FINAL PERFORMANCE REPORT

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IONIZING RADIATION-INDUCED DNA DAMAGE AND ITS REPAIR IN HUMAN CELLS

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by

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Abbreviations

DPC, DNA-protein cross-link; **GC/MS-SIM**, gas chromatography/mass spectrometry with selected-ion monitoring; **$^{\bullet}$OH**, hydroxyl radical; e_{aq}^{-}, hydrated electron; $O_2^{\bullet-}$, superoxide radical; **J**, joule; **5,6-diHThy**, 5,6-dihydrothymine; **5-OH-5-Me-Hyd**, 5-hydroxy-5-methyl-hydantoin; **5-OH-Hyd**, 5-hydroxyhydantoin; **5-OH-Ura**, 5-hydroxyuracil; **5-OH-Cyt**, 5-hydroxycytosine; **5-OH-6-HThy**, 5-hydroxy-6-hydrothymine; **5-OH-6-HUra**, 5-hydroxy-6-hydrouracil; **5-OH-6-HCyt**, 5-hydroxy-6-hydrocytosine; **5-OHMe-Ura**, 5-(hydroxymethyl)-uracil; **Cyt glycol**, cytosine glycol; **Thy glycol**, thymine glycol; **5,6-diOH-Ura**, 5,6-dihydroxyuracil; **5,6-diOH-Cyt**, 5,6-dihydroxycytosine; **FapyAde**, 4,6-diamino-5-formamidopyrimidine; **8-OH-Ade**, 8-hydroxyadenine; **2-OH-Ade**, 2-hydroxyadenine; **FapyGua**, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; **8-OH-Gua**, 8-hydroxyguanine; **Me_3Si**, trimethylsilyl; **n.d.**, not detected; **NTA**, nitrilotriacetic acid; **SOD**, superoxide dismutase; **Me_2SO** (or **DMSO**), dimethyl sulfoxide; **Thy-Tyr cross-link**, 3-[(1,3-dihydro-2,4-dioxo-pyrimidin-5-yl)methyl]-L-tyrosine.

A. PROGRESS REPORT

ACCOMPLISHMENTS

I. INTRODUCTION

DNA damage in mammalian chromatin *in vitro* and in cultured mammalian cells including human cells was studied. In the first phase of these studies, a cell culture laboratory was established. Necessary equipment including an incubator, a sterile laminar flow hood and several centrifuges was purchased. We have successfully grown several cell lines such as murine hybridoma cells, V79 cells and human K562 leukemia cells. This was followed by the establishment of a methodology for the isolation of chromatin from cells. As was indicated in the research plan of the original proposal (page 11, Section III, 2.a.), this was a very important step, because a routine and successful isolation of chromatin was a prerequisite for the success of the further studies in this project, the aim of which was the measurement of DNA damage in mammalian chromatin *in vitro* and in cultured cells. Chromatin isolation was accomplished using a slightly modified procedure of the one described by Mee & Adelstein (1981). For identification and quantitation of DNA damage in cells, analysis of chromatin was preferred over the analysis of "naked DNA" for the following reasons:

i. DNA may not be extracted efficiently from nucleoprotein in exposed cells, due to formation of DNA-protein cross-links (Smith 1976, Yamamoto 1976, Mee & Adelstein 1979, 1981, Oleinick et al. 1987),

ii. the extractibility of DNA is well known to decrease with increasing doses of radiation (Yamamoto 1976, Mee & Adelstein 1979),

iii. portions of DNA may not be extracted due to fragmentation,

iv. unextracted DNA may contain a significant portion of damaged DNA bases and DNA-protein cross-links.

The technique of gas chromatography/mass spectrometry (GC/MS), which was used in the present project, permits the identification and quantitation of modified DNA bases in chromatin in the presence of proteins without the necessity of first isolating DNA from chromatin. This has been demonstrated previously by the results from our laboratory (Dizdaroglu & Gajewski 1990, also see original proposal, pages 11, 11A, and 11B) and by the results obtained during the course of the present project (see e.g., Gajewski et al. 1990). The quality of isolated chromatin was tested by measurement of its content of DNA, proteins, and RNA, by analysis of its protein components using gel electrophoresis, and by absorption spectral analysis. Generally, the RNA content was $\leq 5\%$ of the amount of DNA, and the ratio of the amount of protein to that of DNA was ≈ 1.8—2 (w/w). Figure 1 illustrates a representative absorption spectrum of isolated chromatin with spectral characteristics of: $A_{258}/A_{280} = 1.58$; $A_{258}/A_{230} = 1.1$; $A_{258}/A_{320} = 8.5$; A(maximum)/A(minimum) = 1.26. Figure 2 illustrates a representative picture of gel electrophoretic patterns of isolated chromatin and commercial histones. Figure 2 indicates the presence of histones H1, H3, H2B, H2A, and H4 in chromatin [for more details on isolation and characterization of chromatin, see Gajewski et al. (1990)]. The absorption spectral characteristics of chromatin given above and the gel electrophoretic patterns of histones of chromatin shown in Figure 2 were typical of all samples of chromatin isolated in subsequent studies.

Having developed a suitable methodology for routine isolation of chromatin from mammalian cells, studies of DNA damage in chromatin *in vitro* and in cultured human cells were pursued.

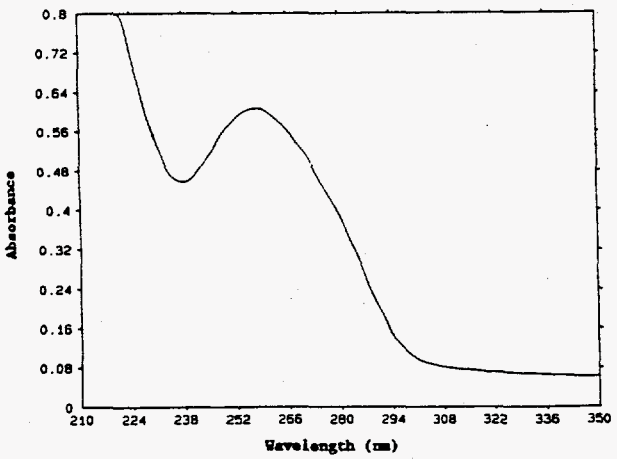


FIGURE 1: Absorption spectrum of isolated chromatin in phosphate buffer.

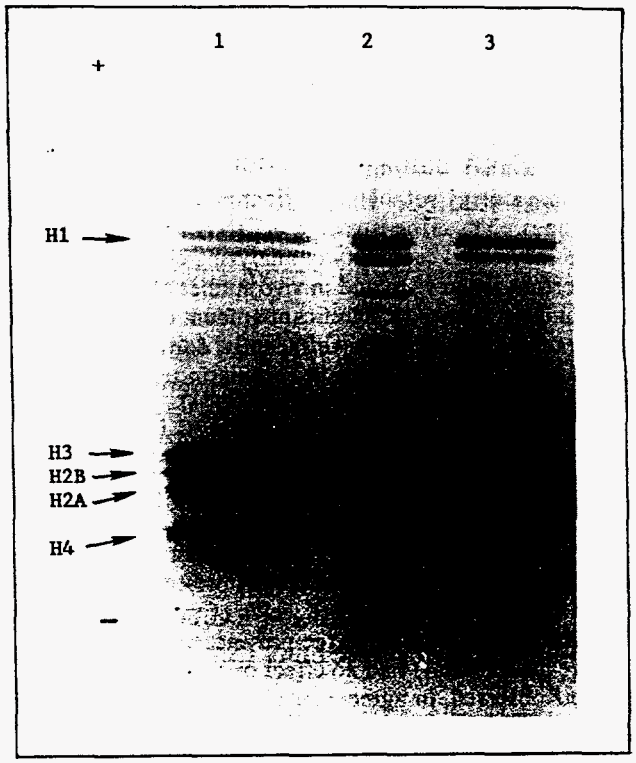


FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of chromatin suspended in Tris-HCl buffer (lane 1), histones H1, H3, H2B, H2A, and H4 (lane 2), and chromatin dialyzed against and suspended in phosphate buffer (lane 3).

II. STUDIES OF DNA DAMAGE IN CHROMATIN *in vitro*

According to the research plan in the original proposal (page 13, Section III, 2.e.), we have studied radiation-induced DNA damage in mammalian chromatin in aqueous suspension in the presence of various gases, which provided different radical environments. Argon, air, N_2O and N_2O/O_2 were used for this purpose. The yields of radical species, i.e., hydroxyl radical ($\cdot OH$), hydrated electron (e_{aq}^-) and H atom, produced from water by ionizing radiation depend on the presence of these gases. Of the radical species, $\cdot OH$ is formed under all four gaseous conditions with the yields of 0.28 μmol/J (argon and air) and 0.56 μmol/J (N_2O and N_2O/O_2); e_{aq}^- is present only in the presence of argon (0.27 μmol/J); H atom is present under argon and N_2O with equal yields (0.057 μmol/J); superoxide radical (O_2^-) is formed under air and N_2O/O_2 with the yields of 0.33 and 0.057, respectively [yields are adapted from the review by von Sonntag (1987)]. The gaseous conditions used facilitate the study of the effects of different radical environments and oxygen on the types and yields of DNA products.

1. Base Damage in Chromatin

a. base damage produced by ionizing radiation

Chromatin was isolated from murine hybridoma cells. Aqueous suspensions of chromatin were exposed to ionizing radiation at doses ranging from 20 to 200 Gy. Samples were hydrolyzed, trimethylsilylated and analyzed by GC/MS with selected-ion monitoring (SIM) (for experimental details see Gajewski et al. 1990). Figure 3 illustrate typical selected-ion current profiles.

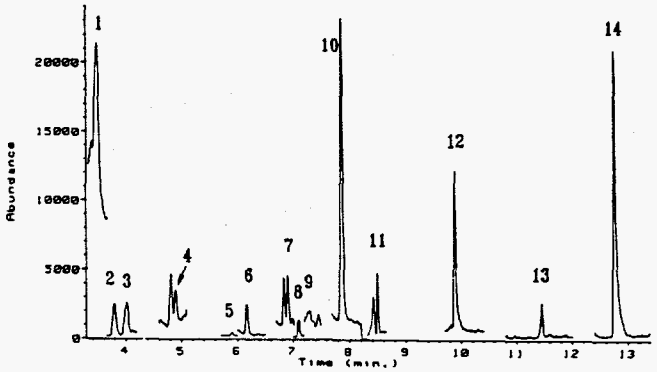


FIGURE 3: Ion-current profiles obtained during the GC/MS-SIM analysis of a trimethylsilylated hydrolysate of mammalian chromatin exposed to ionizing radiation in N_2O/O_2-saturated aqueous suspension. Radiation dose was 40 Gy. The column was programmed from 150 to 260 °C at a rate of 8 °C/min after 2 min at 150 °C. Amount of an aliquot of DNA in chromatin injected onto the column was approximately 0.4 μg. For other details see Experimental Procedures. Peaks (ion monitored): 1, 6-azathymine (*m/z* 256) (internal standard); 2, 5-OH-5-MeHyd (*m/z* 331); 3, 5-OH-Hyd (*m/z* 317); 4, 5-OH-Ura (*m/z* 329); 5, 5-OHMeUra (*m/z* 358); 6, 5-OH-Cyt (*m/z* 343); 7, *cis*-Thy glycol (*m/z* 259); 8, 5,6-dihydroxyuracil (*m/z* 417); 9, *trans*-Thy glycol (*m/z* 259); 10, 8-azaadenine (*m/z* 265) (internal standard); 11, FapyAde (*m/z* 354); 12, 8-OH-Ade (*m/z* 352); 13, FapyGua (*m/z* 442); 14, 8-OH-Gua (*m/z* 440) (all compounds as their trimethylsilyl derivatives).

