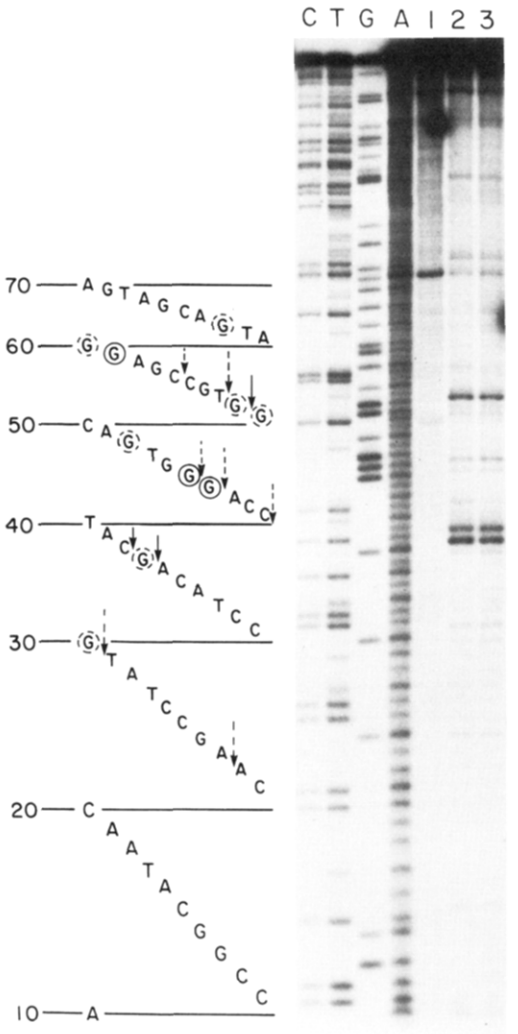

FIGURE 1: Effect of carrier ligand on the sites of platination. The ^{32}P 5′ end labeled (*Hae*III–*Hin*dIII)$_{146}$ fragment of pBR322 DNA was treated with either $PtCl_2$(dach) or $PtCl_2$(en) to give 16 adducts/fragment. 0.31 pmol of these platinated fragments were then incubated with 2.2 pmol of UvrA, 2.4 pmol of UvrB, and 2.1 pmol of UvrC for 30 min at 37 °C and run on a 8% sequencing gel. Untreated 146 base pair DNA was sequenced by the Maxam and Gilbert (1977) procedure to identify the sites of ABC excinuclease cleavage. The details of these assays are given under Experimental Procedures. (Lane 1) Control DNA, no ABC excinuclease treatment; (lane 2) $PtCl_2$(dach)-treated DNA; (lane 3) $PtCl_2$(en)-treated DNA. Lanes C, T, G, and A are Maxam–Gilbert sequencing ladders. The sequence from base 10 to base 70 is shown to the left of the autoradiograph. The positions of cutting by ABC excinuclease are shown by arrows, and the sites of the corresponding adducts are circled (ABC excinuclease cuts seven bases 5′ to the adduct).

incision signals at this level of platination. There did not appear to be any significant effect of carrier ligand on the sites of platination (Figures 1 and 2). However, quantitative interpretation of these data required a knowledge of the relative excision efficiencies of purified ABC excinuclease for dach– and en–platinum adducts of the type observed in this 146 base pair fragment (GG, AG, GNG, and G monoadduct). Thus, the reactivity of ABC excinuclease toward oligonucleotide substrates containing a single, defined platinum adduct was also determined.

Synthesis of Specific Platinum–DNA Adducts as Substrates. We followed the strategy utilized previously by Van Houten et al. (1986) which consists of forming the adduct on a short oligomer, purifying the adducted oligomer, and assembling several oligomers into a piece of duplex DNA large enough to serve as a substrate for ABC excinuclease.

