Picton, C., Woodget, J., Hemmings, B., & Cohen, P. C. (1982) *FEBS Lett. 150*, 191-196.
Proudfoot, J. M., & Brownlee, G. G. (1976) *Nature 265*, 211-214.
Pyerin, W., Burow, E., Michaely, K., Kubler, D., & Kinzel, V. (1987) *Biol. Chem. Hoppe-Seyler 368*, 215-227.
Qi, S.-L., Yukioka, M., Morisawa, S., & Inoue, A. (1986) *FEBS Lett. 203*, 104-108.
Renart, M. F., Sastre, L., & Sebastian, J. (1984) *Eur. J. Biochem. 140*, 47-54.
Richardson, W. D., Roberts, B. L., & Smith, A. E. (1986) *Cell 44*, 77-85.
Rose, K. M., Bell, L. E., Siefken, D. A., & Jacob, S. T. (1981) *J. Biol. Chem. 256*, 7468-7477.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Saxena, A., Padmanabha, R., & Glover, C. V. C. (1987) *Mol. Cell. Biol. 7*, 3409-3417.
Schneider, H. R., & Issinger, O. G. (1988) *Biochem. Biophys. Res. Commun. 156*, 1390-1397.
Sommercorn, J., & Krebs, E. G. (1988) in *Advances in Post-Translational Modifications of Proteins and Aging* (Zappia, V., Galletti, P., Porta, R., & Wold, F., Eds.) pp 403-415, Plenum Publishing Corp., New York.
Sommercorn, J., Mulligan, J. A., Lozeman, F. J., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8834-8838.
Takio, K., Kuenzel, E. A., Walsh, K. A., & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4851-4855.
Thornburg, W., & Lindell, T. J. (1977) *J. Biol. Chem. 252*, 6660-6665.
Thornburg, W., Gamo, S., O'Malley, A. F., & Lindell, T. J. (1979) *Biochim. Biophys. Acta 571*, 35-44.
Tuazon, P. T., & Traugh, J. A. (1990) *Adv. Second Messenger Phosphoprotein Res.* (in press).
Witters, L. A., Tipper, J. P., & Bacon, G. W. (1983) *J. Biol. Chem. 258*, 5643-5648.
Witters, L. A., Watts, T. D., Daniels, D. L., & Evans, J. L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5473-5477.
Zandomeni, R., Zandomeni, M. C., & Weinmann, R. (1988) *FEBS Lett. 235*, 247-251.

Modification of Bases in DNA by Copper Ion-1,10-Phenanthroline Complexes†

Miral Dizdaroglu,*,‡ Okezie I. Aruoma,§,∥ and Barry Halliwell§

Center for Chemical Technology, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, and Biochemistry Department, University of London King's College, Strand Campus, London WC2R 2LS, United Kingdom

Received March 14, 1990; Revised Manuscript Received May 30, 1990

ABSTRACT: Damage to the bases in DNA by the cupric ion-1,10-phenanthroline complex was investigated. Ten base products in DNA were identified and quantitated by the use of gas chromatography/mass spectrometry with selected-ion monitoring. DNA damage by the cupric ion-1,10-phenanthroline complex required the presence of a reducing agent such as ascorbic acid or mercaptoethanol. Products identified were typical hydroxyl radical induced products from the pyrimidines and purines in DNA, well-known from previous studies using various hydroxyl radical producing systems such as ionizing radiation, hypoxanthine/xanthine oxidase, or hydrogen peroxide in the presence of transition metal ions. Product formation was not significantly inhibited by typical scavengers of hydroxyl radical such as mannitol and sodium formate, but there was partial inhibition by dimethyl sulfoxide. Catalase substantially decreased formation of base products, and added hydrogen peroxide stimulated it, indicating the hydrogen peroxide dependency of DNA base damage. Superoxide dismutase afforded only a partial reduction in product yields in systems containing ascorbic acid. On the basis of the types of base products formed, the hydrogen peroxide dependency of product formation, and a previous report suggesting that DNA damage is due to a diffusible species [Williams, L. D., Thivierge, J., & Goldberg, I. H. (1988) *Nucleic Acids Res. 16*, 11607-11615], we propose that DNA base damage is caused by hydroxyl radical.

