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Characterization of in Vitro Deoxyribonucleic Acid Breakage and Cross-Linking Induced by Bis(isopropylamine)-*trans*-dihydroxy-*cis*-dichloroplatinum(IV)†

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ABSTRACT: Bis(isopropylamine)-*trans*-dihydroxy-*cis*-dichloroplatinum(IV) (CHIP or JM-9), a derivative of Cisplatin, was found to have DNA breakage and interstrand cross-linking activities in vitro. DNA breakage was detected by alkaline and neutral sucrose gradient analysis, agarose gel electrophoresis, and alkaline ethidium bromide fluorescence assay employing covalently closed circular PM2 DNA. DNA cross-linking activity was detected by alkaline sucrose gradient analysis and by the "snap-back" assay employing PM2 DNAs. Non-sulfhydryl-containing reducing agents, e.g., $NaBH_4$ and NADPH, stimulated both cross-linking and breakage activities. Alkaline buffers, cyanide, or sulfhydryl group containing agents inhibited both types of activities. The hydroxyl free radical scavenger sodium benzoate (100 mM) was found to inhibit 99% and 25% of DNA breakage and cross-linking activities, respectively, suggesting DNA breakage and cross-linking may be independently mediated.

Platinum coordination complexes have been shown to be active antitumor agents in many in vitro and in vivo systems (Rosenberg et al., 1969; Prestayko et al., 1979; Cleare et al., 1978). The mechanism of cytotoxicity of platinum complexes has been attributed to their interaction with tumor cell DNA, since it was found that the compound *cis*-diamminedichloroplatinum(II) (CDDP)[1] can bind to cellular DNA and inhibit cellular DNA biosynthesis (Rosenberg et al., 1969; Roberts & Thomson, 1979). Using isolated purified covalently closed circular (CCC) DNA, Cohen et al. (1979) and this laboratory reported that CDDP induced tertiary conformational changes in CCC DNA (Mong et al., 1980a). CDDP apparently binds to the CCC DNA in vitro causing intrastrand cross-linking and results in an unwinding and then subsequent rewinding-like phenomenon as evidenced by the reversal of the DNA viscosity and the gel electrophoretic mobility (Macquet & Butour, 1978; Mong et al., 1980a,b, 1981). DNA interstrand cross-linking induced by CDDP was also detected (Horaček & Drobnik, 1971; Macquet & Theophonides, 1975; Pascoe & Roberts, 1974a,b). It was concluded, however, that CDDP does not induce DNA breakage and that DNA intrastrand cross-linking is probably the predominant mode of DNA binding in vitro (Mong et al., 1980a; Kleinwachter, 1978; Roberts & Thomson, 1979).

When the so-called "second generation platinum analogues" were examined, several octahedral platinum(IV) compounds were shown to induce DNA breakage (Mong et al., 1980b) and DNA interstrand cross-linking in vitro (Mong et al., 1981) instead of intrastrand cross-linking, DNA shortening, unwinding, and rewinding as did CDDP and other platinum(II) analogues. Since DNA degradation has been considered an important mechanism of action of many antineoplastic agents and CDDP has *not* been shown to induce DNA breakage in vitro or in vivo, platinum(IV) compounds may represent a distinct class of platinum drugs potentially having a different mechanism of action from CDDP. The purposes of this study were to characterize the mechanism(s) of DNA breakage and cross-linking induced by CHIP and to determine whether the primary types of damage induced by CHIP are different from those induced by CDDP, i.e., DNA shortening, unwinding, and rewinding. Using several highly sensitive assay systems tailored for each type of DNA interaction, we have also investigated the conditions and cofactors that either stimulated or inhibited the DNA breakage and cross-linking effects. The results suggest that the mechanism(s) of action of CHIP is

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[1] Abbreviations: EB, ethidium bromide; CDDP, *cis*-diamminedichloroplatinum(II) or Cisplatin; CHIP or JM-9, bis(isopropylamine)-*trans*-dihydroxy-*cis*-dichloroplatinum(IV); JM-28, bis(isopropylamine)-*trans*-(dihydroxymalonato)platinum(IV); Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; 2ME, 2-mercaptoethanol; CCC PM2 or form I PM2 DNA, covalently closed circular supercoiled PM2 DNA; form II, single-strand broken circular form PM2 DNA; form III, double-strand broken linear duplex form PM2 DNA; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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(are) clearly different from those of CDDP and that DNA interstrand cross-linking and breakage are probably the major types of damage observed in vitro.

Materials and Methods

Materials

Ethidium bromide (EB) (2,7-diamino-10-ethyl-*O*-phenylphenanthridinium bromide) was purchased from Sigma Chemical Co. (St. Louis, MO). S1 nuclease (from *Aspergillus oryzae*) and agarose–ME were purchased from Miles Laboratories (Elkhart, IN). Tricine buffer [*N*-tris(hydroxymethyl)methylglycine], Mops buffer [3-(*N*-morpholino)-propanesulfonic acid], and CHES buffer [2-(cyclohexylamino)ethanesulfonic acid] were purchased from Boehringer Mannheim Co. (Indianapolis, IN). *cis*-Diamminedichloroplatinum(II) (Cisplatin) and *cis*-bis(isopropylamine)-*trans*-dihydroxy-*cis*-dichloroplatinum(IV) (JM-9) were provided by the Johnson and Matthey Co. (Sonning Common, Reading, England). Both the reduced and oxidized forms of NAD and NADP (nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate) were obtained from Calbiochem Co. (La Jolla, CA).