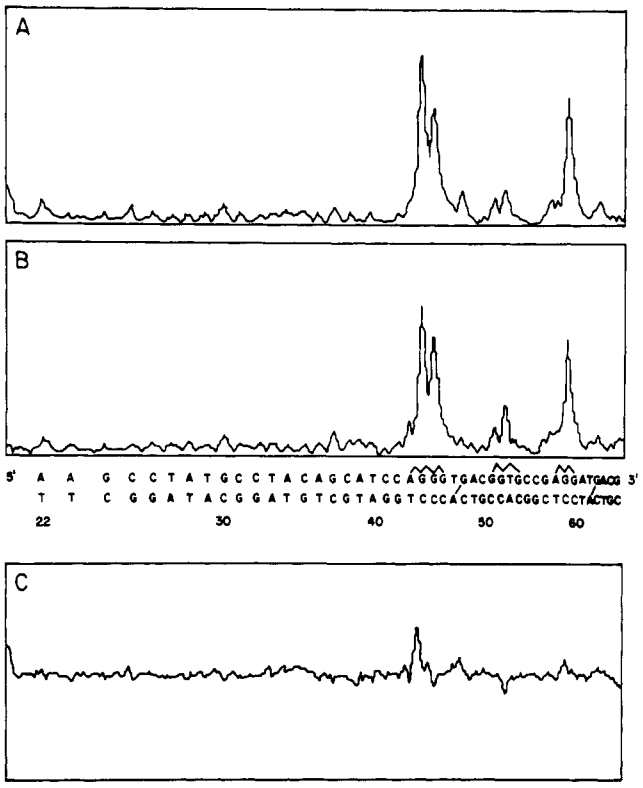

FIGURE 2: Quantitation of diaminocyclohexane and ethylenediamineplatinum adducts. Lanes 2 and 3 from the autoradiogram shown in Figure 1 were scanned with a Zeineh Model SLR-2D/1D laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA). (A) Scan of ABC digest of $PtCl_2$(dach)-treated DNA; (B) scan of ABC digest of $PtCl_2$(en)-treated DNA; (C) difference, (A) − (B). The corresponding sequence and identification of likely adducts are also shown.

Figure 3 shows the sequences of the four types of substrates we constructed. Each central 12-mer was used to form the desired adduct, with the type of platinum–DNA adduct formed being determined by the sequence, e.g., the 12-mer for the G monoadduct contained only a single G flanked by C and T, which have been shown not to react with the platinum drugs (Eastman, 1983). This central 12-mer was incubated with either $PtCl_2$(dach) or $PtCl_2$(en). After removal of excess drug, the platinum-adducted 12-mer was end labeled with [γ-^{32}P]ATP and run on a gel to separate the platinated and unplatinated oligomers (Figure 4). Purification of the platinated 12-mer at this stage was essential to assure that all the 43-mer substrates subsequently constructed had the specific platinum adduct they were designed to have. To demonstrate that the 12-mers were fully platinated at the appropriate sites, the Maxam–Gilbert sequencing reactions for G and A+G were performed on both the unplatinated and the platinated 12-mer for each of the four types of adduct. These sequencing reactions work by alkylating bases at precisely the same atoms where the platinum binds, the N7 of guanine and adenine, so if platinum is already bound at that site, the sequencing reaction is blocked for that base. Figure 5 shows the results of these experiments. Clearly, the unplatinated 12-mers in each case gave either one or two bands corresponding to the G or A present in the oligomer. The platinated 12-mers, however, showed no reaction at the G or A nucleotides in the sequence. The purified 12-mers were then ligated into the 43-mer substrates, as indicated in Figure 3, and used as substrates for ABC excinuclease. Each sequence was constructed with no platination as a control and with both the dach- and en–platinum adducts. The reactive site of the dach- and en–G monoadducts was blocked by treating with dithiothreitol (10 mM, 30 min at 37 °C) prior to separation of the platinated and unplatinated 12-mers. In addition, the G monoadduct was also constructed with [PtCl(dien)]$^+$, which has only one leaving group and thus can only form monoadducts.

Excision of the Platinum Adducts from Synthetic Oligomers. The effectiveness of the different platinum–DNA adducts as substrates for ABC excinuclease was determined by the excision assay described under Experimental Procedures. As with other bulky adducts, ABC excinuclease excised these platinum–DNA adducts as 12-mers. These excised 12-mers are not identical with the 12-mers used to synthesize the substrates for these reactions. In the original 12-mers the platinum adduct was 4–6 bases from the 5′ end (Figure 3), while ABC excinuclease always cuts 7 bases 5′ to the adduct. In each experiment, the time course of excision was performed on two of the platinated substrates in parallel. To control for day to day variation in ABC excinuclease activity, excision of the [AG]Pt(en) substrate was included in each experiment