In the presence of H_2O_2, a 2:1 complex of the chelating agent 1,10-phenanthroline (OP)[1] with Cu^+ ions [$(OP)_2Cu^+$] produces strand cleavage of DNA after binding to the minor groove (Sigman et al., 1979; Sigman, 1986). The nuclease activity of the $(OP)_2Cu^+$ complex has been widely used for structural studies upon DNA (Sigman, 1986; Marshall et al., 1981; Pope & Sigman, 1984; Veal & Rill, 1988). In addition, measurement of DNA damage in the presence of an excess of DNA and of OP has been employed as a sensitive assay to measure the availability of copper ions in human body fluids (Gutteridge, 1984; Gutteridge et al., 1985; Evans et al., 1989). During experimentation, Cu^{2+} ions are often mixed with the OP so that a reducing agent has to be added to reduce Cu^{2+} ions to Cu^+ ions and form the DNA-cleaving species. The reducing agents that have been employed in vitro to facilitate

† We are grateful to the Association for International Cancer Research, Uxbridge, U.K., for partial financial support.

* To whom correspondence should be addressed.
‡ National Institute of Standards and Technology.
§ University of London King's College.
∥ Part of this work was done when O.I.A. was a guest scientist at the National Institute of Standards and Technology.

[1] Abbreviations: OP, 1,10-phenanthroline; O_2^-, superoxide radical; •OH, hydroxyl radical; GC/MS-SIM, gas chromatography/mass spectrometry with selected-ion monitoring; 5-OH-5-MeHyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; 5-OHMeUra, 5-(hydroxymethyl)uracil; 5,6-diOH-Cyt, 5,6-dihydroxycytosine; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 8-OH-Gua, 8-hydroxyguanine; ME, mercaptoethanol; SOD, copper-zinc superoxide dismutase; asc, ascorbic acid; TBA, thiobarbituric acid; Me_2SO, dimethyl sulfoxide.

0006-2960/90/0429-8447$02.50/0

the DNA degradation by $(OP)_2Cu^{2+}$ include ascorbic acid, thiols (such as mercaptoethanol), NADH, and superoxide radical (O_2^-) (Sigman et al., 1979; Sigman 1986; Marshall et al., 1981; Pope & Sigman, 1984; Reich et al., 1981; Que et al., 1980). It is often unnecessary to add H_2O_2 to such reaction mixtures, since it can be generated by the reactions taking place (Sigman, 1986; Marshall et al., 1981; Gutteridge & Halliwell, 1982).

Despite the frequent use of $(OP)_2Cu^+$ as a cleaving agent, the mechanism of DNA cleavage is unclear. A popular view is that the $(OP)_2Cu^+$ complex binds to DNA and reacts with H_2O_2 to form hydroxyl radical (•OH) (Sigman et al., 1979; Sigman, 1986; Marshall et al., 1981; Pope & Sigman, 1984; Thederahn et al., 1989; Gutteridge & Halliwell, 1982):

$$(OP)_2Cu^+ + H_2O_2 \rightarrow (OP)_2Cu^{2+} + {}^\bullet OH + OH^-$$

However, serious questions have arisen as to whether $(OP)_2Cu^+$ complexes do form •OH upon reaction with H_2O_2 or whether an oxo–copper ion complex is responsible for the DNA damage (Johnson & Nazhat, 1987). Mixtures of unchelated Cu ions, H_2O_2, and reducing agents (to convert Cu^{2+} to Cu^+) have also been suggested to form •OH, but again this proposal has been challenged, and the debate continues in the literature (Johnson & Nazhat, 1987; Johnson et al., 1985; Sutton & Winterbourn, 1989; Czapski et al., 1988; Masarwa et al., 1988; Rowley & Halliwell, 1983; Yamamoto & Kawanishi, 1989; Eberhardt et al., 1989; Halliwell & Gutteridge, 1990). The chemical characterization of any base damage produced in DNA by $(OP)_2Cu^+$ complexes has not been reported.

Attack of ionizing radiation generated •OH upon DNA produces a wide range of products from the pyrimidine and purine bases [for reviews, see Teoule and Cadet (1978) and von Sonntag (1987)]. By contrast, H_2O_2 and O_2^- do not form any base products in DNA unless transition metal ions are present (Aruoma et al., 1989a,b). The measurement of the pattern of the DNA base products produced when DNA was exposed in aqueous solution to the hypoxanthine/xanthine oxidase system or to H_2O_2 in the presence of certain ferric ion complexes strongly suggested that •OH was responsible for the DNA damage (Aruoma et al., 1989a,b). Base damage in DNA can be accurately and sensitively measured by the use of gas chromatography/mass spectrometry with selected-ion monitoring (GC/MS-SIM) after hydrolysis of DNA followed by derivatization (Dizdaroglu, 1985; Fuciarelli et al., 1989; Aruoma et al., 1989a,b; Dizdaroglu & Gajewski, 1990; Blakely et al., 1990).

In the present paper, we have investigated $(OP)_2Cu^+$-mediated base damage in DNA. The base modifications in DNA were analyzed by GC/MS-SIM, to see what, if any, base-derived products are generated in DNA by the $(OP)_2Cu^+$ system and to gain information about the reactive species that attacks the DNA.