The modified bases identified and their yields under four different gaseous conditions are listed in Table I. Figure 4 illustrates representative dose-yield plots of some products. Linear dose-yield plots were obtained for all products at the dose range used. The results obtained in this study clearly indicated that DNA base products identified previously in model systems such as bases, nucleosides, and nucleotides, and in DNA (reviewed in Teoule & Cadet 1978, von Sonntag 1987) were also formed in mammalian chromatin in aqueous suspension upon exposure to ionizing radiation. The types of modified bases and their quantities depended on the radical environment and the gas present in the aqueous system. The higher yields of the products in the presence of N_2O/O_2 than in the presence of air indicate that O_2^- (see also Table I) had no effect on the production of the products observed, in agreement with previous observations (Aruoma et al. 1989). In the presence of oxygen (air and N_2O/O_2), 5,6-diHThy, 5-OH-6-HThy, and 5-OH-6-HCyt were not detected. 5,6-DiHThy arises from reactions of e_{aq}^- and H atom with the thymine moiety of DNA (reviewed in Teoule & Cadet 1978, and von Sonntag 1987). Thus this compound is not formed in the presence of oxygen. As expected, the yield of 5,6-diHThy in the presence

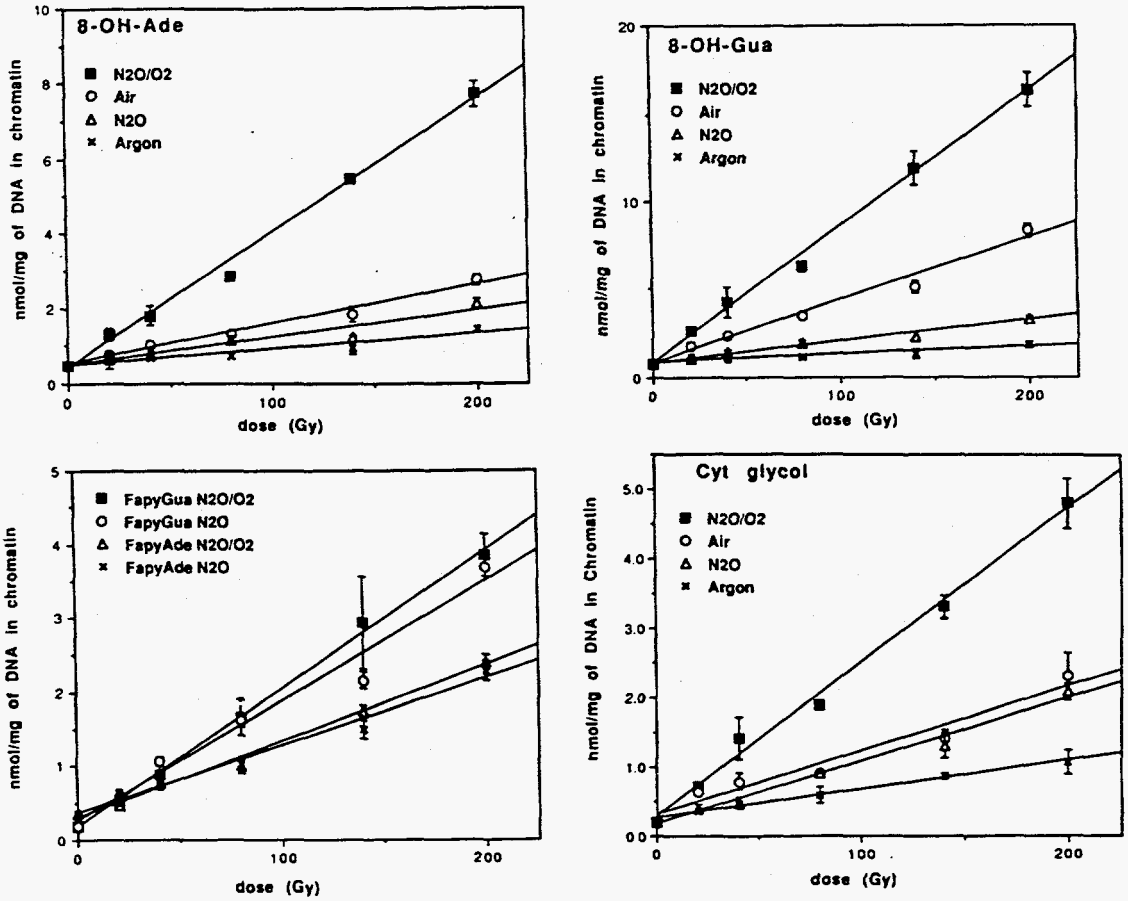


FIGURE 4: Dose–yield plots of some products. Error bars represent standard deviations of the means from three independent measurements.

TABLE I: Yields ($nmol \cdot J^{-1}$) of the DNA Base Products in Chromatin

product	argon	air	N_2O	N_2O/O_2	amount (μmol/mol of nucleotides) in control chromatin
5,6-diHThy	0.48 ± 0.04	nd[a]	0.26 ± 0.03	nd	nd
5-OH-5-MeHyd	*b*	0.21 ± 0.02	*b*	0.42 ± 0.02	64 ± 6.4
5-OH-Hyd	*b*	*b*	*b*	0.22 ± 0.02	73.6 ± 4.5
Cyt glycol	0.50 ± 0.025	1.10 ± 0.04	0.95 ± 0.03	2.30 ± 0.18	64 ± 19.2
5-OH-6HThy	0.30 ± 0.03	nd	0.30 ± 0.03	nd	nd
5-OH-6-HCyt	0.17 ± 0.02	nd	0.17 ± 0.02	nd	nd
5-OHMeUra	0.045 ± 0.005	0.02 ± 0.003	0.064 ± 0.008	0.052 ± 0.007	3.2 ± 1.28
Thy glycol	0.045 ± 0.004	0.18 ± 0.02	0.094 ± 0.01	0.40 ± 0.03	35.2 ± 2.56
5,6-diOH-Cyt	*b*	0.14 ± 0.007	*b*	0.33 ± 0.03	25.6 ± 3.2
FapyAde	0.82 ± 0.04	0.60 ± 0.04	0.96 ± 0.06	1.02 ± 0.08	48.0 ± 1.9
8-OH-Ade	0.45 ± 0.02	1.10 ± 0.08	0.80 ± 0.07	3.50 ± 0.30	89.6 ± 5.8
FapyGua	0.96 ± 0.03	1.11 ± 0.05	1.81 ± 0.18	1.81 ± 0.18	16.0 ± 5.4
8-OH-Gua	0.55 ± 0.03	3.85 ± 0.20	1.35 ± 0.12	8.05 ± 0.65	182 ± 4.2

[a] Not detected. [b] No increase in the amount above the background level was observed at doses ranging from 20 to 200 Gy.

of argon is higher than that in the presence of N_2O because of the diffusion-controlled reaction of e_{aq}^- with N_2O. 5-OH-6-HThy and 5-OH-6-HCyt are produced by reactions of $^{\bullet}OH$ with the thymine and

cytosine moieties of DNA, respectively, followed by reduction of so-formed C-5 OH-adduct radicals (reviewed in von Sonntag 1987). Their formation is inhibited in the presence of oxygen due to the diffusion-controlled reaction of oxygen with the OH-adduct radicals of thymine and cytosine. An increase in the amount of 5,6-diOH-Cyt was observed only in the presence of oxygen, and its yield was 2-fold higher in the presence of N_2O/O_2 than in the presence of air. This is because 5,6-diOH-Cyt is produced by addition of $^{\bullet}OH$ to the 5,6-double bond of cytosine followed by addition of oxygen to the OH-adduct radical and subsequent oxidation (reviewed in Teoule & Cadet 1978). An increase in the amount of 5-OH-Hyd due to irradiation was observed only in the presence of N_2O/O_2. This is presumably because 5-OH-Hyd is not a primary product and results from acid-induced modification of 5,6-diOH-Cyt. Other remaining products of pyrimidines Cyt glycol, Thy glycol and 5-OHMe-Ura, and all the products of purines were observed under all four gaseous conditions. The yields of Cyt glycol and Thy glycol were higher in the presence of oxygen than in its absence. This is expected because oxygen inhibits the formation of 5-OH-6-hydropyrimidines, which result from OH-adduct radicals of pyrimidines as do pyrimidine glycols. The formation of 8-hydroxypurines was affected substantially by the presence of oxygen in the system. Much higher yields of both 8-OH-Ade and 8-OH-Gua were observed with oxygen than without oxygen in the system. By contrast, the yields of formamidopyrimidines were not affected by addition of oxygen to the system, and were even higher than those of 8-hydroxypurines in the absence of oxygen. The addition of N_2O to the system increased the yields of both formamidopyrimidines and 8-hydroxypurines, indicating the role of $^{\bullet}OH$ in their formation. These two types of compounds result from addition of $^{\bullet}OH$ to the C-8 of purines followed by respective one-electron reduction and oxidation of the C-8 OH-adduct radicals (reviewed in Steenken 1989). Apparently, the presence of oxygen in the system strongly favors the oxidation of the C-8 OH-adduct radicals of purines as the ratios of the yields of 8-hydroxypurines to those of formamidopyrimidines suggest: 8-OH-Gua/FapyGua = 0.57 in argon vs. 3.47 in air, and 0.75 in N_2O vs. 4.45 in N_2O/O_2; 8-OH-Ade/FapyAde = 0.56 in argon vs. 1.83 in air, and 0.83 in N_2O vs. 3.43 in N_2O/O_2. In the case of Ade, the higher yield of 8-OH-Ade than that of FapyAde in the presence of oxygen might be explained by the addition of oxygen in a diffusion-controlled reaction to the C-8 OH-adduct radical of Ade followed by $HO_2^{\bullet}$ elimination (loss of O_2^{-} followed by deprotonization) as was described by Vieira & Steenken (1987). Whether this mechanism holds for the formation of 8-OH-Gua as well, is not known. No satisfactory explanation can be given for the formation of formamidopyrimidines in the presence of oxygen. The fact that the ring opening reaction of the C-8 OH-adduct radical of adenine, which precedes the formation of FapyAde, is suppressed by oxidants such as oxygen (Vieira & Steenken 1987), is in agreement with the findings here. This might also hold for the C-8 OH-adduct radical of guanine, because the values of the ratio 8-OH-Gua/FapyGua had an analogous trend to those of the ratio 8-OH-Ade/FapyAde. Nevertheless, the formation of formamidopyrimidines is not suppressed, and these compounds have appreciable yields in the presence of oxygen, compared with the yields of the other products. This is in contrast to pyrimidines because 5-OH-6-HThy and 5-OH-6-HCyt, which result similarly from reduction of C-5 OH-adduct radicals of Thy and Cyt, respectively, were not formed in the presence of oxygen. The overall yields of the modified bases in chromatin irradiated in aqueous suspension are much lower than the yields of the same products determined in DNA irradiated in aqueous solution under similar conditions (Fuciarelli et al. 1990). This is expected because of the presence of histones in chromatin. Besides the simple scavenging of free radicals by the histones, the nucleosome structure might also play an important role in the production of the products. Because chromatin is in suspension rather than in solution, only those radicals formed in the immediate vicinity of DNA in chromatin might cause formation of the products. Furthermore, the