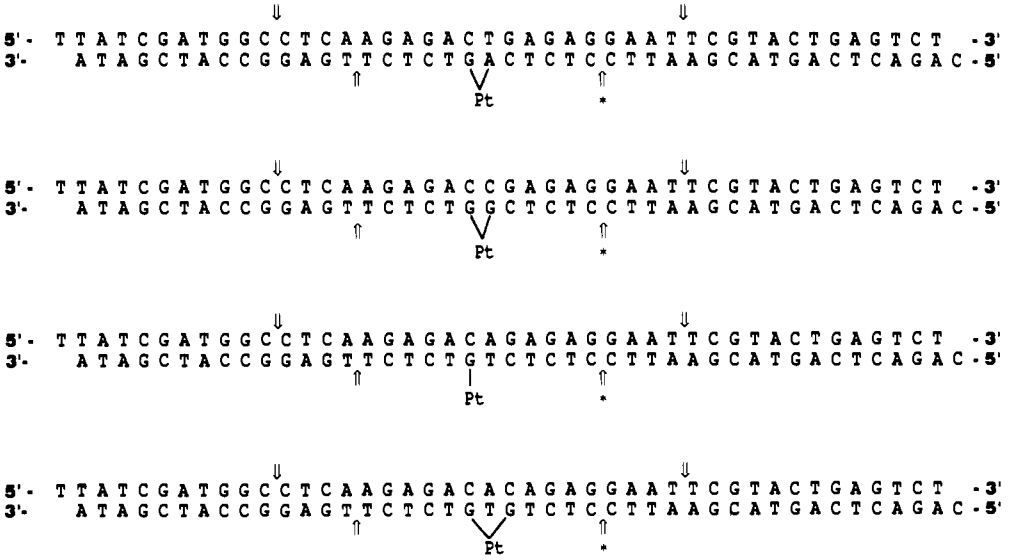

FIGURE 3: Synthetic substrates for ABC excinuclease. The arrows denote the sites of ligation of the individual oligomers used to construct the substrate. The site of platination of the central 12-mer is indicated. This 12-mer was ^{32}P 5′ end labeled at the position shown with an asterisk, platinated, and purified to homogeneity. It was then mixed with the other oligomers, annealed, and ligated as described under Experimental Procedures.

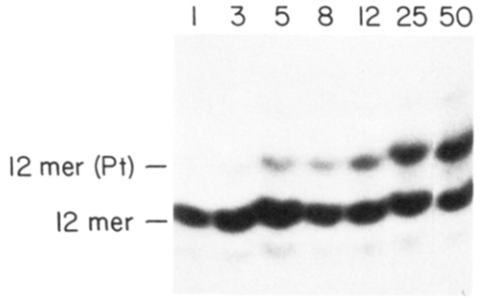

FIGURE 4: Platination of central 12-mer. The [GG]Pt 12-mer shown in Figure 3 was ^{32}P 5′ end labeled and incubated with $PtCl_2$(dach) at 37 °C as described under Experimental Procedures. Aliquots of the reaction were removed after 1, 3, 5, 8, 12, 25, and 50 h of incubation and separated on a 20% polyacrylamide sequencing gel.

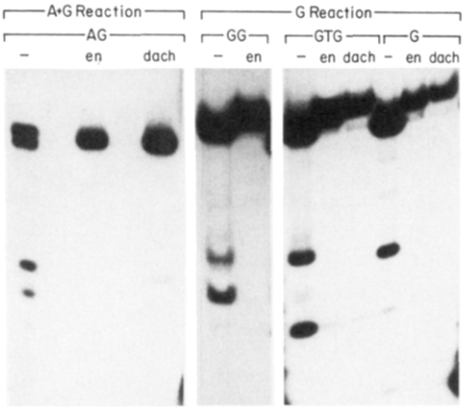

FIGURE 5: Partial sequencing of platinated 12-mers. The 12-mers shown in Figure 3 were reacted with either $PtCl_2$(dach) or $PtCl_2$(en) and the platinated 12-mers purified as described under Experimental Procedures. Following purification both the platinated and unplatinated 12-mers were subjected to either the G or A+G sequencing reactions (Maxam & Gilbert, 1977).

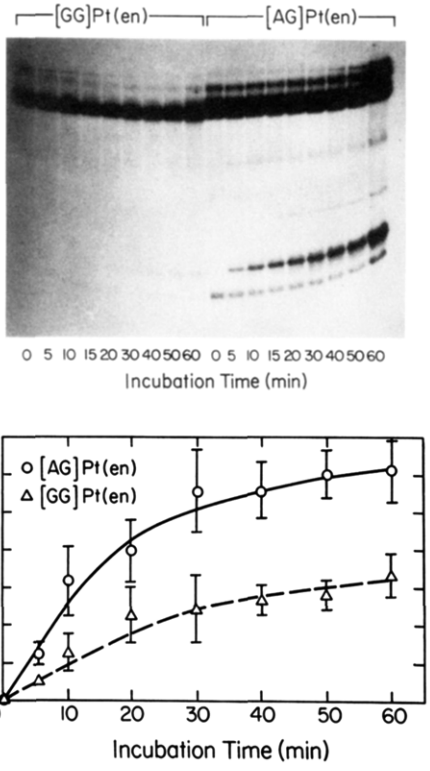

FIGURE 6: Excision of the [AG]Pt(en) and [GG]Pt(en) substrates by ABC excinuclease. The [AG]Pt(en) and [GG]Pt(en) substrates were digested with ABC excinuclease and run on a 20% sequencing gel as described under Experimental Procedures. For the ABC excinuclease digestion 0.5–1.0 pmol of oligomer was incubated with 10 pmol of UvrA, 12.5 pmol of UvrB, and 7.5 pmol of UvrC. (Top) Autoradiograph of a single experiment. (Bottom) Summary of five excision experiments with three different preparations of these substrates. The error bars indicate the standard error of the mean.