Experimental Procedures

Materials.[2] Calf thymus DNA, 1,10-phenanthroline, mercaptoethanol, ascorbic acid, mannitol, bovine copper/zinc-containing superoxide dismutase, catalase (type C-40, thymol free), dimethyl sulfoxide, sodium formate, and sodium azide were purchased from Sigma Chemical Co. Units of superoxide dismutase were as defined by the cytochrome *c* assay (McCord & Fridovich, 1969). One unit of catalase decomposes 1 μmol of H_2O_2/min at pH 7.0 at 25 °C, as defined by Sigma. Dialuric acid (5-hydroxyuracil) was purchased from American Tokyo Kasei, Inc. Other reagents were as described previously (Dizdaroglu, 1985; Fuciarelli et al., 1989; Aruoma et al., 1989a).

Treatment of DNA. Reaction mixtures contained in a volume of 2 mL the following reagents at the final concentration given: DNA (0.25 mg/mL), $CuCl_2$ (60 μM), 1,10-phenanthroline (88 μM), mercaptoethanol (where used, 11.5 mM), ascorbic acid (where used, 60 μM), sodium azide (where used, 10 mM), and NaH_2PO_4/Na_2HPO_4 buffer at pH 6.7 (25 mM). Copper ions and phenanthroline were premixed before addition to the assay mixture so as to avoid precipitation of copper ions by the phosphate buffer [used as a physiological buffer system and because most other available buffers react with •OH with rate constants of $\geq 10^9$ $M^{-1} \cdot s^{-1}$ (Halliwell et al., 1987)]. 1,10-Phenanthroline crystals were first dissolved in 50 μL of absolute ethanol prior to the addition of the required volume of water to make up the stock solution. Control experiments showed that ethanol did not interfere with the assay. Mercaptoethanol or ascorbic acid was added to start the reaction. Mixtures were incubated for 1 h at 37 °C. Mannitol, sodium formate, dimethyl sulfoxide (Me_2SO), and superoxide dismutase (SOD) or catalase were added to the reaction mixtures, where appropriate, at the final concentrations given in the tables. After the incubation, 0.1 mL of 0.1 M EDTA was added to stop the reaction (Gutteridge & Halliwell, 1982). The mixtures were extensively dialyzed against water at 4 °C. The absorbance at 260 nm of each sample was measured in order to calculate the amount of DNA (absorbance of 1 = 50 μg of DNA/mL). In parallel experiments, formation of deoxyribose fragments was assayed by the thiobarbituric acid test (Gutteridge & Halliwell, 1982).

Hydrolysis of DNA samples, derivatization of hydrolysates, and identification and quantitation of derivatized products by GC/MS-SIM were performed as described previously (Dizdaroglu, 1985; Fuciarelli et al., 1989; Aruoma et al., 1989a). The column used was a fused silica capillary column (12.5 m × 0.2 mm i.d.) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness 0.33 μm). Approximately 0.4 μg of hydrolyzed and derivatized DNA was injected onto the column for each analysis.

Results

Hydrolyzed and derivatized DNA samples were analyzed by GC/MS-SIM. The use of this technique for measurement of base damage in DNA has been described previously (Dizdaroglu, 1985; Fuciarelli et al., 1989; Dizdaroglu & Gajewski, 1990). Figure 1 illustrates ion-current profiles obtained during the GC/MS-SIM analysis of a trimethylsilylated hydrolysate of DNA treated with $(OP)_2Cu^{2+}$/ascorbic acid. Peak identification is given in the figure legend. 5-Hydroxy-5-methylhydantoin (5-OH-5-MeHyd) and 5-hydroxyhydantoin (5-OH-Hyd) are believed to result from acid-induced modification of 5-hydroxy-5-methylbarbituric acid and 5,6-dihydroxycytosine, respectively (Teoule & Cadet, 1978). 5-Hydroxycytosine (5-OH-Cyt) and 5-hydroxyuracil (5-OH-Ura) result from the acid-induced modification of cytosine glycol, the former by dehydration and the latter by both dehydration and deamination (Dizdaroglu et al., 1986). 5,6-Dihydroxyuracil [dialuric acid and/or isodialuric acid] (5,6-diOH-Ura) is formed by the acid-induced deamination of 5,6-dihydroxycytosine (5,6-diOH-Cyt), which is, like cytosine glycol, the actual product produced by attack of •OH

[2] Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified is necessarily the best available for the purpose.