DNA bases might participate in the formation of DNA-protein cross-links in mammalian chromatin (reviewed in Oleinick et al. 1987), resulting in the decreased yields of the modified bases. A paper describing the results on the effect of ionizing radiation on mammalian chromatin under various gaseous conditions has been published in *Biochemistry* (Gajewski et al. 1990).

b. base damage produced by hydrogen peroxide and metal ions

As an extension of the studies on radiation-induced DNA damage in chromatin, we have studied the effect of H_2O_2 in the presence of transition metal ions on chromatin DNA. Hydrogen peroxide is ubiquitous in biological systems and is formed in all aerobic cells as a result of normal cellular metabolism (reviewed in Halliwell & Gutteridge 1990). Hydrogen peroxide is also formed from radiolysis of water with a yield of 0.073 μmol/J (anoxic conditions) (reviewed in von Sonntag 1987). Unlike •OH, which has a short diffusion distance in cells due its high reactivity toward organic molecules and thus reacts at or close to its site of formation, H_2O_2 can diffuse long distances in cells to reach DNA and can also cross biological membranes. Most of the •OH generated *in vivo*, except during exposure to ionizing radiation, results from transition-metal ion-catalyzed conversion of H_2O_2 (Haber-Weiss reaction) (reviewed in Halliwell & Gutteridge 1990). In the past, H_2O_2 has been used as a source of •OH and as a radiomimetic agent, because it produces lesions in DNA similar to those produced by ionizing radiation (see e.g., Ward et al. 1985, Lesko et al. 1982). However, DNA damage in chromatin by H_2O_2 in the presence of metal ions has not been characterized. We studied DNA base modifications in mammalian chromatin by H_2O_2 in the presence of Fe(III), Cu(II), Co (II) and Ni(II) ions. Fe(III) and Cu(II) ions are ubiquitous in biological systems (see e.g., George et al. 1987, Halliwell & Gutteridge 1990). Co(II) and Ni(II) ions are known to be carcinogenic to humans and animals (Costa 1991, Leonard & Lauwerys 1990).

Isolated chromatin was treated with H_2O_2 in the presence of chelated or unchelated Fe(III) and Cu(II) ions. Chromatin samples were then hydrolyzed, trimethylsilylated and analyzed by GC/MS-SIM. Figure 5 illustrates representative selected-ion current profiles. A comparison of this Figure with Figure 3 indicates that the nature of DNA base products were the same as in chromatin exposed to ionizing radiation in the presence of oxygen. The yields of DNA base products in chromatin treated with H_2O_2 in the presence of unchelated and chelated Fe(III) ions are listed in Tables II and III. Incubation of chromatin with H_2O_2 or with Fe(III) ions alone did not produce any increase in the amount of the compounds identified in untreated chromatin. H_2O_2/Fe(III) produced a slight increase in the amounts of all the compounds over the background levels, and also caused formation of traces of 5-OHMeUra (Table II). By contrast, the treatment of chromatin with H_2O_2/Fe(III) chelated with EDTA or NTA produced significant increases in the amounts of the products (Table III). H_2O_2/Fe(III)-NTA produced the highest amount of base damage. In all cases, addition of ascorbic acid to reaction mixtures caused a substantial increase in product yields with a particularly high proportional increase in the yields of formamido-pyrimidines over the background levels.

The yields of DNA base products formed in chromatin by H_2O_2/Cu(II) ions are shown in Table IV. Treatment of chromatin with Cu(II) ions alone caused an increase in the amounts of all the compounds with a substantial increase in the amounts of 8-hydroxypurines. The chelation of Cu(II) ions with NTA decreased the product formation; background levels of the amounts of most products were observed. Treatment of chromatin with H_2O_2/Cu(II) caused extensive damage to the DNA bases, much

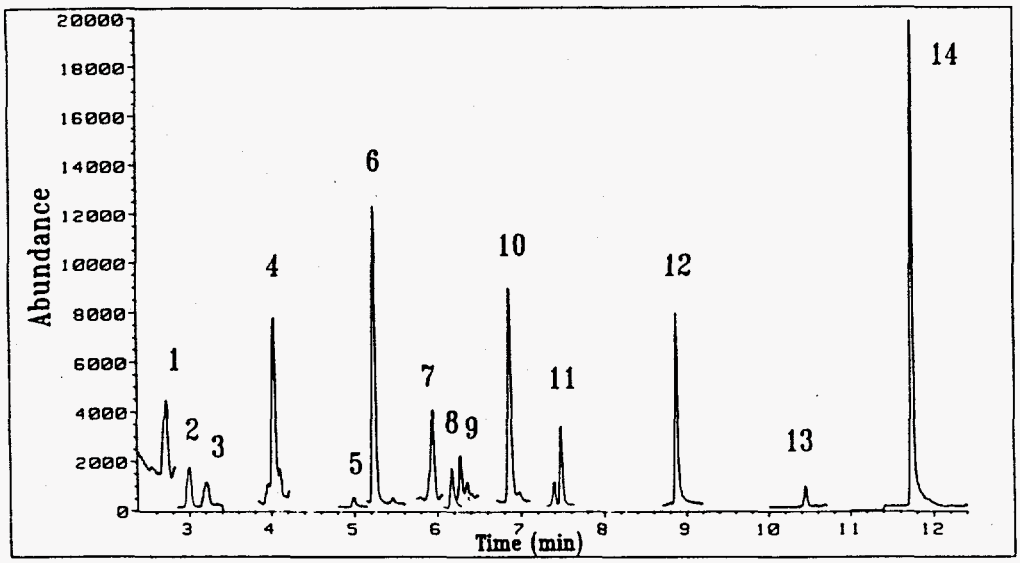


FIGURE 5: Ion-current profiles obtained during the GC/MS-SIM analysis of a trimethylsilylated hydrolysate of mammalian chromatin treated with H_2O_2 in the presence of Fe^{3+}-NTA. For experimental details see Materials and Methods. The column was programmed from 150 to 260°C at a rate of 8°C/min after 2 min at 150°C. The amount of an aliquot of DNA in chromatin injected onto the column was approximately 0.4 μg. Peaks (ion monitored): 1, 6-azathymine (*m/z* 271) (internal standard); 2, 5-OH-5-MeHyd (*m/z* 331); 3, 5-OH-Hyd (*m/z* 317); 4, 5-OH-Ura (*m/z* 329); 5, 5-OHMeUra (*m/z* 358); 6, 5-OH-Cyt (*m/z* 343); 7, *cis*-Thy glycol (*m/z* 259); 8, 5,6-dihydroxyuracil (*m/z* 417); 9, *trans*-Thy glycol (*m/z* 259); 10, 8-azaadenine (*m/z* 265) (internal standard); 11, FapyAde (*m/z* 354); 12, 8-OH-Ade (*m/z* 352); 13, FapyGua (*m/z* 442); 14, 8-OH-Gua (*m/z* 440) (all compounds as their trimethylsilyl derivatives). [It should be noted that 5-OH-Ura (peak 4) and 5-OH-Cyt (peak 6) arise by acid-induced modification of Cyt glycol. Similarly, 5,6-dihydroxyuracil (peak 8) is produced by acid-induced deamination of 5,6-diOH-Cyt. 5-OH-5-MeHyd (peak 2) and 5-OH-Hyd (peak 3) are believed to result from acid-induced modification of 5-methyl-5-hydroxybarbituric acid and 5,6-diOH-Cyt, respectively.]

TABLE II: Yields (nmol/mg of DNA[a]) of DNA Base Products Formed in Chromatin by Treatment with the H_2O_2/Fe^{3+} System

	Treatment							
Product	1[b]	2	3	4	5	6	7	8
5-OH-5-MeHyd	0.11 ± 0.02	0.10 ± 0.005	0.11 ± 0.016	0.19 ± 0.006	0.60 ± 0.015	0.58 ± 0.05	0.25 ± 0.03	0.28 ± 0.07
5-OH-Hyd	0.23 ± 0.014	0.24 ± 0.014	0.22 ± 0.018	0.29 ± 0.027	0.39 ± 0.02	0.52 ± 0.05	0.32 ± 0.03	0.30 ± 0.03
Cyt glycol	0.55 ± 0.06	0.56 ± 0.06	0.54 ± 0.02	1.30 ± 0.155	4.38 ± 0.10	4.48 ± 0.18	1.60 ± 0.15	1.79 ± 0.29
5-OHMeUra	n.d.[c]	n.d.	n.d.	0.03 ± 0.01	0.09 ± 0.013	0.13 ± 0.01	0.03 ± 0.004	0.04 ± 0.01
Thy glycol	0.08 ± 0.015	0.12 ± 0.03	0.07 ± 0.007	0.16 ± 0.025	0.79 ± 0.07	0.85 ± 0.146	0.28 ± 0.03	0.23 ± 0.06
5,6-diOH-Cyt	0.11 ± 0.001	0.09 ± 0.003	0.07 ± 0.005	0.30 ± 0.016	0.84 ± 0.05	0.62 ± 0.085	0.28 ± 0.02	0.24 ± 0.013
FapyAde	0.15 ± 0.006	0.15 ± 0.02	0.30 ± 0.05	0.30 ± 0.04	2.18 ± 0.084	2.58 ± 0.37	0.84 ± 0.07	0.79 ± 0.097
8-OH-Ade	0.28 ± 0.018	0.28 ± 0.01	0.33 ± 0.01	0.91 ± 0.057	2.57 ± 0.06	2.64 ± 0.19	0.98 ± 0.10	0.94 ± 0.22
FapyGua	0.05 ± 0.017	0.06 ± 0.02	0.20 ± 0.05	0.21 ± 0.06	1.99 ± 0.33	1.95 ± 0.20	1.02 ± 0.11	0.96 ± 0.25
8-OH-Gua	0.57 ± 0.013	0.53 ± 0.04	0.83 ± 0.06	1.73 ± 0.38	12.0 ± 0.77	12.4 ± 0.18	5.60 ± 0.60	5.89 ± 0.37

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

[b] 1, chr; 2, chr/H_2O_2; 3, chr/H_2O_2/asc; 4, chr/H_2O_2/Fe^{3+}; 5, chr/H_2O_2/Fe^{3+}/asc; 6, chr/H_2O_2/Fe^{3+}/asc/SOD; 7, chr/H_2O_2/Fe^{3+}/asc/mannitol; 8, chr/H_2O_2/Fe^{3+}/asc/DMSO.