Methods

PM2 DNA Isolation and Purification. Bacteriophage PM2 was isolated and purified as described by Salditt et al. (1972). It was lysed with 0.5% Sarkosyl NL927 (60 °C, 10 min). DNA was extracted with equal volumes of chloroform–isoamyl alcohol (24:1) at 4 °C and precipitated with 2 volumes of ethanol. CCC PM2 DNA (form I) was purified by cesium chloride–ethidium bromide isopycnic gradients (Strong & Crooke, 1978). After EB was removed with NaCl-saturated 2-propanol, DNA was extensively dialyzed against 0.15 M NaCl or 0.05 M $NaNO_3$ before use. Each batch of PM2 DNA was analyzed by gel electrophoresis; only those batches containing greater than 90% CCC PM2 DNA were used in the assays. The nicked relaxed form of DNA (form II) was obtained from the upper bands of the EB–CsCl gradients and a subsequent preparative neutral sucrose gradient purification or from S1 nuclease relaxation and purification.

pBR322 DNA Isolation and Purification. pBR322 DNA was isolated from *Escherichia coli* RR1 as described previously (Clewell & Helinski, 1970; Bolivar et al., 1977).

Alkaline Fluorescence Assay. The alkaline fluorescence assay was based on the fact that DNA breakage renders the CCC PM2 DNA alkali denaturable and thus unable to bind the intercalative dye EB. The DNA–CHIP reaction was performed in a reaction mixture of 1.1 mL containing PM2 DNA, CHIP, 2 mM EDTA, 12.5 mM of various buffers, and 100 or 150 mM NaCl. After incubation at various time points, 60 μL of the reaction mixture was withdrawn and mixed with 0.9 mL of the denaturation buffer (40 mM Na_3PO_4, 100 mM NaCl, and 10 mM EDTA, pH 12.1, adjusted with 0.15 M NaOH). Following denaturation, 0.1 mL of EB solution (22 μg/mL in denaturation buffer) was added. Fluorescence determination and data calculation were described previously (Mong et al., 1980a,b). Under the conditions employed, no significant decrease of fluorescence or DNA breakage was detected by $NaBH_4$, 2-mercaptoethanol, dithioerythritol, sodium benzoate, and bovine serum albumin.

Agarose Gel Electrophoresis. The products of drug–PM2 DNA interactions were electrophoretically separated on 1% agarose gels under nondenaturing conditions. The reactions were slowed or terminated by adding 100 μL of bromophenol blue (0.1% w/v) in 75% glycerol–50 mM CHES buffer (pH 9.5) to 30 μL of the reaction mixture and chilling on an ice bath. Approximately 1 μg of DNAs in the resultant mixtures was electrophoretically separated on 1% agarose gels in a horizontal slab gel apparatus (Aquebogue Machine Shop, Aquebogue, NY). The gel electrophoresis and staining conditions were described previously (Mong et al., 1980a,b).

Alkaline and Neutral Sucrose Gradient Separation of PM2 DNA Conformational Isomers. The reaction mixture containing PM2 DNA and drug was applied directly to a linear 5–20% alkaline sucrose gradient (0.3 M NaOH, 0.7 M NaCl, and 1 mM EDTA) and centrifuged for 7.5 h at 23 000 rpm with a SW27 rotor (Beckman Instruments, St. Louis, MO). The drug–DNA mixture was also sedimented on 5–20% linear neutral sucrose gradients (0.7 M NaCl, 2 mM EDTA, and 50 mM Tris, pH 7.5) for 16 h at 23 000 rpm at 4 °C. The gradients were fractionated with an ISCO density gradient fractionator, and the absorbance profiles at 254 nm were recorded. The DNAs used for DNA cross-linking assay and gel electrophoresis were obtained from fractions 4 and 7 of the alkaline gradients. They were isolated, pooled, and neutralized to pH 7.5 by adding concentrated phosphoric acid at 4 °C and then concentrated by reversed dialysis with poly(ethylene glycol) 6000. The neutralized, concentrated fractions were dialyzed against Mes buffer (25 mM Mops, pH 7.5, 100 mM NaCl, and 2 mM EDTA) before being used for gel electrophoresis and/or DNA interstrand cross-linking assay.

Ethidium Bromide Fluorescence Assay for DNA Interstrand Cross-Linking. EB fluorescence has been used extensively for detecting DNA interstrand cross-linking (Lown et al., 1976, 1978; Lown & Weir, 1978; Morgan et al., 1979; Mong et al., 1980a,b). Briefly, after form II or III PM2 DNA was reacted with CHIP, 50-μL aliquots of reaction mixture were mixed with 950 μL of 50 mM sodium phosphate buffer (pH 11.7) containing 0.5 μg/mL EB and 0.4 mM EDTA, and the fluorescence was recorded (preheated fluorescence). The solutions were then heated to 96 °C for 2–3 min and rapidly chilled to 0 °C by immersion into an ice bath, and the fluorescence was recorded after the solutions reached room temperature (postheating fluorescence). Under these conditions, the DNA interstrand cross-links provide nucleation points and renature the DNA upon rapid cooling, resulting in a recovery of EB-induced fluorescence. Form II or III DNA without cross-links will not renature under these conditions and result in a loss of fluorescence. So that possible interference with the assay due to extensive DNA breakage could be avoided, only relatively low concentrations of drugs were employed, and the initial rates of DNA cross-linking were estimated.

Cross-links in form I PM2 DNA can also be detected after the drug-bound DNA is nicked by S1 nuclease. In this case, the drug-bound form I PM2 DNA was precipitated from the reaction mixture by adding 2 volumes of ethanol at −20 °C and then redissolved in S1 buffer (33 mM sodium acetate, pH 5, 100 mM NaCl, and 0.5 mM $ZnCl_2$) and nicked with 2×10^3 units of S1 nuclease at 37 °C for 60 min. After the reaction was stopped by adding 10× TNE buffer (500 mM Tricine, pH 7.5, 500 mM NaCl, and 150 mM EDTA) to $^1/_{10}$ of the total volume, the nicked PM2 DNA was extracted with phenol (TNE saturated) and precipitated with ethanol. The DNA samples were then dissolved in TNE buffer and aliquoted in pH 11.7 phosphate buffer to assay for the cross-linking effect.