Table I: Yields (nmol/mg of DNA) of Base Products Formed in DNA by Treatment with Cu^{2+}–Phenanthroline[a]

	treatment					
product	DNA alone	DNA/$(OP)_2Cu^{2+}$	DNA/Cu^{2+}/asc	DNA/$(OP)_2Cu^{2+}$/asc	DNA/Cu^{2+}/ME	DNA/$(OP)_2Cu^{2+}$/ME
5-OH-5-MeHyd	0.28 ± 0.001	0.24 ± 0.11	0.21 ± 0.03	0.47 ± 0.03	1.08 ± 0.27	1.95 ± 0.26
5-OH-Hyd	0.23 ± 0.037	0.11 ± 0.005	0.18 ± 0.02	0.35 ± 0.02	0.33 ± 0.01	1.82 ± 0.12
5-OHMeUra	≤0.01	≤0.01	≤0.01	0.10 ± 0.008	≤0.01	0.31 ± 0.02
cytosine glycol	0.14 ± 0.03	0.20 ± 0.03	0.24 ± 0.03	1.02 ± 0.15	0.33 ± 0.07	3.51 ± 0.52
thymine glycol	0.24 ± 0.027	0.19 ± 0.01	0.33 ± 0.056	1.42 ± 0.08	0.69 ± 0.03	9.01 ± 0.52
5,6-diOH-Cyt	≤0.01	≤0.01	≤0.01	0.23 ± 0.02	≤0.01	0.53 ± 0.03
FapyAde	≤0.01	0.05 ± 0.001	0.08 ± 0.002	0.14 ± 0.02	0.21 ± 0.01	0.19 ± 0.02
8-OH-Ade	0.40 ± 0.08	0.82 ± 0.05	0.53 ± 0.04	1.36 ± 0.19	1.34 ± 0.07	2.07 ± 0.11
FapyGua	≤0.01	≤0.01	0.10 ± 0.005	0.41 ± 0.06	≤0.01	0.24 ± 0.06
8-OH-Gua	1.02 ± 0.09	1.49 ± 0.15	2.95 ± 0.21	10.4 ± 0.75	4.59 ± 0.71	34.2 ± 3.3

[a] All values represent the mean ± standard deviation from triplicate measurements.

Table II: Yields (nmol/mg of DNA) of Base Products Formed in DNA by Treatment with Cu^{2+}–Phenanthroline[a]

	treatment				
product	DNA/$(OP)_2Cu^{2+}$/asc	DNA/$(OP)_2Cu^{2+}$/asc/mannitol (50 mM)	DNA/$(OP)_2Cu^{2+}$/asc/formate (50 mM)	DNA/$(OP)_2Cu^{2+}$/asc/SOD (625 units/mL)	DNA/$(OP)_2Cu^{2+}$/asc/catalase (10^3 units/mL)
5-OH-5-MeHyd	0.47 ± 0.03	0.42 ± 0.025	0.48 ± 0.01	0.66 ± 0.13	0.43 ± 0.08
5-OH-Hyd	0.35 ± 0.02	0.31 ± 0.02	0.35 ± 0.01	0.60 ± 0.02	0.28 ± 0.08
5-OHMeUra	0.10 ± 0.008	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.09 ± 0.008
cytosine glycol	1.02 ± 0.15	0.98 ± 0.06	1.25 ± 0.12	0.63 ± 0.01	0.31 ± 0.09
thymine glycol	1.42 ± 0.08	1.34 ± 0.05	1.64 ± 0.05	1.24 ± 0.23	0.29 ± 0.06
5,6-diOH-Cyt	0.23 ± 0.02	0.27 ± 0.028	0.31 ± 0.034	0.19 ± 0.03	0.05 ± 0.002
FapyAde	0.14 ± 0.02	0.14 ± 0.05	0.17 ± 0.03	0.21 ± 0.03	≤0.01
8-OH-Ade	1.36 ± 0.19	1.32 ± 0.11	1.63 ± 0.18	0.69 ± 0.11	0.37 ± 0.09
FapyGua	0.41 ± 0.06	0.53 ± 0.077	0.58 ± 0.09	0.37 ± 0.10	≤0.01
8-OH-Gua	10.4 ± 0.75	11.5 ± 1.1	12.5 ± 0.4	4.99 ± 1.0	2.06 ± 0.26

[a] All values represent the mean ± standard deviation from triplicate measurements.

upon Cyt in DNA (Dizdaroglu, 1985). The chemical structures of the products identified are shown in Figure 2. The base products and their yields in various DNA samples are given in Tables I–III. All these compounds are typical products that arise from attack of •OH upon bases in DNA, as previously identified by radiation studies [for reviews, see Teoule and Cadet (1978) and von Sonntag (1987)].