[c] Not detected.

TABLE III:

Yields (nmol/mg of DNA[a]) of DNA Base Products Formed in Chromatin by Treatment with the H_2O_2/Chelated Fe^{3+} System

	Treatment						
Product	9[b]	10	11	12	13	14	15
5-OH-5-MeHyd	0.29 ± 0.025	0.48 ± 0.006	0.56 ± 0.05	0.84 ± 0.005	0.44 ± 0.035	0.15 ± 0.001	0.16 ± 0.015
5-OH-Hyd	0.30 ± 0.01	0.35 ± 0.017	0.41 ± 0.026	0.47 ± 0.017	0.52 ± 0.12	0.36 ± 0.03	0.31 ± 0.017
Cyt glycol	2.77 ± 0.26	3.45 ± 0.21	5.54 ± 1.14	6.68 ± 0.50	4.31 ± 0.68	0.85 ± 0.07	0.74 ± 0.038
5-OHMeUra	0.07 ± 0.009	0.07 ± 0.004	0.10 ± 0.014	0.15 ± 0.026	0.10 ± 0.02	0.06 ± 0.005	0.05 ± 0.01
Thy glycol	0.17 ± 0.048	0.59 ± 0.09	0.82 ± 0.18	1.78 ± 0.08	0.62 ± 0.03	0.15 ± 0.01	0.13 ± 0.014
5,6-diOH-Cyt	0.45 ± 0.06	0.71 ± 0.045	0.85 ± 0.08	1.23 ± 0.015	0.37 ± 0.10	0.11 ± 0.005	0.07 ± 0.016
FapyAde	0.57 ± 0.09	1.66 ± 0.013	1.77 ± 0.09	2.68 ± 0.40	0.94 ± 0.138	0.25 ± 0.02	0.26 ± 0.06
8-OH-Ade	2.06 ± 0.23	1.06 ± 0.015	2.65 ± 0.25	3.34 ± 0.08	1.54 ± 0.086	0.40 ± 0.05	0.36 ± 0.065
FapyGua	0.38 ± 0.006	2.03 ± 0.07	0.78 ± 0.15	1.75 ± 0.22	1.23 ± 0.04	0.20 ± 0.025	0.18 ± 0.047
8-OH-Gua	3.40 ± 0.34	4.95 ± 0.11	6.60 ± 0.34	9.57 ± 0.61	5.03 ± 0.15	0.88 ± 0.09	0.75 ± 0.07

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

[b] 9, chr/H_2O_2/Fe^{3+}-EDTA; 10, chr/H_2O_2/Fe^{3+}-EDTA/asc; 11, chr/H_2O_2/Fe^{3+}-NTA; 12, chr/H_2O_2/Fe^{3+}-NTA/asc; 13, chr/H_2O_2/Fe^{3+}-NTA/asc/SOD; 14, chr/H_2O_2/Fe^{3+}-NTA/asc/mannitol; 15, chr/H_2O_2/Fe^{3+}-NTA/asc/DMSO.

TABLE IV:

Yields (nmol/mg of DNA[a]) of DNA Base Products Formed in Chromatin by Treatment with the H_2O_2/Cu^{2+} System

	Treatment							
Product	1[b]	2	3	4	5	6	7	8
5-OH-5-MeHyd	0.11 ± 0.02	0.15 ± 0.014	0.10 ± 0.002	0.36 ± 0.06	0.52 ± 0.013	0.56 ± 0.02	0.44 ± 0.034	0.36 ± 0.04
5-OH-Hyd	0.23 ± 0.014	0.30 ± 0.017	0.30 ± 0.016	0.38 ± 0.026	0.56 ± 0.136	0.60 ± 0.012	0.38 ± 0.023	0.44 ± 0.027
Cyt glycol	0.55 ± 0.06	1.92 ± 0.02	0.56 ± 0.01	5.59 ± 0.54	10.7 ± 0.75	10.8 ± 0.30	7.48 ± 0.214	9.61 ± 0.33
5-OHMeUra	n.d.[c]	0.07 ± 0.01	n.d.	0.10 ± 0.006	0.21 ± 0.011	0.24 ± 0.02	0.23 ± 0.02	0.20 ± 0.023
Thy glycol	0.08 ± 0.015	0.22 ± 0.05	0.07 ± 0.007	0.55 ± 0.19	0.95 ± 0.11	1.00 ± 0.15	1.07 ± 0.16	1.05 ± 0.05
5,6-diOH-Cyt	0.11 ± 0.001	0.05 ± 0.005	0.03 ± 0.005	0.15 ± 0.018	0.29 ± 0.05	0.25 ± 0.05	0.29 ± 0.04	0.25 ± 0.02
FapyAde	0.15 ± 0.006	0.21 ± 0.01	0.15 ± 0.017	0.59 ± 0.13	1.51 ± 0.024	1.71 ± 0.12	1.42 ± 0.155	1.52 ± 0.26
8-OH-Ade	0.28 ± 0.018	3.03 ± 0.19	0.70 ± 0.06	8.48 ± 0.97	15.4 ± 1.21	17.6 ± 0.7	13.1 ± 1.10	13.8 ± 1.12
FapyGua	0.05 ± 0.017	0.14 ± 0.03	0.07 ± 0.002	0.35 ± 0.10	1.11 ± 0.17	1.17 ± 0.06	0.45 ± 0.19	0.84 ± 0.19
8-OH-Gua	0.57 ± 0.013	3.54 ± 0.27	0.76 ± 0.14	15.1 ± 1.12	34.0 ± 1.85	35.1 ± 1.99	34.3 ± 3.15	36.0 ± 4.07

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

[b] 1, chr; 2, chr/Cu^{2+}; 3, chr/Cu^{2+}-NTA; 4, chr/H_2O_2/Cu^{2+}; 5, chr/H_2O_2/Cu^{2+}/asc; 6, chr/H_2O_2/Cu^{2+}/asc/SOD; 7, chr/H_2O_2/Cu^{2+}/asc/mannitol; 8, chr/H_2O_2/Cu^{2+}/asc/DMSO.

[c] Not detected.

more than the treatment with H_2O_2/Fe(III). Addition of ascorbic acid to the reaction mixture caused approximately a 2-fold increase in the yields of most products. By contrast to Fe(III) ions, chelation of Cu(II) ions with EDTA or NTA provided a marked inhibition of product formation (data not shown here; see Dizdaroglu et al. 1991b). DNA base products identified in chromatin were typical products of •OH, indicated the involvement of •OH in their formation. Inhibition of product formation in the presence of chelated Fe(III) and Cu(II) ions by typical scavengers of •OH supports this idea. When unchelated ions were used, however, scavengers provided partial or no inhibition. This might be due to binding of the

unchelated ions to chromatin and formation of $^{\bullet}OH$ in a site-specific manner at the binding sites in close proximity of DNA, so that the $^{\bullet}OH$ reacts with DNA bases in chromatin rather than with scavengers (Samuni et al. 1983, Ward et al. 1985). Superoxide dismutase (SOD) provided some inhibition of product formation only when chelated Fe(III) ions were used, implicating the involvement of O_2^- in the generation of $^{\bullet}OH$. There were marked quantitative differences between the effects of the chelated and unchelated forms of the same ion, and between the effects of Fe(III) and Cu(II) ions on product formation. Fe(III) and Cu(II) ions, their chelation and the presence of ascorbic acid had a pronounced effect on the ratio of the yields of 8-hydroxypurines to those of formamidopyrimidines. The presence of Cu(II) ions favored the oxidation of the C-8 OH-adduct radicals of purines. The results suggest that ascorbic acid increases the reduction of the C-8 OH-adduct radicals of purines, in addition to its role as a reducing agent for Fe(III) and Cu(II) ions. As was observed in radiation studies (see above), the overall yields of the modified DNA bases were substantially lower than the yields of the same modified bases found in isolated calf thymus DNA treated with H_2O_2 in the presence of Fe(III) and Cu(II) ions under the same reaction conditions (data with "naked DNA" are not shown here, see Dizdaroglu et al. 1990b). A paper describing the effect of H_2O_2 in the presence of Fe(III) and Cu(II) ions on DNA bases in mammalian chromatin has been published in *Archives of Biochemistry and Biophysics* (Dizdaroglu et al. 1991b).

The yields of DNA base products formed in mammalian chromatin by treatment with H_2O_2 in the presence of Co(II) and Ni(II) ions are given in Tables V and VI. In addition to the base products found

TABLE V: *Yields (nmol/mg of DNA) of DNA base products formed in chromatin by treatment with the Co(II)/H_2O_2 system*

	Treatment[a]					
Product	1	2	3	4	5	6
5-OH-5-Me-Hyd	0.074 ± 0.043[b]	0.505 ± 0.081[c]	0.390 ± 0.035[d]	0.218 ± 0.011[d]	0.278 ± 0.046[d]	1.38 ± 0.31[d]
5-OH-Hyd	0.250 ± 0.025	0.548 ± 0.062[c]	0.652 ± 0.069	0.380 ± 0.092[d]	0.319 ± 0.061[d]	1.03 ± 0.06[d]
5-OHMe-Ura	0.024 ± 0.003	0.227 ± 0.041[c]	0.080 ± 0.010[d]	0.081 ± 0.007[d]	0.170 ± 0.044[d]	0.485 ± 0.134[d]
Cytosine glycol	0.082 ± 0.013	1.87 ± 0.45[c]	0.484 ± 0.204[d]	0.478 ± 0.145[d]	3.75 ± 0.24[d]	3.01 ± 0.48[d]
Thymine glycol	0.017 ± 0.002	0.289 ± 0.002[c]	0.238 ± 0.120	0.088 ± 0.039[d]	0.225 ± 0.001	0.409 ± 0.156
5,6-diOH-Cyt	0.030 ± 0.003	0.344 ± 0.044[c]	0.077 ± 0.038[d]	0.095 ± 0.014[d]	0.302 ± 0.023	0.636 ± 0.076[d]
FapyAde	0.115 ± 0.028	0.895 ± 0.255[c]	0.380 ± 0.113[d]	0.318 ± 0.055[d]	0.735 ± 0.108	1.47 ± 0.044[d]
8-OH-Ade	0.113 ± 0.035	1.02 ± 0.29[c]	0.290 ± 0.042[d]	0.293 ± 0.050[d]	0.513 ± 0.031[d]	2.20 ± 0.066[d]
2-OH-Ade	0.078 ± 0.016	0.177 ± 0.056[c]	0.189 ± 0.030	0.100 ± 0.009[d]	0.108 ± 0.017	0.297 ± 0.067[d]
FapyGua	0.471 ± 0.073	1.86 ± 0.53[c]	—	1.09 ± 0.23	3.76 ± 0.374[d]	2.99 ± 0.31[d]
8-OH-Gua	0.526 ± 0.165	5.71 ± 1.99[c]	1.73 ± 0.59[d]	2.40 ± 0.57[d]	4.78 ± 0.145	12.20 ± 2.62[d]

[a] 1, chromatin; 2, chromatin/Co(II)/H_2O_2; 3, chromatin/Co(II)/H_2O_2/mannitol (50 mM); 4, chromatin/Co(II)/H_2O_2/DMSO (50 mM); 5, chromatin/Co(II)/H_2O_2/glutathione (1 mM); 6, chromatin/Co(II)/H_2O_2/SOD (200 units/ml).
[b] Mean ± SD (n = 3).
[c] Values significantly different from those of Column 1 ($P < 0.05$).
[d] Values significantly different from those of Column 2 ($P < 0.05$).