as a control. Figures 6 and 7 show the resulting autoradiograms from two such experiments. The top portion of Figure 6 shows the excision of the [AG]Pt(en) diadduct vs the excision of the [GG]Pt(en) diadduct. The autoradiogram shows a clear time-dependent appearance of the platinated 12-mer band (second band from the bottom), and it is clear that the [AG]Pt(en) adduct was cut more efficiently than the [GG]-Pt(en) adduct. These substrates contained a small amount of contaminating unplatinated 12-mer which appears as the bottom band in this gel. The amount of unplatinated 12-mer remained constant from 0 to 50 min and was not included in estimates of percent cutting. Subsequent preparations of the substrate were run on a second preparative denaturing gel and did not show this contamination. These reactions also exhibit a characteristic which has been reported previously for incision by ABC excinuclease, that is, some of the adducted strands were only partially cut by the enzyme, leaving longer fragments which contained the internal label. This uncoupled incision was seen predominantly with the [GG]Pt and [AG]Pt substrates, but was not observed with either the [GNG]Pt or [G]Pt substrates. These partially excised adducts were included in the estimates of percent cutting. The bottom portion of Figure 6 shows the results of five ABC excinuclease digestion experiments with three different preparations of these two adducts. Control experiments with unplatinated substrate showed no excision by ABC excinuclease (data not shown).

Figure 7 shows a comparison of the excision of the [G]-Pt(en) and [G]Pt(dach) substrates. A time-dependent removal of both platinated monoadducts is seen with a clear preference for [G]Pt(dach) compared to [G]Pt(en). This autoradiogram shows no contaminating band of unplatinated 12-mer in the unreacted sample. However, small amounts of two faster migrating bands accumulate with time. The band with the greatest mobility probably represents unplatinated 12-mer resulting from the displacement of platinum from the monoadducted 12-mer by the 10 mM dithiothreitol in the assay buffer. A time-dependent increase in unplatinated 12-mer was seen only with the monoadduct substrates. In the case of the [G]Pt(dach) adduct, a faint band of slightly greater mobility appeared below the excised platinated 12-mer. This is characteristic of all the Pt(dach) substrates and probably results from the fact that the $PtCl_2$(dach) used for these experiments contained both the trans-*d* and trans-*l* isomeric forms (Inagaki & Kidani, 1986). Because they increased with time of incubation, both faster moving bands were included in the estimate of percent excision. [AG]Pt(en) was included as a control. The results from four experiments with two different substrate preparations are shown graphically in the bottom portion of Figure 7.

The ABC excinuclease excision experiments were carried out with three different preparations of substrate. There was some difference in the extent of excision from one preparation to the next, probably because of unavoidable differences in substrate concentration. Within each preparation of substrates, the substrate concentration was controlled by kinasing all 12-mers with [γ-^{32}P]ATP of the same specific activity and using the same counts per minute of substrate in each excision assay. There was also some day to day variation in ABC excinuclease activity, but this was controlled by including the [AG]Pt(en) substrate in each cutting experiment. With substrate concentration and ABC excinuclease activity con-

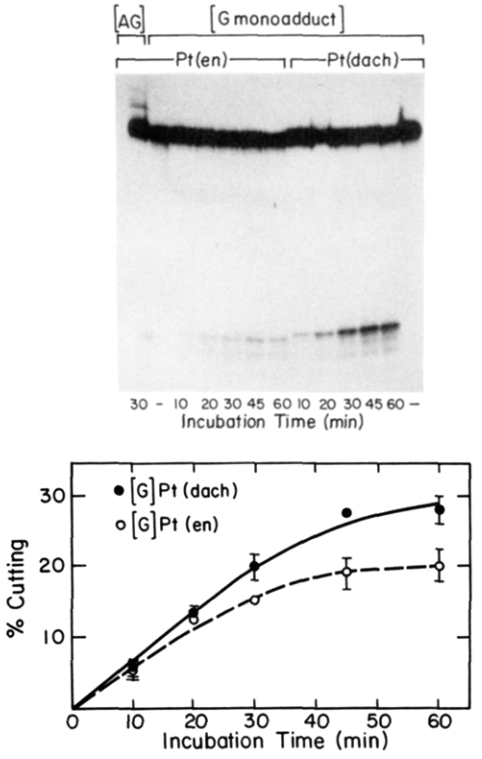

FIGURE 7: Excision of the [G]Pt(en) and [G]Pt(dach) substrates by ABC excinuclease. The conditions were the same as Figure 6 except that the [G]Pt(en) and [G]Pt(dach) substrates were used. The [AG]Pt(en) subsatrate was included as an internal control to adjust for day to day variations in ABC excinuclease activity. (Top) Autoradiogram from a single experiment. (Bottom) Summary of four experiments with two different preparations of substrates.