The commercial calf thymus DNA used in the present study already contained small amounts of modified bases (Table I), as described previously (Aruoma et al., 1989a,b). Incubation of DNA in phosphate buffer with mercaptoethanol (ME) alone, Cu^{2+} ions alone, ascorbic acid (asc) alone, or $(OP)_2Cu^{2+}$ alone at the concentrations used in this study produced no significant changes in the amounts of base products. However, incubation of DNA with $(OP)_2Cu^{2+}$/asc and $(OP)_2Cu^{2+}$/ME produced extensive modification of the DNA bases (Table I). The presence of asc promoted much more extensive DNA damage than that of ME over a wide range of concentrations (data not shown). For example, a much higher concentration of ME than that of asc in the reaction mixture was necessary to produce more damage than asc as shown in Table I. Addition of H_2O_2 (final concentration, 1.2 mM) to the reaction mixtures increased the yields of the base products approximately 2-fold (data not shown). Inclusion of the •OH scavengers mannitol and sodium formate at 50 mM final concentrations in the reaction mixture containing $(OP)_2Cu^{2+}$/asc had no significant effect on the yield of the products (Table II). Similarly, a mannitol concentration of 500 mM inhibited DNA degradation only slightly in the $(OP)_2Cu^{2+}$/ME system, whereas 500 mM Me_2SO had a somewhat more inhibitory action on the formation of most base products (Table III). Similar results were obtained in the $(OP)_2Cu^{2+}$/asc system. The addition of SOD inhibited somewhat the formation of cytosine glycol, thymine glycol, 5,6-diOH-Cyt, 8-OH-Ade, and 8-OH-Gua in the $(OP)_2Cu^{2+}$/asc system (Table II). By contrast, the presence of catalase in the reaction mixture

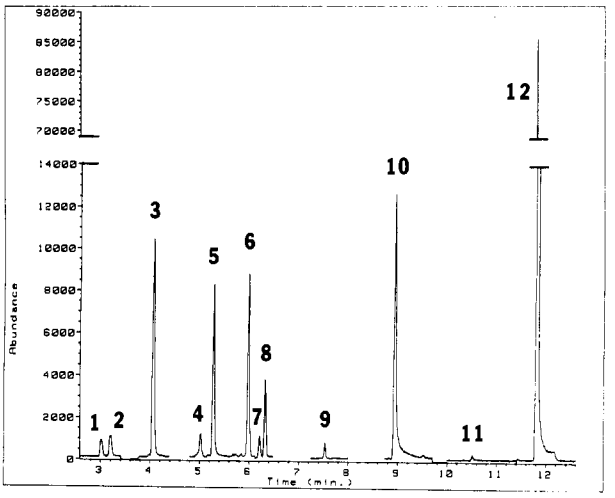

FIGURE 1: Ion-current profiles obtained during the GC/MS-SIM analysis of a trimethylsilylated hydrolysate of DNA treated with $(OP)_2Cu^{2+}$/ascorbic acid. The column was programmed from 150 to 260 °C at a rate of 8 °C/min after 2 min at 150 °C. For other details, see Experimental Procedures. Peaks (ions monitored for unequivocal identification with the first ion being illustrated in the figure): 1, 5-OH-5-MeHyd (*m/z* 331, 346, 216, 203); 2, 5-OH-Hyd (*m/z* 317, 332, 202, 189); 3, 5-OH-Ura (*m/z* 329, 344, 343, 255); 4, 5-OHMeUra (*m/z* 358, 343, 270, 255); 5, 5-OH-Cyt (*m/z* 343, 342, 328, 240); 6, *cis*-thymine glycol (*m/z* 259, 448, 433, 301); 7, 5,6-diOH-Ura (*m/z* 417, 432, 343, 328); 8, *trans*-thymine glycol (*m/z* 259, 448, 433, 301); 9, FapyAde (*m/z* 354, 369, 368, 280); 10, 8-OH-Ade (*m/z* 352, 367, 353, 264); 11, FapyGua (*m/z* 442, 457, 368, 352); 12, 8-OH-Gua (*m/z* 440, 455, 366, 352) (all compounds as their trimethylsilyl derivatives).

caused a substantial decrease in the yields of all the products (Table II). The yields of some products were even decreased to the background levels (compare Tables I and II). Comparable results with catalase were obtained with the $(OP)_2Cu^{2+}$/ME system, but SOD had very little inhibitory effect, thus ruling out any nonspecific effect of protein on the