High-Pressure Liquid Chromatography Purification of CHIP. The highly purified CHIP was obtained by preparative high-pressure liquid chromatography with a 25 cm × 10 mm (internal diameter) stainless steel μBondapak NH_2 column

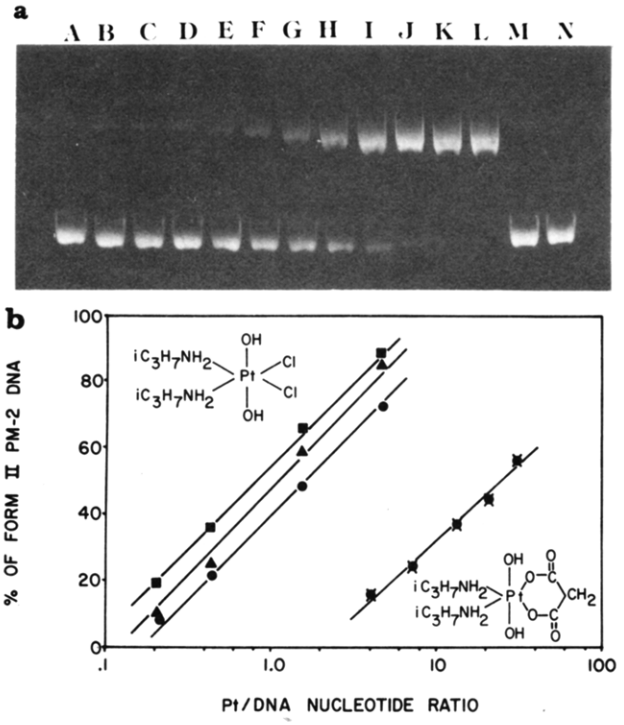

FIGURE 1: (a) PM2 DNA breakage by CHIP. CCC PM2 DNA (>90%) was incubated with CHIP in the presence of 15 mm $NaNO_3$, 25 mM Tricine (pH 7.1), and 100 mM NaCl at 37 °C for 9 h; the PM2 DNA conformation isomers were then separated by gel electrophoresis as described under Materials and Methods. The concentrations of CHIP were expressed in both Pt/DNA nucleotide ratio and molarity: (A) 0 (control), (B) 0.03 (18.2 μM), (C) 0.08 (48.5 μM), (D) 0.15 (91 μM), (E) 0.18 (170 μM), (F) 0.43 (212 μM), (G) 0.8 (394 μM), (H) 1.2 (591 μM), (I) 2.0 (985 μM), (J) 3.2 (1.57 mM), (K) 4.5 (2.2 mM), (L) 5.4 (2.66 mM), and (M and N) 0 (control). (b) CCC PM2 DNA was incubated with CHIP (■) (structure shown in the upper corner) as described in (a). The incubation mixtures were heated to 95 °C for 8 min and then added with PM2 DNA (▲), or 5 mM EDTA (●) was maintained for the entire 9 h of incubation. A crossed closed circle indicates the result of another Pt(IV) compound JM-28. The amount of form II PM2 DNA was calculated by quantitative densitometric scanning of the EB (0.5 μg/mL)-stained agarose gel. Form II PM2 DNA stained 1.4 times better than form I PM2 DNA (Lloyd et al., 1978) and thus corrected (see text).

(No. 84155, Waters Associates). An acetonitrile–H_2O mixture was used as the solvent system. Separation of the minor contaminant and CHIP was achieved by using an 11 to 88% (H_2O) linear gradient at a flow rate of 10 mL/min. Eluates were detected at 254-nm absorption with a Model 440 detector.

Results

DNA Breakage by CHIP. The systems used in this study are based on the superhelicity of PM2 DNA and on the marked conformational changes induced by single- and double-strand breaks as detected by agarose gel electrophoresis. Also, DNA breakage resulted in strand separation and loss of EB-induced fluorescence in pH 12.1 alkaline denaturation buffer (Strong & Crooke, 1978; Mong et al., 1979) as detected by a decrease of EB-induced fluorescence. Figure 1a demonstrates that when form I PM2 DNA was incubated with CHIP, it was converted into relaxed form II DNA characteristic of single-strand breakage without the intermediate diffuse staining bands characteristic of unwinding and rewinding effects induced by CDDP (Mong et al., 1980a). Previously, it was demonstrated that DNA breakage by CHIP was independent of DNA superhelicity and was not inhibited by 100 mM NaCl (Mong et al., 1980b). Figure 1b shows DNA breakage vs. the Pt/DNA ratio from several experiments. The mass fractions of different forms of DNA species were obtained by quantitative densitometric gel scanning and appropriate correction for differential stainability of form I PM2 DNA (Lloyd et al., 1978). The results in Figure 1b suggest the following: (a) DNA breakage cannot be solely contributed by contaminating DNases since 5 mM EDTA or heating inhibited only 5–15% of the DNA breakage activity induced by the drug; (b) under the conditions used in these experiments, the concentrations of both CHIP and JM-28 required to break 50% of form I DNA molecules were relatively high (600 μM and 1.4 mM) when compared with those of other DNA breakage agents; (c) the DNA breakage induced was primarily single strand since only form II DNA accumulated whereas the double-strand broken linear form III DNA did not accumulate to an appreciable extent (Figure 1a) (Mong et al., 1979). In contradistinction to CDDP, the results in Figure 1, however, do not demonstrate any conformational changes or modifications in PM2 DNA species other than form I PM2 DNA transformation into form II. No DNA tertiary conformational alterations (e.g., DNA shortening, unwinding, and rewinding) were observed in the CHIP-treated form I or form II PM2 DNA when compared with those treated with CDDP (Mong et al., 1980a,b, 1981).