TABLE VI: *Yields (nmol/mg of DNA) of DNA base products formed in chromatin by treatment with the Ni(II)/H_2O_2 system*

	Treatment[a]						
Product	1	2	3	4	5	6	7
5-OH-5-Me-Hyd	0.176 ± 0.052[b]	0.407 ± 0.062[c]	0.539 ± 0.074[c]	1.97 ± 1.09[d]	0.796 ± 0.153[d]	1.14 ± 0.310[e]	1.69 ± 0.177
5-OH-Hyd	1.05 ± 0.042	1.33 ± 0.123	1.22 ± 0.064	1.69 ± 0.82	1.33 ± 0.204	0.829 ± 0.270[e]	1.74 ± 0.126
5-OHMe-Ura	0.097 ± 0.022	0.144 ± 0.015[c]	0.204 ± 0.097[c]	0.632 ± 0.127[d]	0.631 ± 0.211	0.378 ± 0.049[e]	1.53 ± 0.212[e]
Cytosine glycol	0.153 ± 0.006	0.146 ± 0.042	1.04 ± 0.133[c]	3.37 ± 0.716[d]	2.99 ± 0.396	2.00 ± 0.629	5.70 ± 0.637[e]
Thymine glycol	0.031 ± 0.004	0.084 ± 0.049	0.578 ± 0.095[c]	1.02 ± 0.178[d]	0.603 ± 0.209	0.544 ± 0.152[e]	1.14 ± 0.036
5,6-diOH-Cyt	0.079 ± 0.016	0.185 ± 0.028[c]	0.151 ± 0.053	0.597 ± 0.250[d]	0.178 ± 0.074[e]	0.668 ± 0.054	0.550 ± 0.007
FapyAde	0.340 ± 0.049	0.308 ± 0.056	0.728 ± 0.009[c]	2.33 ± 0.169[d]	1.45 ± 0.432	1.44 ± 0.217[e]	3.41 ± 0.403[e]
8-OH-Ade	0.277 ± 0.037	0.591 ± 0.152[c]	0.646 ± 0.024[c]	4.02 ± 0.649[d]	3.29 ± 0.10	0.508 ± 0.052[e]	9.28 ± 1.06[e]
2-OH-Ade	0.140 ± 0.005	0.419 ± 0.047[c]	0.374 ± 0.009[c]	0.567 ± 0.057	0.653 ± 0.120	0.193 ± 0.029[e]	0.971 ± 0.132[e]
FapyGua	1.88 ± 0.074	2.11 ± 0.030	2.55 ± 0.315	6.39 ± 0.789[d]	3.14 ± 0.658[e]	14.7 ± 3.89[e]	5.05 ± 0.628
8-OH-Gua	1.14 ± 0.108	2.73 ± 0.406[c]	4.62 ± 0.131[c]	30.2 ± 1.77[d]	13.8 ± 1.34[e]	18.9 ± 2.67[e]	30.3 ± 3.10

[a] 1, chromatin; 2, chromatin/Ni(II); 3, chromatin/H_2O_2; 4, chromatin/Ni(II)/H_2O_2; 5, chromatin/Ni(II)/H_2O_2/DMSO (50 μM); 6, chromatin/Ni(II)/H_2O_2/glutathione (1 mM); 7, chromatin/Ni(II)/H_2O_2/SOD (200 units/ml).
[b] Mean ± SD (n = 3).
[c] Values significantly different from those of Column 1 ($P < 0.05$).
[d] Values significantly different from those of Columns 2 and 3 ($P < 0.05$).
[e] Values significantly different from those of Column 4 ($P < 0.05$).

in chromatin previously, the formation of 2-hydroxyadenine (2-OH-Ade) was also observed. The DNA base products identified are typical products arising from reactions of •OH with the DNA bases. This pattern of products suggests that reaction of Co(II) or Ni(II) with H_2O_2 produces •OH, which attacks the DNA bases. Partial inhibition of base modification by typical •OH scavengers is consistent with this view. The failure of scavengers to prevent DNA damage in chromatin completely might be due to "site-specific" formation of •OH (Samuni et al. 1983, Goldstein & Czapski 1986, Halliwell & Gutteridge 1990). In contrast to Co(II) alone, Ni(II) alone caused significant rises in the background amounts of modified DNA bases in chromatin. This may be due to the ability of complexes of Ni(II) with certain peptide sequences in chromatin to generate free radicals in the presence of oxygen (Kasprzak & Bare 1989). Failure of catalase to inhibit the damage by Ni(II) alone indicates that H_2O_2 may not be required for such reactions. On the other hand, Co(II)/H_2O_2 caused more damage in chromatin than Ni(II)/H_2O_2. In the latter case, a substantial increase in Ni(II) concentration and in treatment time was necessary to produce significantly higher amounts than the background amounts of base products in chromatin. Addition of ascorbic acid had little effect on product yields. By contrast, ascorbic acid greatly stimulates DNA base modification produced by Cu(II)/H_2O_2 or Fe(III)/H_2O_2, as discussed above. The observed increase in the yield of FapyGua in the case of both Co(II) and Ni(II) in the presence of glutathione may result from increased reduction by glutathione of the C-8 OH-adduct radical of guanine. The site-specificity of •OH-generation may account for the inability of glutathione to markedly inhibit the DNA damage. These results suggest that glutathione may not be able to prevent the DNA damage mediated by bound-metal ions *in vivo*. The inability of SOD to inhibit the product formation suggests that O_2^- was not required in generation of •OH by Co(II)/H_2O_2 or by Ni(II)/H_2O_2. The observed increase in product yields by addition of SOD to Co(II)/H_2O_2 or to Ni(II)/H_2O_2 may indicate additional generation of •OH in these systems by an unknown mechanism. Inhibition of product formation by chelation of Co(II) and Ni(II) with EDTA is analogous to the results obtained with Cu(II), but in contrast to those obtained with Fe(III) under similar reaction conditions (see above). These results describing the effect of H_2O_2 in the presence of Co(II) and Ni(II) ions on DNA bases in mammalian chromatin have been published in *Cancer Research* (Nackerdien et al. 1991a).

2. DNA-Protein Cross-Links in Chromatin

a. DNA-protein cross-links produced by ionizing radiation

Recently, we have reported on the chemical nature, and the gas chromatographic and mass spectrometric characteristics of various DNA-protein cross-links (DPCs) involving DNA bases thymine and cytosine, and a number of amino acids in calf thymus nucleohistone, which was exposed to ionizing radiation in deoxygenated aqueous solution (Gajewski et al. 1988, Margolis et al. 1988, Dizdaroglu et al. 1989, Dizdaroglu & Gajewski 1989, Gajewski & Dizdaroglu 1990; see also the original proposal page 7, Section III, 1.b.). In the present project (see the original proposal, page 13, Section III, 2.e.), we have studied radiation-induced DNA-protein cross-linking in mammalian chromatin in aqueous suspension in the presence of N_2O and N_2O/O_2. Under these conditions, $^{\bullet}OH$ is formed almost exclusively as a radical species with a yield of 0.56 μmol/J (see above), and the N_2O/O_2 bubbling permits us to study the effect of oxygen. With the information obtained in the previous work (see above), the GC/MS-SIM technique was used here to search for the DPCs in trimethylsilylated hydrolysates of

chromatin. Analysis of irradiated chromatin samples by GC/MS-SIM showed that DPCs identified previously in calf thymus nucleohistone upon γ-irradiation under anoxic conditions were also formed in mammalian chromatin γ-irradiated under anoxic conditions (with N_2O bubbling). However, only 3-[(1,3-dihydro-2,4-dioxopyrimidin-5-yl)methyl]-L-tyrosine (Thy-Tyr cross-link) was detected in chromatin γ-irradiated under oxic conditions (with N_2O/O_2 bubbling). Figures 6A and 6B illustrate representative selected-ion current profiles of three DPCs obtained during GC/MS-SIM analyses of irradiated samples of chromatin. A comparison of Figure 6A with Figure 6B clearly shows that only the Thy-Tyr cross-link is formed in the presence of oxygen.

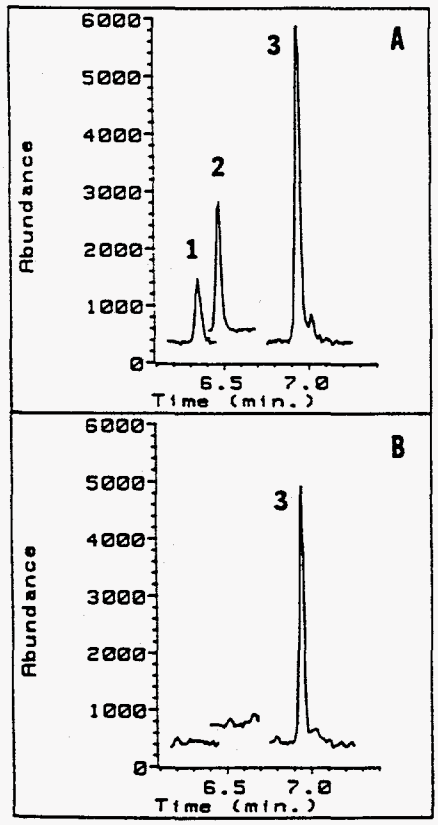


FIGURE 6: Ion-current profiles of the ions at *m/z* 585, 433, and 548, representing Thy-Lys, Cyt-Tyr, and Thy-Tyr cross-links, respectively. Data were obtained during GC/MS-SIM analysis of trimethylsilylated hydrolysates of chromatin. (A) Chromatin γ-irradiated under anoxic conditions; (B) chromatin γ-irradiated under oxic conditions. Peaks: 1, Thy-Lys (*m/z* 585); 2, Cyt-Tyr (*m/z* 433); 3, Thy-Tyr (*m/z* 548).

b. DNA-protein cross-links produced by H_2O_2 and metal ions

Formation of DPCs in isolated chromatin by H_2O_2 and Fe(II)-EDTA has been demonstrated previously (Lesko et al. 1982); however their chemical nature has not been elucidated. In the present project, we have investigated the formation of DPCs in mammalian chromatin by H_2O_2 in the presence of Fe(III) and Cu(II) ions under the conditions used for the study of DNA base damage (see above I, 1.b.). Chromatin samples were analyzed by GC/MS-SIM. Among the DPCs known to occur in

chromatin upon exposure to ionizing radiation under anoxic conditions (see above), only the Thy-Tyr cross-link was detected in the present work in chromatin upon exposure to H_2O_2 in the presence of various forms of Fe(III) or Cu(II) ions (for more details on identification see Nackerdien et al. 1991b). Figures 7, 8 and 9 illustrate the yields of the Thy-Tyr cross-link in chromatin samples, which were treated with H_2O_2 in the presence of various forms of Fe(III) and Cu(II) ions. In Figure 7, the yields of the Thy-Tyr cross-link in irradiated chromatin are also included. Equal yields of the Thy-Tyr cross-link were observed under both oxic and anoxic conditions.