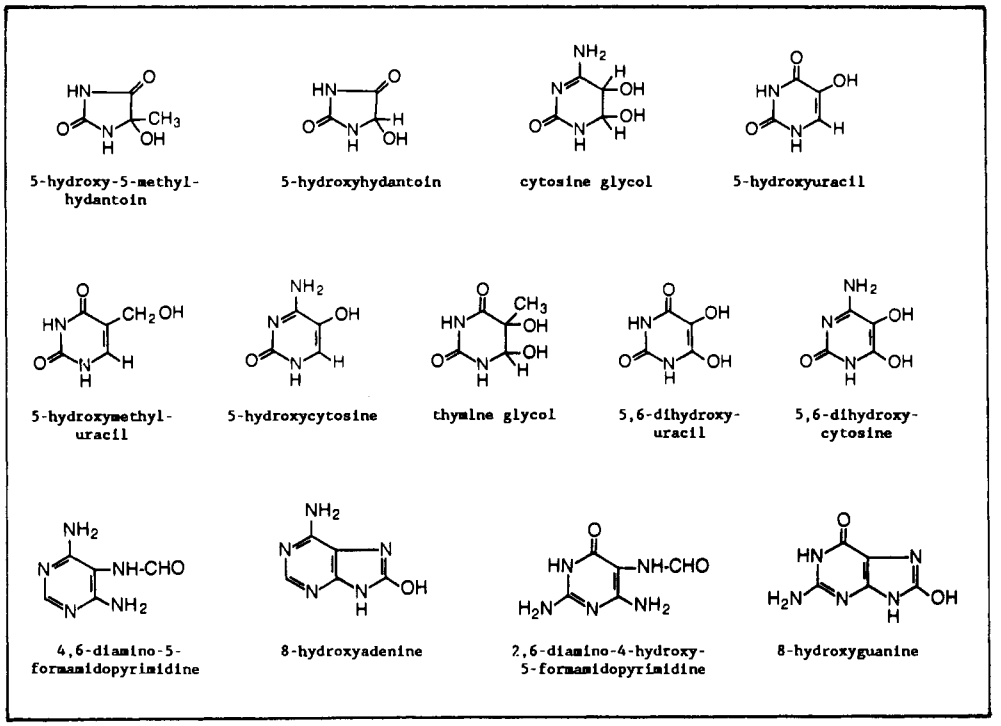

FIGURE 2: Chemical structures of DNA base products identified in the present work.

Table III: Yields (nmol/mg of DNA) of Base Products Formed in DNA by Treatment with Cu^{2+}–Phenanthroline[a]

	treatment				
product	DNA/$(OP)_2Cu^{2+}$/ME	DNA/$(OP)_2Cu^{2+}$/ME/mannitol (500 mM)	DNA/$(OP)_2Cu^{2+}$/ME/Me_2SO (500 mM)	DNA/$(OP)_2Cu^{2+}$/ME/SOD (625 units/mL)	DNA/$(OP)_2Cu^{2+}$/ME/catalase (10^3 units/mL)
5-OH-5-MeHyd	1.95 ± 0.26	1.62 ± 0.17	1.35 ± 0.14	1.15 ± 0.06	1.17 ± 0.04
5-OH-Hyd	1.82 ± 0.12	1.36 ± 0.14	1.20 ± 0.05	1.47 ± 0.13	1.18 ± 0.16
5-OHMeUra	0.31 ± 0.02	0.29 ± 0.02	0.25 ± 0.01	0.21 ± 0.02	0.27 ± 0.02
cytosine glycol	3.51 ± 0.52	2.99 ± 0.37	3.53 ± 0.30	3.32 ± 0.76	2.97 ± 0.87
thymine glycol	9.01 ± 0.52	7.23 ± 1.00	6.30 ± 0.98	6.61 ± 0.88	7.15 ± 1.24
5,6-diOH-Cyt	0.53 ± 0.03	0.47 ± 0.08	0.43 ± 0.06	0.49 ± 0.01	0.39 ± 0.07
FapyAde	0.19 ± 0.02	0.17 ± 0.004	≤0.01	0.35 ± 0.01	0.14 ± 0.01
8-OH-Ade	2.07 ± 0.11	1.63 ± 0.29	1.32 ± 0.10	1.48 ± 0.49	1.12 ± 0.21
FapyGua	0.24 ± 0.06	≤0.01	≤0.01	0.47 ± 0.03	≤0.01
8-OH-Gua	34.2 ± 3.3	26.8 ± 4.2	18.5 ± 2.3	30.0 ± 7.3	9.10 ± 2.44

[a] All values represent the mean ± standard deviation from triplicate measurements.

assay of base products (Table III).

Average sugar degradation in DNA by $(OP)_2Cu^{2+}$/ME as measured by the deoxyribose assay and calculated as malondialdehyde equivalents was approximately 7–10 nmol/mg of DNA. This is approximately one-seventh of the total amount of base damage measured (53.8 nmol/mg of DNA; see Table I).