Neutral and Alkaline Sucrose Gradient Analysis. Since many DNA reactive agents introduce alkaline labile damage (e.g., depurination and depyrimidation) as well as DNA interstrand cross-links, neutral and alkaline sucrose gradients were used to investigate whether CHIP produced similar types of damage in DNA. Figure 2a shows the results of the neutral and alkaline sucrose density gradient analysis of CCC PM2 DNA incubated with several concentrations of CHIP. The rapidly sedimenting peaks (alkaline gradient fractions 14–16 in panels A–C and neutral gradient fractions 16 and 17 in panels D–F) represent the CCC PM2 DNA with sedimentation coefficients of 74 and 27 S in alkaline and neutral sucrose gradients, respectively. The more slowly sedimenting peaks represent the denatured single-strand circular and linear PM2 DNA species (fraction 5 in panels A–C with sedimentation coefficients from 24 to 27 S) and form II DNA (fraction 13 in panels D–F with a sedimentation coefficient of 21 S) (Thielmann, 1977). The area under each peak was calculated and plotted against the concentrations of CHIP employed as shown in Figure 2b. These results confirmed the DNA breakage shown in Figure 1 and showed that no significant amount of alkaline labile damage could be detected. The shoulder area in panels B and C of Figure 2a (indicated by the arrow) probably represents the denatured cross-linked form II DNA (see DNA Cross-Linking).

To better understand the mechanism(s) involved in DNA breakage, we have also investigated the various conditions and cofactors that either inhibited or stimulated DNA breakage by CHIP. The alkaline EB fluorescence assay was carried out in 150 mM NaCl, 25 mM Mops buffer (pH 7.1), and 2 mM EDTA. DNA breakage was found to be temperature dependent; a temperature vs. rate of fluorescence decrease plot (Arrhenius plot) indicates that the energy of activation is approximately 8.7 kcal (data not shown). The pH but not the species of the buffer significantly influenced the rate of reaction (Figure 3). These results were all confirmed by agarose gel electrophoretic results (data not shown but similar to those shown in Figure 1a).

It is known that reducing agents potentiate the DNA degradation induced by many agents (Mong et al., 1979). Also, cyanide and sulfhydryl-containing agents are known to bind the CDDP and inhibit subsequent DNA binding (Cleare et

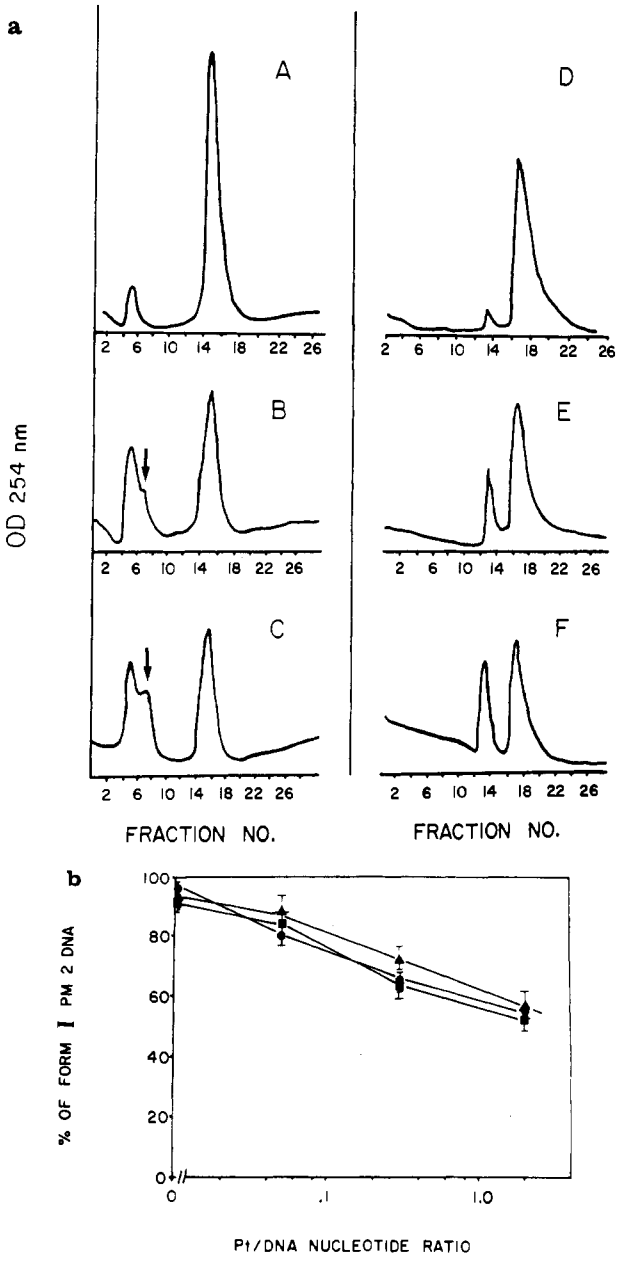

FIGURE 2: Neutral and alkaline sucrose gradient separation of PM2 DNA conformation isomers. (a) CCC PM2 DNA was incubated with CHIP as described in the text, and the different forms of PM2 DNA were separated by neutral (pH 7.5) or alkaline (pH 12.7) 5–20% sucrose gradients (Materials and Methods). Panels A, B, and C were alkaline sucrose gradients of PM2 DNA that was treated with 0, 136 μM, and 2.3 mM CHIP. The same samples were also fractionated by neutral sucrose gradients (panels D–F). Sedimentation is from left to right. Note the shoulder area indicated by the arrow. (b) Each peak area was calculated from the alkaline gradients (■) and the neutral gradients (▲) and plotted against the concentration of the drug used. An aliquot of each sample was also analyzed by gel electrophoresis and by densitometric scanning of the gel (●).