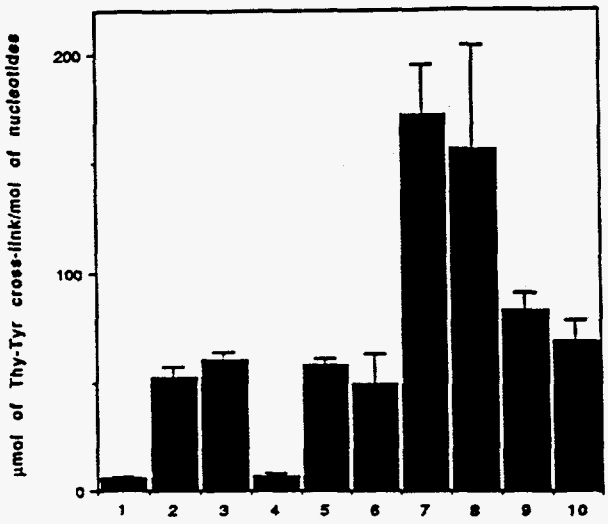


FIGURE 7: Yields of the Thy-Tyr cross-link in chromatin under various conditions: 1, chr; 2, chr, γ-irradiated with N_2O bubbling (dose 200 Gy); 3, chr, γ-irradiated with N_2O/O_2 bubbling (dose 200 Gy); 4, chr/Fe^{3+}; 5, chr/H_2O_2; 6, chr/H_2O_2/Fe^{3+}; 7, chr/H_2O_2/Fe^{3+}/asc; 8, chr/H_2O_2/Fe^{3+}/asc/SOD; 9, chr/H_2O_2/Fe^{3+}/asc/mannitol; 10, chr/H_2O_2/Fe^{3+}/asc/Me_2SO. Graphs represent the mean ± SD from triplicate measurements.

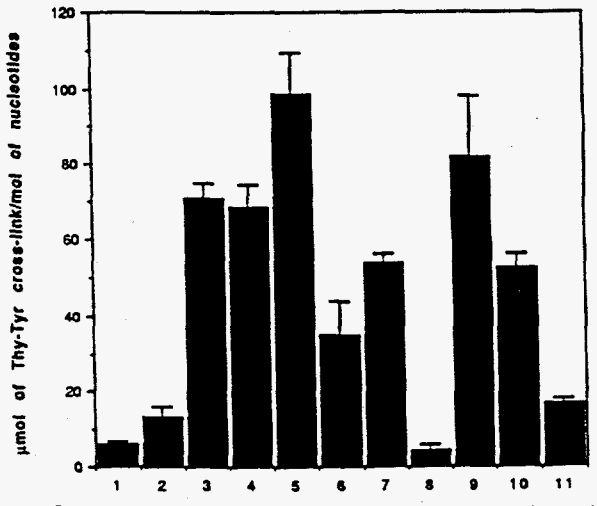


FIGURE 8: Yields of the Thy-Tyr cross-link in chromatin under various conditions: 1, chr; 2, chr/Cu^{2+}; 3, chr/H_2O_2/Cu^{2+}; 4, chr/H_2O_2/Cu^{2+}/asc; 5, chr/H_2O_2/Cu^{2+}/asc/SOD; 6, chr/H_2O_2/Cu^{2+}/asc/mannitol; 7, chr/H_2O_2/Cu^{2+}/asc/Me_2SO; 8, chr/H_2O_2/Cu^{2+}–NTA; 9, chr/H_2O_2/Cu^{2+}–NTA/asc; 10, chr/H_2O_2/Cu^{2+}–NTA/asc/SOD; 11, chr/H_2O_2/Cu^{2+}–NTA/asc/Me_2SO. Graphs represent the mean ± SD from triplicate measurements.

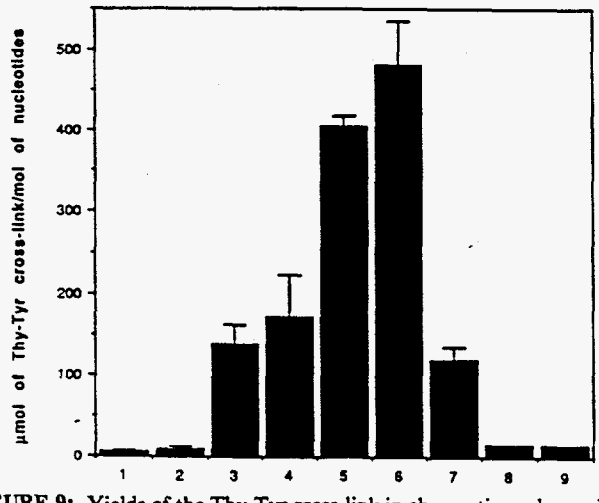


FIGURE 9: Yields of the Thy-Tyr cross-link in chromatin under various conditions: 1, chr; 2, chr/Fe^{3+}–NTA; 3, chr/H_2O_2/Fe^{3+}–EDTA; 4, chr/H_2O_2/Fe^{3+}–EDTA/asc; 5, chr/H_2O_2/Fe^{3+}–NTA; 6, chr/H_2O_2/Fe^{3+}–NTA/asc; 7, chr/H_2O_2/Fe^{3+}–NTA/asc/SOD; 8, chr/H_2O_2/Fe^{3+}–NTA/asc/mannitol; 9, chr/H_2O_2/Fe^{3+}–NTA/asc/Me_2SO. Graphs represent the mean ± SD from triplicate measurements.

The results indicate that oxygen had no effect on the formation of the Thy-Tyr cross-link in chromatin γ-irradiated in the presence of oxygen although oxygen completely inhibited the formation of other DPCs observed under anoxic conditions (see above Figures 6A and 6B). The results also indicate that the Thy-Tyr cross-link is also formed in H_2O_2/metal ion-treated chromatin despite the presence of oxygen. Generally, oxygen reacts with carbon-centered radicals at diffusion-controlled rates (reviewed in von Sonntag 1987), and thus inhibits cross-linking (dimerization) reactions of radicals. In fact, oxygen has been reported to markedly reduce the yields of DPCs formed by ionizing radiation in isolated chromatin (Mee & Adelstein 1981). In mammalian cells, formation of DPCs is also reduced by oxygen (Oleinick et al. 1987). The results obtained using ionizing radiation show that DPCs involving thymine and aliphatic amino acids, and cytosine and tyrosine were not formed in chromatin under oxic conditions, indicating the inhibition of their formation by oxygen. These DPCs were not formed in chromatin upon treatment with H_2O_2 and metal ions, either. Hence, oxygen also appears to be the main factor in inhibition of formation of those DPCs under the conditions of H_2O_2-treatment. By contrast, oxygen does not inhibit the formation of the Thy-Tyr cross-link. This DPC is thought to result from addition of the $^{\bullet}$OH-generated allyl radical of thymine to carbon-3 of tyrosine followed by oxidation of the resulting adduct radical and/or from combination of the allyl radical of thymine with $^{\bullet}$OH-generated tyrosine radicals (Dizdaroglu et al. 1989). Oxygen may not be able to interfere with these reactions. The formation of the Thy-Tyr cross-link by H_2O_2 was affected differently by various forms of Fe(III) and Cu(II) ions. Apparently, Fe(III) ions chelated with NTA or EDTA are very effective in producing cross-linking, whereas NTA- or EDTA-chelated Cu(II) ions are not effective. Unchelated Cu(II) ions were also very effective in producing DPCs. The patterns of the yields of the Thy-Tyr cross-link produced by H_2O_2 and Fe(III) and Cu(II) ions were analogous to those of the yields of modified DNA bases in mammalian chromatin, which was treated under similar conditions (see above I, 1.b., and Dizdaroglu et al. 1991b). The presence of ascorbic acid in reaction mixtures generally caused an increase in the yield of the Thy-Tyr cross-link. Ascorbic acid is an antioxidant, but it also acts as a prooxidant in the presence of metal ions, most likely by reducing them (reviewed in Halliwell & Gutteridge 1990). Partial inhibition by mannitol and Me_2SO of the DPC formation in the case of H_2O_2 and unchelated Fe(III) and Cu(II) ions might be due to "site-specific" generation of $^{\bullet}$OH upon reaction with H_2O_2 (Samuni et al. 1983, Goldstein & Czapski 1986, Halliwell & Gutteridge 1990). This means that $^{\bullet}$OH produced this way will immediately attack nearby components of chromatin before it can be scavenged. Thus, inability of mannitol and Me_2SO to completely inhibit the formation of the Thy-Tyr cross-link might be due to generation of $^{\bullet}$OH near thymine and tyrosine molecules in chromatin. The site-specific generation of $^{\bullet}$OH by metal ions bound to chromatin is more likely to occur *in vivo* than generation of $^{\bullet}$OH in "free solution". Therefore, it appears from our results that copper ion-dependent DPC formation might be more relevant *in vivo* than iron ion-dependent DPC formation, unless reducing agents are available to reduce Fe(III) ions. Significant inhibition by SOD of cross-linking caused by H_2O_2/chelated metal ions implicates the involvement of O_2^- in generation of $^{\bullet}$OH. By contrast, O_2^- might not be involved in production of $^{\bullet}$OH in reactions of H_2O_2 with unchelated Fe(III) and Cu(II) ions because of the failure of SOD to inhibit cross-linking. Alternatively, the site-specificity of the reaction of H_2O_2 with bound metal ions might prevent scavenging of O_2^- by SOD. A paper describing the formation of DPCs in mammalian chromatin by ionizing radiation in the presence of oxygen and by H_2O_2-treatment in the presence of Fe(III) and Cu(II) ions has been published in *Biochemistry* (Nackerdien et al. 1991b).

III. STUDIES OF DNA DAMAGE IN CULTURED HUMAN CELLS

Having completed most of the *in vitro* studies proposed in the original proposal, we have turned our attention to the *in vivo* studies using cultured mammalian cells including human cells. According to the research plan (see the original proposal, Section III, 2.a), radiation-induced DNA base damage and DNA-protein cross-links in cells were studied.