Table I: Substrate Preference of ABC Excinuclease

substrate	extent of excision[a] (%)	initial rate of excision[a] (%/min)
[GG]Pt(en)	6.5 ± 3.4 (n = 5)	0.20 ± 0.14 (n = 4)
[GG]Pt(dach)	6.9 ± 4.7 (n = 5)	0.14 ± 0.03 (n = 4)
[AG]Pt(en)	12.2 ± 3.1 (n = 10)	0.70 ± 0.05 (n = 6)
[AG]Pt(dach)	9.7 ± 3.1 (n = 5)	0.17 ± 0.05 (n = 4)
[G]Pt(en)	22.3 ± 4.1 (n = 4)	0.6 ± 0.1 (n = 3)
[G]Pt(dach)	28.0 ± 2.1 (n = 4)	0.6 ± 0.2 (n = 3)
[G]Pt(dien)	19.1 ± 5.0 (n = 5)	0.7 ± 0.2 (n = 3)
[GNG]Pt(en)	25.7 ± 3.0 (n = 3)	1.3 ± 0.2 (n = 3)
[GNG]Pt(dach)	33.8 ± 2.2 (n = 3)	1.9 ± 0.2 (n = 3)

	analysis of variance[b]	
comparisons	extent of excision	initial rate of excision
[AG] vs [GG]	$P < 0.02$	NS
[GNG] vs [GG]	$P < 0.001$	$P < 0.001$
[G] vs [GG]	$P < 0.001$	$P < 0.005$
[GNG] vs [AG]	$P < 0.001$	$P < 0.001$
[G] vs [AG]	$P < 0.001$	NS
[G] vs [GNG]	NS	$P < 0.001$
[GG], (dach) vs (en)	NS	NS
[AG], (dach) vs (en)	NS	$P < 0.02$
[GNG], (dach) vs (en)	$P < 0.02$	$P < 0.05$
[G], (dach) vs (en)	$P = 0.05$	NS
[G], (dien) vs (en)	NS	NS
[G], (dien) vs (dach)	$P < 0.005$	NS

[a] The extent and initial rate of excision for each of the substrates by ABC excinuclease were determined from summary figures such as those shown at the bottom of Figures 6 and 7. Each value is reported plus or minus the standard deviation. The number of experiments is shown in parentheses. [b] For statistical analysis the data from all of the individual experiments were paired either according to adduct type or according to the carrier ligand for a particular adduct type. Analysis of variance was carried out with the SYSTAT program. NS = not statistically different in the two data sets. For all other data sets the probability that the two data sets could be the same is indicated.

trolled in this manner, the pattern of excision between substrates was very reproducible. The results of all the repair experiments are summarized in Table I along with the statistical analysis of the data.

There were significant differences seen in both the extent and rate of excision for the different types of adducts. The GG diadducts were removed with the lowest efficiency of the four types of adducts studied. Only about 7% of these diadducts were excised from these substrates. The AG diadducts were the next in excision efficiency with 10–12% of this adduct being excised. The G monoadducts and GNG diadducts were excised with 20–30% efficiency, with no statistically significant difference between these two types of adducts. There were also similar differences in the initial rate of cutting for these adducts. The [G]Pt(dach) and [G]Pt(en) monoadducts used in these experiments were blocked with dithiothreitol to stabilize the adducts during the subsequent ligation and purification steps. In vivo, however, the excision repair system has to deal with a mixture of unblocked monoadducts, monoadducts blocked with glutathione, and monoadducts that have been cross-linked to protein sulfhydryl. To rule out the possibility that the bulky dithiothreitol group was creating an artificially good substrate for ABC excinuclease, the [G]Pt monoadduct was also prepared with the dien ligand, which more closely resembles the unblocked monoadduct in structure. The [G]Pt(dien) adduct was almost as good a substrate as the other two monoadducts treated, suggesting that the ABC excinuclease was recognizing the [G]Pt monoadduct rather than the bulky dithiothreitol group.

With respect to the effect of carrier ligands, no difference in either extent or rate of excision was seen between dach and en for the GG diadduct. The AG diadduct was excised significantly faster with the en carrier ligand compared to the dach carrier ligand, but the extent of excision between these two was not statistically different. The [G]Pt(dach) adduct had a significantly greater extent of excision than either [G]Pt(en) or [G]Pt(dien), but was not excised at a rate different from those of the other two. Finally, the [GNG]Pt(dach) adduct was excised both faster and to a greater extent than the [GNG]Pt(en) adduct.