al., 1978). Therefore the effects of these agents on CHIP-induced DNA breakage were investigated. Figure 4 shows that the fluorescence decrease induced by CHIP was inhibited by cyanide and sulfhydryl-containing reducing agents but not by sodium borohydride ($NaBH_4$). The initial slight stimulation (Figure 4, at first- and second-hour time points with 2ME and DTT) by sulfhydryl reducing agents was reproducible, suggesting that these agents may be involved in a complex DNA–drug interaction. The non-sulfhydryl reducing agent $NaBH_4$, however, stimulated DNA breakage 5–6-fold (Figure 4). Sodium benzoate inhibited DNA breakage while reducing

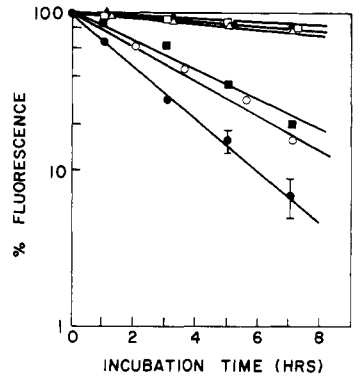

FIGURE 3: Buffer and pH effects on PM2 DNA breakage by CHIP. A standard incubation mixture contains 265 μg of CHIP (576 μM), 2 mM EDTA, 150 mM NaCl, 12.5 mM of various kinds of buffer, and 145 μg of CCC PM2 DNA incubated at 37 °C. Alkaline DNA breakage assays were described in previous figures (Materials and Methods). Standard deviations when not shown were smaller than the symbols used. Mops, pH 6.8 (●); Tricine, pH 7.1 (○); Mops, pH 7.6 (■); CHES, pH 8.8 (▲); CHES, pH 9.5 (△); borate buffer, pH 9.5 (□).

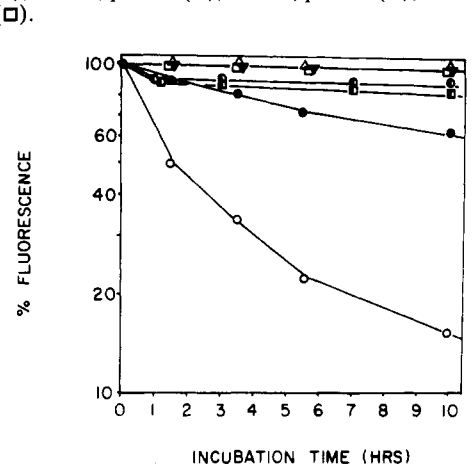

FIGURE 4: Conditions and cofactors affecting PM2 DNA breakage by CHIP. Standard incubation reaction mixtures, set up as described in Figure 5, contained 360 μM CHIP in Mops, pH 7.6, buffer (●) with 20 mM 2ME (◐), 5 mM DTT (◧), 20 mM $NaBH_4$ (○), 100 mM sodium benzoate (□), and 40 mM NaCN (△) included; control reaction without CHIP added (▼).

agents NADH and NADPH stimulated the rate of reaction. Table I summarizes the stimulatory or inhibitory effects of various agents on DNA cross-linking or breakage. The numbers indicate the ratios of either stimulation or inhibition of the rates of DNA breakage or cross-linking effects to those observed under standard conditions (Table I).

DNA Cross-Linking. Figure 5a shows the gel electrophoresis pattern of the DNA in fractions 4 and 7 (Figure 2a, panels A–C) after isolation, neutralization, and concentration from alkaline sucrose gradients as described under Materials and Methods. These results suggest that fraction 7 contained denatured DNA that, upon neutralization, renatured to assume a double-strand circular conformation. Form II type DNA from fraction 7, however, was different from the purified bona fide form II DNA or from the neutralized fraction 4 DNA. Figure 5b shows that under low-salt conditions (50 mM Na_3PO_4, 0.4 mM EDTA, and 0.8–1.0 μg/mL EB, at pH 11.2) fraction 7 DNA renatured after a heat–cool cycle as indicated by the nearly 90% recovery of EB-induced fluorescence. Untreated form II DNA lost greater than 90% of the EB fluorescence after heating and cooling. Fraction 4 DNA, however, did not enter the agarose gel (Figure 5a, channel B). In the snap-back assay (Materials and Methods) the fraction 4 DNA intercalated EB only slightly before heating, suggesting

Table I: Stimulatory or Inhibitory Effects of Various Agents on DNA Cross-Linking or Breakage

agents or conditions tested	concn (mM)	DNA cross-linking[a]	DNA breakage[a]
buffers and pH effect			
Mops, pH 7.0[b]	12.5	1.0	1.0
Mops, pH 6.8	12.5	1.25	1.36
Mops, pH 7.6	12.5	0.70	0.80
CHES, pH 8.7	12.5	0.30	0.14
CHES, pH 9.5	12.5		0.10
borate, pH 8.7	12.5	0.08	
borate, pH 9.5	12.5	0.08	0.08
Tricine, pH 7.1	12.5		1.10
reducing agents			
2ME	20	0.32	0.28
DTT	20	0.47	0.45
$NaBH_4$	20	2.40	6.2
NADPH[c]	3	1.08	3.2
free radical scavenger			
sodium benzoate	100	0.75	0.01
others			
NaCN	40	0	0
$NaNO_3$	25	0.95	1.0
phosphate[d]	25	0.98	1.0