1. DNA Base Damage

a. DNA base damage in γ-irradiated cultured human cells

In the past, a number of modified bases in the DNA of cells exposed to ionizing radiation have been identified and quantitated by the use of various measurement techniques (Hariharan & Cerutti 1972, Mattern et al. 1975, Frenkel et al. 1981, 1985, Leadon & Hanawalt 1983, Teebor et al. 1984, Breimer & Lindahl 1985, Patil et al. 1985, Kasai et al. 1986, Furlong et al. 1986, Leadon 1990). Generally, one modified base or a small number of modified bases have been measured at a time with no specific structural evidence. Overall, there exist no comprehensive studies on the simultaneous measurement with structural evidence of both pyrimidine-derived and purine-derived lesions in chromatin DNA of irradiated cells. In the present project, we have studied the formation of pyrimidine- and purine-derived lesions in chromatin of γ-irradiated cultured human cells. The technique of GC/MS-SIM was used for the measurement of base lesions. Unlike other techniques that are available for measurement of a limited number of modified DNA bases, this technique permits the structural identification and the quantitation of various products of all four bases in DNA, and also directly in chromatin, as was mentioned earlier in this text. The preparation of DNA samples for an analysis by GC/MS-SIM involves hydrolysis with formic acid followed by derivatization. A recent study from our laboratory (Fuciarelli et al. 1989) has reported on the stability in formic acid of a number of modified bases. In the present work prior to analysis of chromatin from γ-irradiated cells, we examined the stability of modified bases and their release from DNA at different concentrations of formic acid in a quest for further optimizing hydrolysis conditions. Furthermore, we investigated the possibility that some modified bases may be formed in DNA by treatment with formic acid. Occurrence of some modified bases in isolated DNA and in isolated chromatin, which were not exposed to free radical-generating systems, has been shown previously using the GC/MS technique after hydrolysis with formic acid of DNA or of chromatin followed by derivatization of hydrolysates (reviewed in Dizdaroglu 1991). Our results have shown that formic acid treatment did not contribute to the presence of modified bases in DNA. As a conclusion of the results of these studies, hydrolysis with 60% formic acid was chosen to be optimal for DNA hydrolysis. The details of these studies are given in the attached manuscript by Nackerdien et al. (1991c).

For measurement of modified DNA bases in cultured human cells, chromatin was isolated from unirradiated or γ-irradiated cells, and then hydrolyzed with 60% formic acid. The hydrolysates were derivatized and analyzed by GC/MS-SIM. Twelve modified bases were identified in chromatin samples from cells irradiated at five different radiation doses as well as in those from unirradiated cells. An example of identification by GC/MS-SIM of modified bases in chromatin isolated from irradiated and unirradiated cells is illustrated in Figure 10.

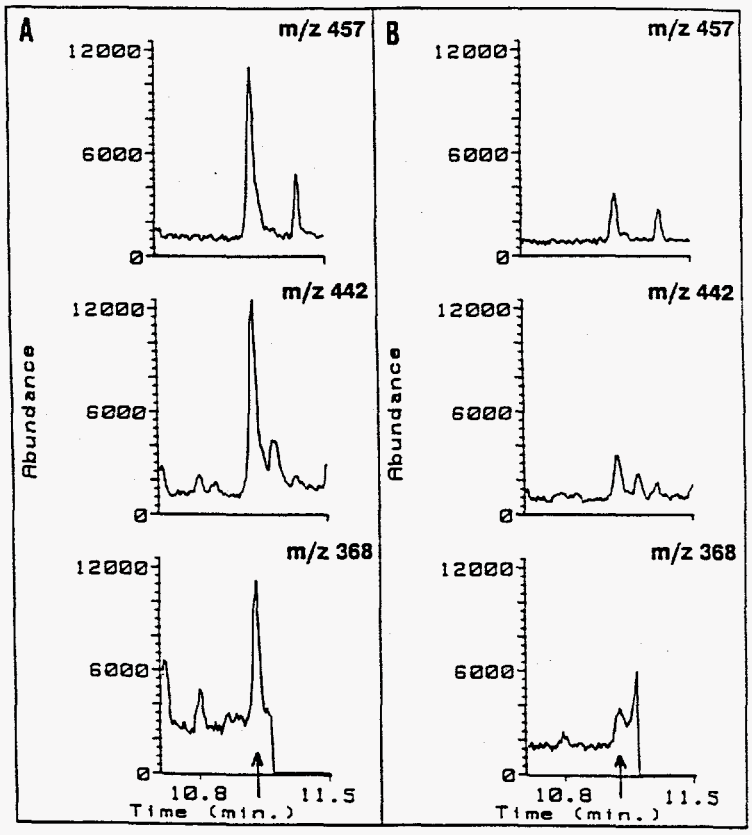


FIGURE 10: Selected-ion current profiles of the ions at *m/z* 368, 442, and 457 obtained during GC/MS-SIM analysis of trimethylsilylated hydrolysates of chromatin. (A) Chromatin isolated from γ-irradiated cells (dose, 116 Gy); (B) chromatin isolated from unirradiated cells.

Shown are selected-ion current profiles of three characteristic ions of the Me_3Si derivative of FapyGua from GC/MS-SIM analyses of hydrolyzed and derivatized chromatin samples. Several more ions were monitored in the same time interval; however, profiles of three ions are illustrated in Figure 10 for practical reasons. Signals of the three monitored ions are seen at the expected retention (indicated with an arrow) of the Me_3Si derivative of FapyGua in both Figure 10A and Figure 10B. A partial mass spectrum was obtained on the basis of the signals of the monitored ions and their relative abundances. This mass spectrum was then compared with that of the authentic material for unequivocal identification [(for a detailed description of this technique see Dizdaroglu (1990, 1991) and Dizdaroglu & Gajewski (1990)]. Modified bases identified and their amounts in control chromatin and in chromatin from irradiated cells are given in Table VII. Significant increases in the yields of a number of modified bases in chromatin over background levels were observed at a dose of as low as 42 Gy.

TABLE VII: *Yields[a] of Modified Bases (molecules/10^5 DNA bases) Formed in Chromatin of γ-Irradiated Cultured Human Cells*

Base	Radiation Dose				
	control	42 Gy	116 Gy	214 Gy	420 Gy
5-OH-5-Me-Hyd	2.91±0.38	4.19±0.64	3.04±0.45	2.78±0.32	3.07±0.39
5-OH-Hyd	10.40±1.38	-	15.74±1.73*	17.50±1.44*	23.23±2.88*
5-OHMe-Ura	0.77±0.09	1.28±0.22	1.89±0.51*	2.21±0.19*	2.85±0.35*
5-OH-Ura	0.38±0.05	0.76±0.14*	1.08±0.19*	1.59±0.27*	1.77±0.06*
5-OH-Cyt	2.44±0.38	3.03±0.59	3.01±0.22	3.26±0.51	4.67±0.54*
Thy glycol	1.63±0.22	3.65±0.51*	6.56±0.61*	6.72±0.96*	10.24±0.83*
5,6-diOH-Cyt	0.32±0.09	1.15±0.08*	1.89±0.22*	2.78±0.11*	4.13±0.86*
FapyAde	3.26±0.51	4.48±1.18	6.98±0.99*	8.33±0.67*	10.02±0.16*
8-OH-Ade	2.98±0.58	4.51±0.99	6.08±0.64*	4.22±0.74	5.54±0.77*
2-OH-Ade	1.95±0.27	3.03±0.10*	4.42±0.38*	3.84±0.64*	4.86±0.58*
FapyGua	2.56±0.48	5.34±0.54*	14.91±3.33*	20.90±2.08*	34.24±1.60*
8-OH-Gua	7.71±1.18	12.54±2.43	15.74±2.24*	15.58±1.22*	23.30±3.07*

[a]Each value represents the mean ± standard error from five independent experiments.
*Significantly different from the value in column 1 ($p \leq 0.05$).

Of the modified bases, 5-OH-5-Me-Hyd, 5-OHMe-Ura,Thy glycol and 8-OH-Gua have been identified previously in DNA of irradiated cells (see above). As for the remaining modified bases, the present work represents the first demonstration of their formation in chromatin of γ-irradiated cultured human cells. Modified bases identified here (except for 2-OH-Ade) have also been shown to be formed in isolated chromatin upon γ-irradiation in aqueous suspension (Gajewski et al. 1990). 2-OH-Ade has been identified in chromatin treated *in vitro* with H_2O_2 in the presence of metal ions (see above and Nackerdien et al. 1991a). The yields of 5-OH-Hyd, Thy glycol, 5,6-diOH-Cyt, FapyGua and 8-OH-Gua were increased linearly by increasing doses of radiation up to 420 Gy. As examples, Figure 11 illustrates the linear dose-yield plots of two modified bases. In the case of 5-OHMe-Ura, 5-OH-Ura and FapyAde, a linear dose-yield relationship was obtained up to 214 Gy. At 420 Gy, no further significant increase in the yields of these modified bases was observed. A dose of 822 Gy was also applied to check whether higher yields would be obtained at high radiation doses. At this dose, the yields of modified bases were not increased significantly over the levels obtained at 420 Gy. The amounts of 5-OH-Cyt and 8-OH-Ade were not increased over the control levels below 420 Gy. The amount of 5-OH-5-Me-Hyd observed in control chromatin was not increased significantly in chromatin of irradiated cells. The yields of products were increased over the backgrounds levels in different ratios. For example at 214 Gy, the highest ratio of increase was ≈8- to 9-fold for 5,6-diOH-Cyt and FapyGua followed by ≈4-fold for 5-OH-Ura and Thy glycol. The yields of modified bases measured here were much lower than their yields measured in isolated chromatin, which was γ-irradiated in air-saturated aqueous suspension (Gajewski et al. 1990). In the previous study, chromatin fully expanded in a low ionic strength buffer has been used. DNA in fully expanded chromatin is expected to be more susceptible to free radical attack than DNA in chromatin of the intact cell, which has a higher order structure. In fact, the susceptibility of isolated chromatin to ionizing radiation-induced damage has been reported to be greater than that of chromatin in cells with respect to DNA lesions such as base damage, DNA-protein cross-links, and strand breaks (Roti Roti et al. 1974, Mee & Adelstein 1979, Heussen et al. 1987). Our results are consistent with the results

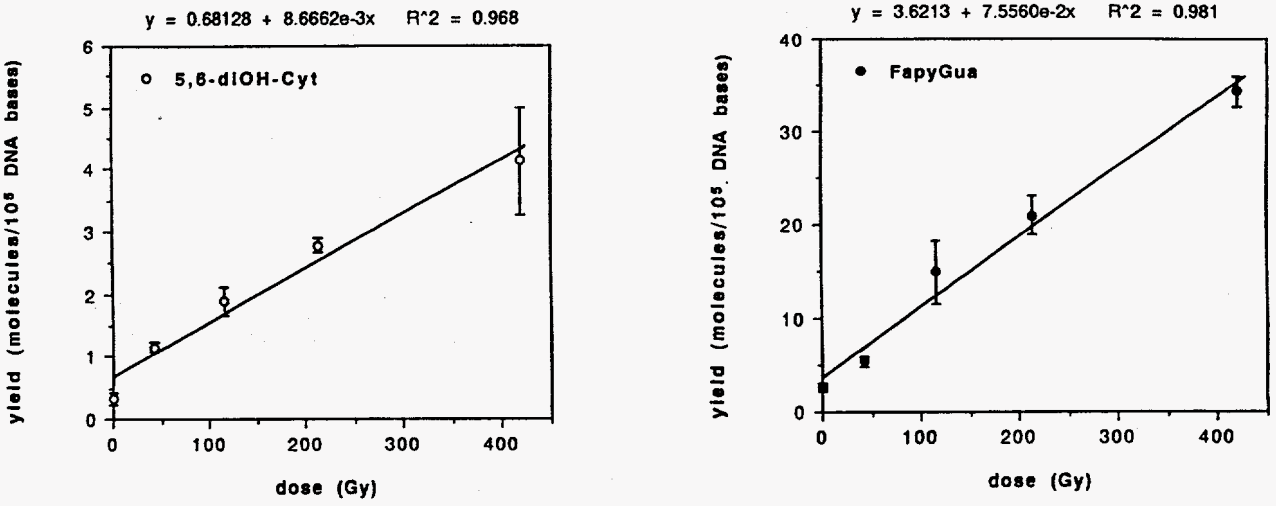


FIGURE 11: Dose-yield plots of 5,6-diOH-Cyt and FapyGua in chromatin of γ-irradiated cells. Error bars represent standard errors of the means from five independent experiments. Lines were drawn by linear regression analysis.