Discussion

DNA damage by the $(OP)_2Cu^{+}$ complex has previously been measured as strand breakage (Sigman et al., 1979; Sigman, 1986) or degradation of deoxyribose to thiobarbituric acid (TBA) reactive material (Gutteridge & Halliwell, 1982). In the present work, we have, for the first time, measured damage to the pyrimidine and purine bases. Formation of modified pyrimidine and purine bases required the presence of a reducing agent. It was also H_2O_2 dependent, as shown by a substantial inhibition of product formation by catalase and its stimulation by addition of H_2O_2 to the reaction mixtures. The •OH scavengers mannitol and formate produced no marked inhibition, suggesting that DNA damage is not mediated by •OH that is accessible to these scavengers. It could be argued that any •OH responsible for the DNA damage is formed by the $(OP)_2Cu^{+}$ complex so close to the DNA bases that •OH scavengers cannot be present at sufficient concentrations to scavenge it (Gutteridge & Halliwell, 1982). It may be that these hydrophilic scavengers cannot approach the hydrophobic DNA bases sufficiently closely. Very high concentrations of the powerful •OH scavenger Me_2SO partially inhibited the base modification (Table III), consistent with a role for •OH. However, these data must be interpreted with caution because Me_2SO is not specific as an •OH scavenger (Puppo & Halliwell, 1989).

When either asc or ME was used as a reducing agent, DNA damage by the $(OP)_2Cu^{+}$ complex largely resulted in formation of 8-OH-Gua and, to a lesser extent, of 8-OH-Ade, thymine glycol, and cytosine glycol. However, the amounts of all modified DNA bases increased substantially, showing that the radical species formed in the $(OP)_2Cu^{2+}$/asc or $(OP)_2Cu^{2+}$/ME system has considerable reactivity toward the bases in DNA. This evidence for high reactivity suggests that this species is •OH. The fact that the base products identified are well-known •OH-induced products of bases in DNA supports the involvement of •OH in product formation. The inability of other researchers to detect •OH during reactions of $(OP)_2Cu^{+}$ complexes in the absence of DNA (Johnson & Nazhat, 1987) does not preclude a mechanism involving •OH, since binding of the $(OP)_2Cu^{+}$ complex to DNA may well alter

the reaction pathway so as to allow •OH formation (Goldstein & Czapski, 1986). We therefore propose that the species responsible for formation of DNA base products by the $(OP)_2Cu^+$ system is •OH, formed upon DNA itself and so not easily accessible to "classical" •OH scavengers such as mannitol and formate. This would be consistent with the data of Williams et al. (1988), who concluded that the DNA-damaging species can diffuse over a limited range from the site of $(OP)_2Cu^+$ binding, suggesting that it is unlikely to be an oxo–copper ion complex.

It is interesting to compare formation of DNA base products generated by the $(OP)_2Cu^+$ system with formation of those generated by the bleomycin system. Kohda et al. (1989) reported that small amounts of 8-OH-Gua were formed in DNA upon treatment with the bleomycin/Fe^{2+} system. However, formation of modified bases in the bleomycin/Fe^{2+} system appears to be a minor side reaction in comparison to sugar damage measured by the TBA test. By contrast, the amount of base modification by the $(OP)_2Cu^+$ system determined in the present study was greater than the amount of sugar damage measured by the TBA test.

The ratio of the yields of 8-hydroxypurines to those of formamidopyrimidines is much greater than 1 in DNA treated with $(OP)_2Cu^{2+}$/asc or $(OP)_2Cu^{2+}$/ME. This is in contrast to the ratios of these products observed with other •OH-producing systems such as ionizing radiation (Fuciarelli et al., 1989), hypoxanthine/xanthine oxidase, and Fe^{3+} chelates/H_2O_2 (Aruoma et al., 1989a,b). Since 8-hydroxypurines and formamidopyrimidines are respectively oxidation and reduction products of the purine radical that results from addition of •OH to the C-8 position of the purine ring [for a review, see Steenken (1989)], the $(OP)_2Cu^+$ system appears to favor the oxidation of the C-8–OH adducts of purines despite the presence of a reducing agent such as asc or ME.

In conclusion, we have characterized and quantitated the base products in DNA produced by $(OP)_2Cu^{2+}$/asc and $(OP)_2Cu^{2+}$/ME. The type of products and the inhibition of product formation by catalase strongly suggest the involvement of •OH generated from H_2O_2 in the mechanism underlying product formation.

References

Aruoma, O. I., Halliwell, B., & Dizdaroglu, M. (1989a) *J. Biol. Chem. 264*, 13024–13028.