[a] DNA breakage was assayed by the EB alkaline fluorescence assay method. DNA cross-linking was assayed by the snap-back assay system (Materials and Methods). [b] DNA cross-linking and breakage assays, unless specified, were carried out under the standard conditions: 12.5 mM Mops, buffer, pH 7.1, 150 mM NaCl, 560 μM CHIP, 4 mM EDTA, and 90 μg of form I (breakage assays) or form II (cross-linking assays) PM2 DNA. The initial rates of DNA cross-linking or breakage were calculated and compared with the rates of control reactions carried out under standard conditions and expressed in ratios of stimulation or inhibition. Only initial rates were used to avoid the complications introduced by cross-linking. [c] NADPH and NADH break DNA in long-term incubation; a short period of incubation stimulated DNA breakage by CHIP. [d] DNA cross-linking and breakage carried out in sodium phosphate buffer (pH 7.1).

that the majority of fraction 4 DNA was not double stranded. After the fraction 4 DNA was heated and cooled, about 50% of the preheated EB fluorescence was recovered, probably due to the nonspecific reannealing or short regions of PM2 DNA that had hairpin structures (Figure 5b). These results suggest that CHIP interacted with DNA in a manner similar to that of certain bifunctional alkylating agents (e.g., nitrosoureas and mitomycin C) extensively studied by investigators using this method (Lown et al., 1978; Lown & Weir, 1978; Morgan et al., 1979). These results thus suggest that CHIP induced DNA interstrand cross-linking in addition to DNA breakage.

Comparison of DNA Breakage and Cross-Linking Activities. DNA cross-linking was demonstrated by using the purified form II PM2 DNA. Figure 6 shows that with the use of form II DNA and the snap-back assay system, DNA interstrand cross-linking occurred as a function of incubation time. Compared with CCNU, an alkylating agent that has been shown to cause bifunctional cross-linking in DNA (Lown et al., 1978; Lown & Weir, 1978), CHIP may have also induced cross-linking on form II PM2 DNA. The effects of various agents and conditions on DNA cross-linking were also investigated in this system. As indicated in Table I, alkaline pH, sulfhydryl reducing agents (2ME and DTT), and cyanide inhibited DNA cross-linking. NADPH, $NaBH_4$, and pH 6.8 Mops buffer stimulated DNA cross-linking activity. Sodium benzoate (100 mM) inhibited 99% of DNA breakage but only 25% of the cross-linking activity (Table I).

The fact that DNA breakage but not cross-linking was inhibited by sodium benzoate is further demonstrated by the results shown in Figure 7. In this experiment, DNA breakage

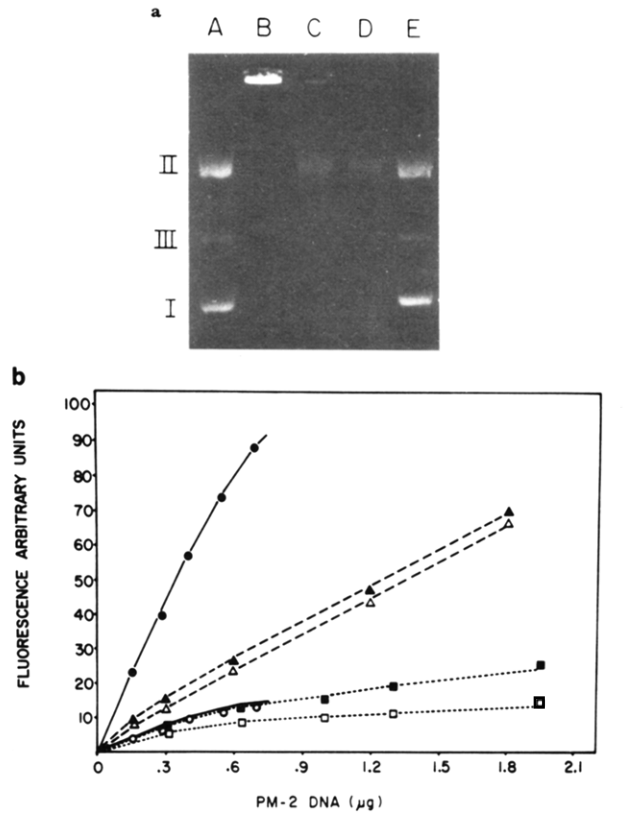

FIGURE 5: Denaturation and renaturation of PM2 DNA with EB as a probe. (a) Fractions 4 and 7 of the B and C gradients were isolated, pooled, neutralized with phosphoric acid, and concentrated by removing the solvent from each dialysis bag with PEG 6000. Each dialysis bag was extensively dialyzed against Mes buffer and analyzed by gel electrophoresis. (A and E) 2 μg of form II, III, and I PM2 DNA standards. (B) Approximately 1.2 μg of fraction 4 DNA. The intense staining in the well indicated that the majority of DNA did not enter the gel. (C and D) Fraction 7 DNA; approximately 1 and 0.5 μg of DNA were loaded in each well. (b) Fractions 4 and 7 and isolated purified form II PM2 were heated to 96 °C (50 mM Na_3PO_4, pH 11.2, 0.4 mM EDTA, and 1 μg/mL EB) for 2–3 min and rapidly chilled in an ice bath (Materials and Methods). (●, ○) indicate the isolated purified form II PM2 DNA before and after the heating–cooling cycle. (▲, △) indicate the fluorescence changes of fraction 7 DNA before and after the heating–cooling cycle. (■, □) are those for fraction 4 DNA fluorescence changes.