Effect of Carrier Ligand on Monoadduct to Diadduct Conversion. Previous studies (Eastman, 1983; data not shown) have shown that under the appropriate conditions thiourea quantitatively reacts with monoadducts without displacing platinum from the DNA. Thus, incorporation [^{14}C]thiourea into DNA under controlled conditions (10 mM thiourea, 10 min, 37 °C) was used to quantitate platinum–DNA monoadducts. In these experiments salmon sperm DNA was treated with either $PtCl_2$(dach), $PtCl_2$(en), or *cis*-DDP for 30 min at 37 °C with drug concentrations designed to produce r_b's of 1×10^{-2}. Following a 30-min incubation, monoadduct to diadduct conversion was blocked by addition of 0.5 M NaCl and the platinum-adducted DNA isolated as described under Experimental Procedures. The total platinum adducts in the DNA were quantitated by flameless atomic absorption. The purified DNA was incubated in 10 mM $NaClO_4$ at 37 °C, and the monoadducts present at various times were quantitated by [^{14}C]thiourea incorporation. The percent monoadduct calculated in this manner was similar to previous estimates (Eastman, 1986). The resulting data are shown in Figure 8A. The $t_{1/2}$ for monoadduct to diadduct conversion was 264 min for dach–platinum adducts compared to only 110 min for en–platinum adducts and 144 min for diammine–platinum adducts. Since a slower rate of monoadduct to diadduct conversion could result from a slower rate of chloride ligand

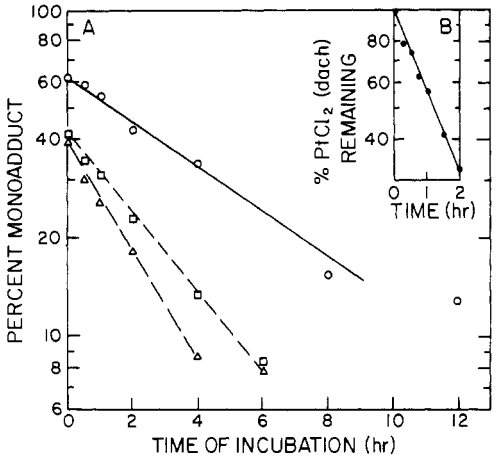

FIGURE 8: Effect of carrier ligand on the rate of hydrolysis and monoadduct to diadduct conversion. (A) Salmon sperm DNA was prepared with dach-, en-, or *cis*-DDP-platinum adducts under conditions designed to maximize platinum monoadduct retention. Total platinum adducts were measured by atomic absorption (r_b = 1.6 × 10^{-2} for $PtCl_2$(dach)-treated DNA, 1.4 × 10^{-2} for $PtCl_2$(en)-treated DNA, and 1.3 × 10^{-2} for *cis*-DDP-treated DNA). After various times of incubation in 10 mM $NaClO_4$ at 37 °C, the r_b for platinum monoadducts was determined by [^{14}C]thiourea incorporation and percent monoadduct calculated as r_b monoadduct/r_b total platinum. The complete protocol is described under Experimental Procedures. (○) $PtCl_2$(dach)-treated DNA; (△) $PtCl_2$(en)-treated DNA; (□) *cis*-DDP-treated DNA. (B) ^{3}H-Labeled $PtCl_2$(dach) in 150 mM NaCl was diluted 1:450 into 10 mM $NaClO_4$ and incubated at 37 °C. At the indicated times aliquots were removed, and the amount of $PtCl_2$(dach) was determined by isocratic HPLC with 5 mM heptanesulfonate, pH 3.4, as described previously (Mauldin et al., 1987).

hydrolysis, the hydrolysis of $PtCl_2$(dach) in 10 mM $NaClO_4$ at 37 °C was also determined (Figure 8B). The rate of hydrolysis of $PtCl_2$(dach) (k = 1.5 × 10^{-4} s^{-1}; $t_{1/2}$ = 75 min) was virtually identical with that previously reported for $PtCl_2$(en) hydrolysis when the temperature dependence of the reaction is taken into account (Coley & Martin, 1973).