Aruoma, O. I., Halliwell, B., Gajewski, E., & Dizdaroglu, M. (1989b) *J. Biol. Chem. 264*, 20509–20512.

Blakely, W. F., Fuciarelli, A. F., Wegher, B. J., & Dizdaroglu, M. (1990) *Radiat. Res. 121*, 338–343.

Czapski, G., Goldstein, S., & Meyerstein, D. (1988) *Free Radical Res. Commun. 4*, 231–236.

Dizdaroglu, M. (1985) *Anal. Biochem. 144*, 593–603.

Dizdaroglu, M., & Gajewski, E. (1990) *Methods Enzymol. 186*, 530–544.

Dizdaroglu, M., Holwitt, E., Hagan, M. P., & Blakely, W. F. (1986) *Biochem. J. 235*, 531–536.

Eberhardt, M. K., Ramirez, G., & Ayala, A. (1989) *J. Org. Chem. 54*, 5922–5926.

Evans, P. J., Bomford, A., & Halliwell, B. (1989) *Free Radical Res. Commun. 7*, 55–62.

Fuciarelli, A. F., Wegher, B. J., Gajewski, E., Dizdaroglu, M., & Blakely, W. F. (1989) *Radiat. Res. 119*, 219–231.

Goldstein, S., & Czapski, G. (1986) *J. Am. Chem. Soc. 108*, 2244–2250.

Gutteridge, J. M. C. (1984) *Biochem. J. 218*, 983–985.

Gutteridge, J. M. C., & Halliwell, B. (1982) *Biochem. Pharmacol. 31*, 2801–2805.

Gutteridge, J. M. C., Winyard, P. G., Blake, D. R., Lunec, J., Brailsford, S., & Halliwell, B. (1985) *Biochem. J. 230*, 517–523.

Halliwell, B., & Gutteridge, J. M. C. (1990) *Methods Enzymol. 186*, 1–85.

Halliwell, B., Gutteridge, J. M. C., & Aruoma, O. I. (1987) *Anal. Biochem. 165*, 215–219.

Johnson, G. R. A., & Nazhat, N. B. (1987) *J. Am. Chem. Soc. 109*, 1990–1994.

Johnson, G. R. A., Nazhat, N. B., & Saadalla-Nazhat, R. A. (1985) *J. Chem. Soc., Chem. Commun.*, 407–408.

Kohda, K., Kasai, H., Ogawa, T., Suzuki, T., & Kawazoe, Y. (1989) *Chem. Pharm. Bull. 37*, 1028–1030.

Marshall, L. E., Graham, D. R., Reich, K. A., & Sigman, D. S. (1981) *Biochemistry 20*, 244–250.

Masarwa, M., Cohen, H., Meyerstein, D., Hickman, D. L., Bakac, A., & Espenson, J. H. (1988) *J. Am. Chem. Soc. 110*, 4293–4297.

McCord, J. M., & Fridovich, I. (1969) *J. Biol. Chem. 224*, 6049–6055.

Pope, L. E., & Sigman, D. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3–7.

Puppo, A., & Halliwell, B. (1989) *Free Radical Res. Commun. 5*, 277–281.

Que, B. G., Downey, K. M., & So, A. G. (1980) *Biochemistry 19*, 5987–5991.

Reich, K. A., Marshall, L. E., Graham, D. R., & Sigman, D. S. (1981) *J. Am. Chem. Soc. 103*, 3582–3584.

Rowley, D. A., & Halliwell, B. (1983) *Arch. Biochem. Biophys. 225*, 279–284.

Sigman, D. S. (1986) *Acc. Chem. Res. 19*, 180–186.

Sigman, D. S., Graham, D. R., D'Aurora, V., & Stern, A. M. (1979) *J. Biol. Chem. 254*, 12269–12271.

Steenken, S. (1989) *Chem. Rev. 89*, 503–520.

Sutton, H. C., & Winterbourn, C. C. (1989) *Free Radical Biol. Med. 6*, 53–60.

Teoule, R., & Cadet, J. (1978) in *Effects of Ionizing Radiation on DNA* (Hütterman, J., Köhnlein, W., & Teoule, R., Eds.) pp 171–203, Springer-Verlag, Berlin.

Thederahn, T. B., Kuwabara, M. D., Larsen, T. A., & Sigman, D. S. (1989) *J. Am. Chem. Soc. 111*, 4941–4946.

Veal, J. M., & Rill, R. L. (1988) *Biochemistry 27*, 1822–1827.

von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor & Francis, London.

Williams, L. D., Thivierge, J., & Goldberg, I. H. (1988) *Nucleic Acids Res. 16*, 11607–11615.

Yamamoto, K., & Kawanishi, S. (1989) *J. Biol. Chem. 264*, 15435–15440.