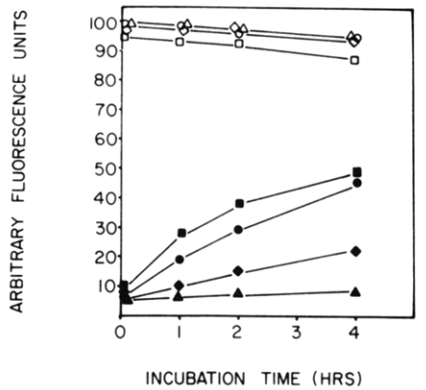

FIGURE 6: DNA cross-linking activity induced by CHIP. 26 μg of form II PM2 DNA was incubated with CHIP at a concentration of 1.28 mg/mL (3 mM) (●) or at 0.128 mg/mL (0.3 mM), 40 °C (♦), in the presence of 50 mM sodium phosphate buffer (pH 7.5), 150 mM NaCl, and 2.5 mM EDTA for increasing times. (▲) indicates the control incubation reaction mixtures. A bifunctional alkylating agent, CCNU, at the concentration of 1 mg/mL in 20% dimethyl sulfoxide was also present (■). The heating–cooling, fluorescence measurements and calculations were performed as described under Materials and Methods. Open symbols indicate the fluorescence before heat denaturation.

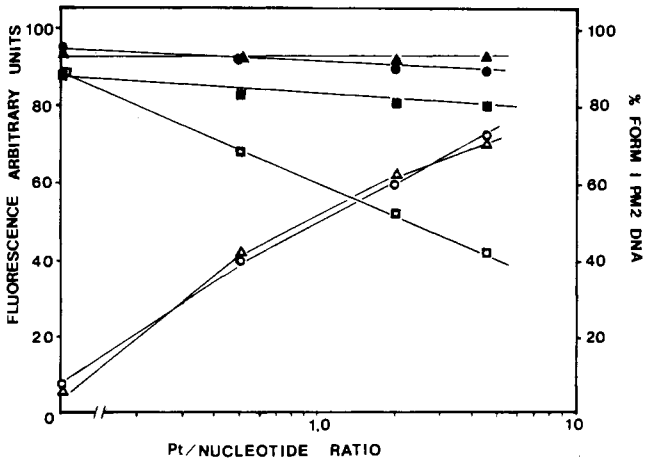

FIGURE 7: Detection of DNA interstrand cross-linking in the presence of sodium benzoate. 100 μg of form I PM2 DNA was incubated with 0, 240, 800, and 1200 μM CHIP (CHIP/nucleotide ratio 0, 0.5, 2.0, and 4.4, respectively) and in the presence or absence of 100 mM sodium benzoate under the standard conditions (Table I). Aliquots of each sample taken were run on agarose gels to evaluate the DNA breakage effect in the presence or absence of sodium benzoate. DNA was precipitated with 2 volumes of ethanol at −20 °C and redissolved in S1 buffer for S1 nuclease digestion. After the DNAs were extracted by phenol (TNE saturated) and precipitated with 2 volumes of ethanol (Materials and Methods), they were redissolved in TNE buffer, and the extent of cross-linking was measured by the snap-back assay. (■, □) indicate the mass fractions of form I PM2 DNA to show the DNA breakage effect in the presence (■) or absence (□) of sodium benzoate. (●, ▲) indicate the "preheating" fluorescence of the nicked DNA samples in the presence (●) or absence (▲) of sodium benzoate. (○, △) indicate "postheating" fluorescence of the DNA samples in the presence (○) or absence (△) of sodium benzoate, respectively. DNA cross-linking is shown as an increase of the fluorescence after the DNA was heat denatured and then quick cooled in alkaline buffer (Materials and Methods).

was detected in the absence of 100 mM sodium benzoate (Figure 7, open squares). DNA degradation was inhibited by sodium benzoate (Figure 7, filled squares). The extent of DNA cross-linking induced by CHIP, however, was not significantly different in either the presence or absence of sodium benzoate (Figure 7, open circles and triangles), confirming the results shown in Table I that sodium benzoate inhibited DNA strand scission preferentially.

Discussion

DNA Breakage. In vitro DNA breakage induced by CHIP or JM28 has been demonstrated by using the alkaline fluorescence, agarose gel electrophoretic, neutral and alkaline fluorescence assay methods. The DNA breakage activity was only minimally inhibited (10–15%) in the presence of 5 mM EDTA or by heating to 95 °C for 8 min before adding form I DNA (Figure 1b), indicating that it is unlikely that traces of contaminating DNases may have induced extensive DNA breakage under the experimental conditions.

It has been suggested by Cleare et al. (1978) that in aqueous solutions, chloride and hydroxyl groups of CHIP may dissociate from the octahedral complexes, giving rise to aquated species of Pt(IV) drugs. Strong nucleophilic ligands such as cyanide or sulfhydryl group containing agents inhibited DNA binding (Howe-Grant et al., 1976; Kelman & Buchbinder, 1978), suggesting that these ligands may prevent DNA degradation by inactivating the drug. Alkaline buffers (pH 8.8 and pH 9.5) but not NaCl (up to 250 mM) inhibited the breakage and cross-linking reactions, suggesting that prior dissociation of transaxial OH groups instead of the equatorial chlorides may be required for DNA breakage and cross-linking.