Effect of Carrier Ligand on Lethality of Platinum Adducts. pBR322 DNA was treated with either $PtCl_2$(dach) or $PtCl_2$(en) to give between 2 and 14 adducts/plasmid. This plasmid DNA was then incubated for an additional 30 h at 37 °C to maximize diadduct formation (see Experimental Procedures). These platinum-treated plasmids were tested in the repair-deficient *E. coli* strain CSR603 (*recA*$^-$ *uvrA*$^-$) to determine the effect of carrier ligand on platinum adduct inactivation of pBR322 transforming efficiency. This experiment was repeated with three different preparations of dach- and en-platinated pBR322. With different preparations of platinated DNA, the difference between dach-platinum adducts and en-platinum adducts on pBR322 inactivation ranged from 2- to 5-fold. However, in every experiment the dach-platinum adducts were significantly more effective at inhibiting pBR322 transformation than en-platinum adducts (data not shown). Control experiments suggest that these differences were not due to an effect of carrier ligand on uptake of platinated plasmid. [^{3}H]Thymidine-labeled pBR322 was treated with either $PtCl_2$(dach) or $PtCl_2$(en) to give 12 or 14 adducts/plasmid, respectively (measured by atomic absorption). Competent CSR603 cells were transformed with the platinated pBR322 preparations as described previously (Husain et al., 1985). Following the expression step, the cell suspensions were treated with 50 μg/mL DNase I for 30 min at 37 °C and washed twice with Hank's balanced salt solution. Under these conditions uptake of plasmid was 0.094 ± 0.008% for both $PtCl_2$(dach)- and $PtCl_2$(en)-treated plasmid. While such control experiments must be considered tentative because of possible contamination by membrane-bound plasmid, we consider it highly unlikely that the carrier ligand would effect uptake of plasmid at a maximum r_b of 3 × 10^{-3} or less.

Since pBR322 transformation requires replication and the recipient strain was repair deficient, it is likely that the dach- and en-platinum adducts had differential effects on DNA replication. These data suggest that while the carrier ligand may have relatively little effect on the type of platinum adducts formed or the repair of the most frequent platinum adducts, it could have a very dramatic effect on processes such as replication or transcription which are essential for cell survival.

Discussion

ABC excinuclease has been used previously to map the sites of platinum adducts (Beck et al., 1985). In this study the reactivity of ABC excinuclease toward the different types of platinum–DNA adducts was determined to allow a more precise quantitation of the cutting pattern. The data obtained with the (*Hae*III–*Hin*dIII)$_{146}$ fragment were essentially consistent with previous mapping studies (Royer-Pokora et al., 1981; Tullius & Lippard, 1981; Pinto & Lippard, 1985a). The GG intrastrand diadducts were much more prevalent than AG or GNG intrastrand diadducts, with the G_3 sequence being a particular hot spot for platinum adduct formation. The minor adducts present at positions 48 and 63 (Figure 2) could be either G monoadducts or AG interstrand diadducts. They are most likely G monoadducts since AG interstrand diadducts have not been previously reported. However, it is not clear that previous experiments have been sensitive enough to detect low levels of AG interstrand diadducts. As reported by Hemminki and Thilly (1985), there is an obvious effect of surrounding bases on platinum adduct formation since the GG adduct at positions 59 and 60 is more than twice as abundant as the one at positions 51 and 52.

Previous studies have demonstrated the usefulness of synthetic oligonucleotides containing single defined adducts for studying the specificity of repair enzymes such as ABC excinuclease (Van Houten et al., 1986) and DNA photolyase (Husain et al., 1987). Our data clearly show that ABC excinuclease recognizes platinum adducts in the order GNG ≥ G > AG > GG. While we do not yet know whether the same specificity will exist for the eukaryotic repair system, these data do provide some insight into how the ABC excision repair system recognizes platinum–DNA adducts. The common theme for the recognition of bulky DNA adducts by ABC excinuclease is thought to be the bending of the DNA helix caused by these agents (Sancar & Sancar, 1988). The differences in the recognition of the GNG and GG adducts are certainly consistent with this view of ABC excinuclease specificity. NMR data and energy minimization considerations suggest that platinum–GNG adducts are more flexible than platinum–GG adducts but result in increased distortion of the DNA helix (Reedijk et al., 1983). Differences in recognition of the AG and GG adducts might be more difficult to explain by helix distortion alone. However, the efficient recognition of G monoadducts by ABC excinuclease is least consistent with a model relying solely on helix distortion for recognition, since platinum monoadducts appear to cause much less distortion of DNA than diadducts (Macquet & Butour, 1978). However, these data are consistent with a recent study showing that ABC excinuclease efficiently excises the O^6-methylguanine adduct (Voigt et al., 1989) even though that adduct causes much less distortion of the DNA helix than either thymine dimers (Husain et al., 1988) or platinum–GG adducts (Rice et al., 1988). These data indicate that factors other than DNA

bending can affect recognition of adducts by ABC excision nuclease.