It is evident from the results of this study that non-sulfhydryl reducing agents such as $NaBH_4$ or NADPH stimulated the DNA breakage reaction. Also free radical scavengers such as sodium benzoate inhibited DNA breakage. These results suggest that a redox-mediated free radical formation may be involved in this mechanism. A similar mechanism that involved hydroxyl radicals (e.g., OH·), superoxide radicals (e.g., O_2·), ferrous cations, and bleomycin in DNA breakage has already been proposed (Sausville et al., 1976; Lown & Sim, 1977). The formation of free radicals in this case was detected by the catalase–superoxide dismutase (SOD) system. This system, however, is not directly applicable in the study of DNA–CHIP interaction for the following reasons: (a) It has been shown that SOD may bind directly to DNA in vitro (Galvan et al., 1981). (b) It has been demonstrated that platinum drugs can bind to many enzymes or proteins (Friedman & Teggins, 1974) and thus may interact with SOD or catalase directly. However, by use of electron spin resonance and phenyl-*tert*-butylnitrone as a spin trap, preliminary experiments suggest that a weak electron spin resonance pattern typical of a hydroxy radical adduct is detectable, suggesting that CHIP may generate hydroxy radicals in aqueous conditions (data not shown). Whether hydroxy radicals are involved in DNA breakage by CHIP remains to be established. The non-sulfhydryl group containing reducing agents and acidic (pH 6.8) buffers might favor the dissociation of such groups to generate free radicals. Whatever the mechanisms may be, depurination, depyrimidation, or alkaline labile site formation can probably be excluded as possible mechanisms for DNA breakage since very little alkaline labile damage was shown by the neutral and alkaline sucrose density gradient studies.

Since CHIP and JM-28 were synthesized by hydrogen peroxide hydroxylation of the planar Pt(II) species, it is conceivable that the minor contaminating species (e.g., peroxide-conjugated Pt(IV) species or free hydrogen peroxide) might be responsible for the DNA breakage effect and account for the variable breakage activity from different lots. However, this seems unlikely because of the following observations: (a) the CHIP preparations used in this study have been reported to be greater than 98% pure; (b) with high-pressure liquid chromatography (HPLC) and other techniques, no demonstrable amount of hydrogen peroxide can be found present in the CHIP preparations; (c) the less than 1% contaminating species isolated by the HPLC method has failed to show DNA breakage activity in vitro; (d) the HPLC-purified CHIP (greater than 99.7% pure), under the optimal in vitro conditions (600 μM CHIP, 1.5 mM $NaBH_4$, 12.5 mM Tricine buffer, pH 7.3, 50 mM $NaNO_3$, and 2.5 mM EDTA, 37 °C incubation for 12 h), retained DNA breakage activity (unpublished results); (e) no other drug-induced DNA conformational changes such as DNA unwinding and rewinding effects were found to be associated with any of the CHIP preparations studied. These observations, although they can only be regarded as inferential evidence, lead us to conclude that DNA breakage is one of the major types of lesions induced by treatment of DNA with high concentrations of CHIP (>600 μM) in vitro. This property makes Pt(IV) analogues distinctly different from CDDP, which induces DNA shortening, unwinding, and rewinding primarily.

DNA Cross-Linking. CHIP-induced DNA cross-linking was demonstrated by two methods in this study. The results obtained from alkaline sucrose density gradient analysis (Figure 2a) suggested that fraction 7 contained cross-linked DNA species so that upon neutralization, the cross-links

provided nucleation sites for DNA reannealing to form form II DNA (Lown et al., 1976; Morgan et al., 1979). Fraction 4 contained primarily alkali-denatured DNA that upon neutralization became partially renatured or nonspecifically aggregated. This interpretation is consistent with the results shown in Figure 5. Alkaline sucrose gradient purified DNA from fraction 7 renatured upon neutralization and migrated to the form II PM2 DNA position (Figure 5a, channels C and D). The neutralized DNA still bound EB although not as efficiently as the control form II DNA. This might be due to local hairpin structures or collapsed regions that did not renature properly. After being heated and rapidly cooled, this fraction of DNA still retained greater than 90% of the EB intercalation induced fluorescence (Figure 5b). DNA from fraction 4, however, did not migrate into the agarose gel (Figure 5a, channel B), did not bind EB efficiently before heat denaturation, and after being heated and cooled, bound even less EB, suggesting that this fraction contained primarily heat-denatured random-coiled DNA.

DNA cross-linking activity was also demonstrated by employing the snap-back assay that has been used extensively in elucidating the mechanism of action of bifunctional alkylating agents. With the exception of sodium benzoate, every factor that stimulated or inhibited DNA cross-linking also stimulated or inhibited DNA breakage activities (Table I). Sodium benzoate (100 mM), however, seems to be inhibitory primarily to the DNA breakage activity, inhibiting cross-linking only minimally (25%, shown in Table I; <5%, shown in Figure 7). While the differences of inhibitory effects by sodium benzoate need to be further investigated, these data suggest that DNA strand breakage and cross-linking may be independently mediated since breakage is preferentially inhibited by sodium benzoate. One possible explanation is that hydroxyl radicals are generated or displaced from the drug when CHIP produces cross-links between the complementary strands of DNA. Sodium benzoate may scavenge the highly reactive hydroxyl radicals and thus prevent DNA breakage but not cross-linking. This hypothesis needs further evidence and is being studied in this laboratory.

CDDP has been demonstrated to cause DNA shortening, unwinding, and subsequently rewinding of CCC DNA in vitro (Cohen et al., 1979; Macquet & Butour, 1978; Mong et al., 1980a,b, 1981) probably by inducing nonseparative denaturative DNA intrastrand cross-linking (Kleinwachter, 1978; Mong et al., 1981.) In contradistinction, the platinum(IV) compounds (e.g., CHIP and JM28) did not produce similar types of DNA tertiary conformational changes in vitro. The data in this study indicated that the major types of damages in vitro are DNA interstrand cross-linking and single-strand breakage, thus making CHIP distinctly different from the parent drug CDDP. Although cellular DNA breakage and interstrand cross-linking have not been demonstrated, CHIP may also cause protein–DNA cross-linking or DNA damage repair that may be in cells, thus masking the DNA breakage effect as detected by alkaline sucrose gradients or other methods (unpublished results). Nevertheless, DNA breakage and interstrand cross-linking are the major effects detected in vitro, making CHIP and other Pt(IV) compounds distinctly different from CDDP and other platinum(II) compounds.

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