In a biological sense these data demonstrate that the most prevalent adduct both in vitro (Fichtinger-Schepman et al., 1985; Eastman, 1986) and in vivo (Fichtinger-Schepman et al., 1987) is the one that is repaired least well by ABC excinuclease. While the specificity of the eukaryotic excision repair system is not known, these data would appear to support recent proposals that the *cis*-DDP–GG adduct is not efficiently repaired in eukaryotic cells (Ciccarelli et al., 1985; Sherman et al., 1985). These data are also consistent with the toxicity and persistence of GG diadducts in vivo (Poirier et al., 1985; Terheggen et al., 1987). Similarly, the efficiency with which ABC excinuclease repairs G monoadducts is consistent with the low toxicity of the monoadducts (Alazard et al., 1982; Pinto & Lippard, 1985b) and the report by Reedijk and Lohman (1985) that platinum monoadducts are repaired very quickly in mammalian cells. The more rapid repair of platinum monoadducts would appear to be particularly significant in light of our data showing that dach–platinum monoadducts are more persistent than either en–platinum or diammine–platinum monoadducts (Figure 8A). Since the rates of $PtCl_2$(dach) and $PtCl_2$(en) hydrolysis are almost identical under these reaction conditions, our data suggest that the slower rate of monoadduct to diadduct conversion is probably due to constraints placed on the G–Pt–G bond angle by the diaminocyclohexane carrier ligand.

One purpose of these experiments was to determine if the diaminocyclohexane (dach) and ethylenediamine (en) carrier ligands had any significant effect on platinum adduct formation, repair, or lethality. Although the data presented here are limited to a single 146 base pair fragment, no significant differences were observed in the sites or frequency of formation for dach–platinum or en–platinum adducts, at least with respect to the two most abundant adducts (GG and AG). Similar results have recently been reported for dach– and *cis*-diammine–platinum adducts (Jennerwein et al., 1989). Our data do not exclude possible differences in the formation of GNG or GG interstrand adducts, but those are both minor adducts in vivo.

Similarly, the dach and en carrier ligands did not have any significant effect on the repair of the two most abundant platinum adducts by ABC excinuclease. Obviously, any effects of the carrier ligand on platinum adduct conformation must be minimized by the constraints placed on the platinum–GG and –AG adducts by the DNA helix. It is interesting to note that the effects of the dach carrier ligand on the more flexible GNG adduct are much more pronounced. However, the effect of carrier ligand on repair of GNG adducts may not be particularly significant in vivo because such adducts are rare and do not appear to be any more lethal than GG or AG adducts.

The differential effect of the dach and en carrier ligand on the repair of G monoadducts in vitro and the persistence of dach–platinum monoadducts suggest several mechanisms that might explain the L1210/DACH phenotype. For example, if the specificity of the eukaryotic excision repair enzyme were similar to that of *E. coli* ABC excinuclease, an enhanced activity of the repair enzyme should lead to a selective removal of dach–platinum adducts, since dach–platinum monoadducts would be removed preferentially. Of course, in experiments with relatively long labeling times this rapid phase of DNA repair would be missed and would be seen as reduced platinum incorporation into DNA. Similarly, platinum monoadducts can readily be quenched by reaction with glutathione (Eastman, 1987) or protein sulfhydryls (Zwelling, 1979). The resulting stable monoadducts or DNA–protein cross-links are likely to be less cytotoxic than platinum diadducts (Eastman, 1987; Zwelling, 1979). Thus, elevations of either glutathione or protein sulfhydryl could lead to selective resistance to dach–platinum compounds. Of course, none of these mechanisms offer a readily apparent explanation of the L1210/DDP phenotype.

The transformation data in repair-deficient *E. coli* cells suggest one other possible model for carrier ligand specific resistance. Those data strongly suggest that the nature of the carrier ligand can affect the lethality of platinum–DNA adducts in ways that have nothing to do with repair. Thus, the carrier ligand specificity in the resistant cell lines described to date could well be independent of the DNA repair capacities of those cell lines, but might instead relate to the effects of carrier ligand on the ability of platinum adducts to inhibit replication, transcription, or cell cycle progression. Sheibani and Eastman (1989) have shown that the presence of a single platinum adduct within a transcription unit is sufficient to block transcription and have postulated that the inhibition of transcription may be a critical step in determining platinum adduct lethality. However, the effect of carrier ligand on inhibition of transcription has not been investigated to date.

In summary, we have shown that the dach and en carrier ligands do not significantly affect the type or sites of platinum adduct formation and have little effect on the repair of the most abundant platinum adducts by *E. coli* ABC excinuclease. Our data have suggested two possible models to explain carrier ligand specific resistance: effects of carrier ligand on the persistence and/or repair of platinum monoadducts or on the lethality of platinum adducts. Our laboratory is currently investigating these possibilities further as well as studying the mechanism for recognition of platinum monoadducts by ABC excision nuclease.

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Registry No. *cis*-DDP, 15663-27-1; $PtCl_2$(dach), 38780-40-4; $PtCl_2$(en), 14096-51-6.

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