reported in those studies. Modified bases detected in chromatin of irradiated cells are known to be the typical ·OH-induced products of DNA bases (reviewed in von Sonntag 1987). Their formation in chromatin of irradiated cells is most likely due to reactions with DNA bases of ·OH produced from cellular water by ionizing radiation. Earlier measurements have shown that ≈70% of radiation-induced lethality and DNA damage in oxic cells is caused by ·OH (Roots & Okada, 1972, 1975, Chapman et al., 1973). Because of its high reactivity toward organic molecules, however, ·OH has a short diffusion distance in cells, and must be generated in close proximity to DNA in order to cause any DNA damage (Roots & Okada 1975). The track model of energy deposition of ionizing radiation in an aqueous medium provides the concept of free radical formation in track entities such as spurs and blobs, which may be formed in the vicinity of DNA bases (reviewed in Chatterjee 1987). The direct effect of radiation on DNA may also account in part for the formation of modified bases through ionization of DNA bases (reviewed in Steenken 1989 and Angelov et al. 1991). Of the modified bases measured in chromatin of irradiated cells, the yields of guanine-derived bases were the highest (e.g., ≈45% of the net total yield of modified bases, when calculated from the yields at 116 Gy after subtraction of background values). The yields of adenine-, cytosine- and thymine-derived bases constituted the rest of the net total yield in almost equal percentages. These results are similar to those previously obtained with isolated chromatin γ-irradiated in aqueous suspension (Gajewski et al. 1990). This may indicate the high reactivity with ·OH of guanine residues in chromatin in cells and *in vitro*. On the other hand, guanine-derived bases FapyGua and 8-OH-Gua may also result in part from reactions of the guanine radical cation formed by direct ionization of guanine residues in chromatin of cells. Thus, the high yield of guanine-derived bases is also in accord with the well-known property of guanine being the most easily oxidized DNA base by the direct effect of ionizing radiation and by other oxidants (reviewed in Steenken 1989). The yields of formamidopyrimidines (FapyAde and FapyGua) were higher than those of 8-hydroxypurines (8-OH-Ade and 8-OH-Gua) in chromatin of irradiated cells. Formamidopyrimidines and 8-hydroxypurines result

from one-electron reduction and oxidation of C-8 OH-adduct radicals, respectively (reviewed in Steenken 1989). The overall pattern of the yields of these compounds are in contrast to that obtained with isolated chromatin γ-irradiated in air-saturated aqueous suspension, and rather resemble that obtained with isolated chromatin γ-irradiated in deoxygenated aqueous suspensions (Gajewski et al. 1990). This may indicate the inability of oxygen to completely interfere with reactions of C-8 OH-adduct radicals of purines in cellular chromatin and/or the hypoxic nature of the environment of the cell nucleus (reviewed in Joenje 1989). A manuscript describing the measurement of modified DNA bases in chromatin of γ-irradiated cultured human cells has been submitted to *Biochemistry* (Nackerdien et al. 1991c).

b. DNA base damage in H_2O_2-treated cultured cells

In addition to radiation-induced damage in cells, we have also investigated DNA damage in H_2O_2-treated mammalian cells. For this purpose, murine hybridoma cells were used. After treatment of cells with H_2O_2, chromatin was isolated, and then hydrolyzed, derivatized and analyzed by GC/MS-SIM as was mentioned above in the case of γ-irradiated cells. Figure 12 illustrates the DNA base products identified in chromatin isolated from H_2O_2-treated cells, and their yields at two different concentrations of H_2O_2. Ten DNA base products were identified and quantitated. The same pattern of products was observed

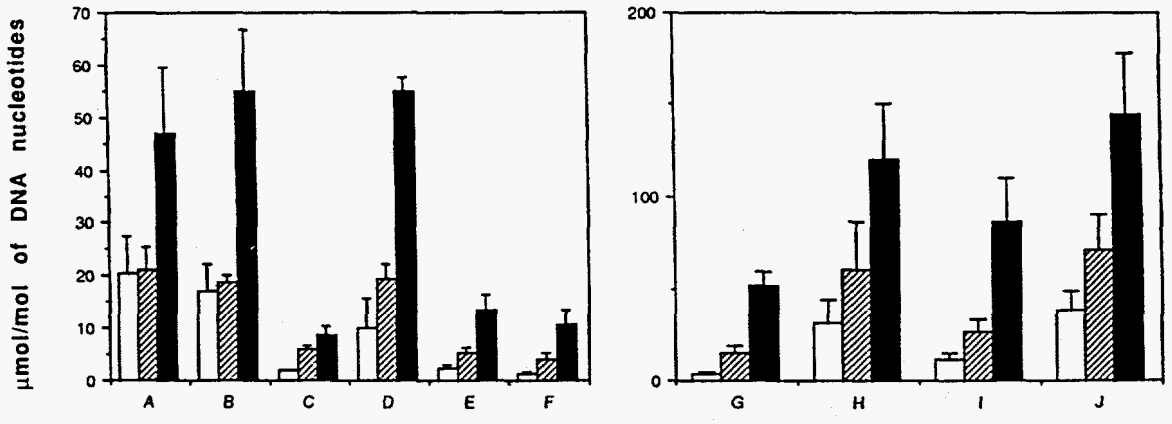


FIGURE 12: Yields of DNA base products in chromatin isolated from cells, as measured by GC/MS-SIM: □, untreated; ▨, treated with 2 mM H_2O_2; ■, treated with 20 mM H_2O_2. A, 5-hydroxy-5-methylhydantoin; B, 5-hydroxyhydantoin; C, 5-hydroxymethyluracil; D, cytosine glycol; E, thymine glycol; F, 5,6-dihydroxycytosine; G, 4,6-diamino-5-formamidopyrimidine; H, 8-hydroxyadenine; I, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; J, 8-hydroxyguanine. Graphs represent the means ± standard deviations from four independent experiments.

as in the case of γ-irradiated cells (see above). Again, the presence of all products was observed in chromatin isolated from untreated cells. In the case of 5-OH-5-Me-Hyd and 5-OH-Hyd, no increase over background amounts was observed in cells treated with 2 mM H_2O_2. The lowest amount of a modified DNA base, i.e., 5,6-diOH-Cyt, detectable in chromatin of untreated cells was approximately 1 in 10^6 DNA nucleotides. The increase of product yields over background levels varied depending on the product type. With 20 mM H_2O_2, the highest ratio of increase over background levels was observed in the yields of 5,6-diOH-Cyt, FapyAde and FapyGua ($\approx$10-fold). The yields of the other products were increased between $\approx$2.5-fold and $\approx$5-fold. As was mentioned above, the products identified in

chromatin of H_2O_2-treated cells are known to be typical $^{\bullet}OH$-induced products of bases in DNA. The pattern of DNA base modification indicates that the reactive species responsible for product formation *in vivo* is unlikely to be anything other than $^{\bullet}OH$. This species might be generated *in vivo* upon treatment of cells with H_2O_2 in a reaction with chromatin-bound transition metal ions (Haber-Weiss reaction) (Mello-Filho & Meneghini 1985, Ward et al. 1985, reviewed in Halliwell & Gutteridge 1990). However, because of its high reactivity, $^{\bullet}OH$ must be generated *in vivo* in close proximity to DNA in a site-specific manner in order to cause any DNA damage (Samuni et al. 1983, Goldstein & Czapski 1986, Ward et al. 1985, Halliwell & Gutteridge 1990). The necessary metal ions might be bound to chromatin, or the oxidative stress might liberate metal ions from their storage sites with subsequent binding to chromatin (reviewed in Halliwell & Gutteridge 1990). Evidence exists for the role of $^{\bullet}OH$ in biological effects of H_2O_2 *in vivo* involving naturally occurring metal ions (Mello-Filho & Meneghini 1985, Ward et al. 1985, Meneghini 1988). Hydrogen peroxide alone does not cause formation of the products identified in the present study (Blakely et al. 1990). A paper describing these results has been published in *Archives of Biochemistry and Biophysics* (Dizdaroglu et al. 1991a).

2. DNA-Protein Cross-Links in γ-Irradiated or H_2O_2-Treated Cultured Human Cells

Identification and quantitation of DPCs produced in isolated chromatin by exposure to ionizing radiation or to H_2O_2/metal ions have been described above. In this part of the project, we have applied the GC/MS-SIM technique in the same manner to examine whether DPCs, which were found in mammalian chromatin in previous *in vitro* studies, are formed in chromatin of γ-irradiated or H_2O_2-treated cultured human cells. Chromatin was isolated from γ-irradiated or H_2O_2-treated cells and also from control cells, and then hydrolyzed and derivatized. Typical ions of the Me_3Si derivatives of DNA base-amino acid cross-links were monitored during the GC/MS-SIM analyses of trimethylsilylated hydrolysates of chromatin samples. Among the DNA-protein cross-links known from previous studies, only the formation of the Thy-Tyr cross-link was observed in cellular chromatin upon γ-irradiation or H_2O_2-treatment of cells. Identification and quantitation of this DPC was carried out, as was described above. Figure 13 shows the yields of the Thy-Tyr cross-link as a function of radiation dose in chromatin of irradiated cells. The insert illustrates the structure of this compound. A linear dose-yield relationship

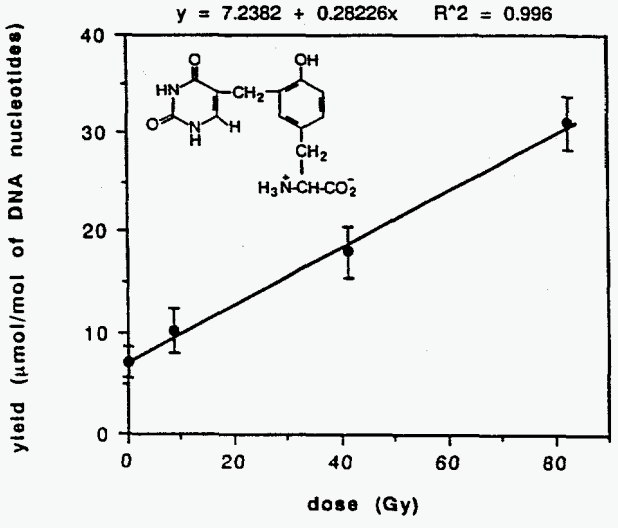
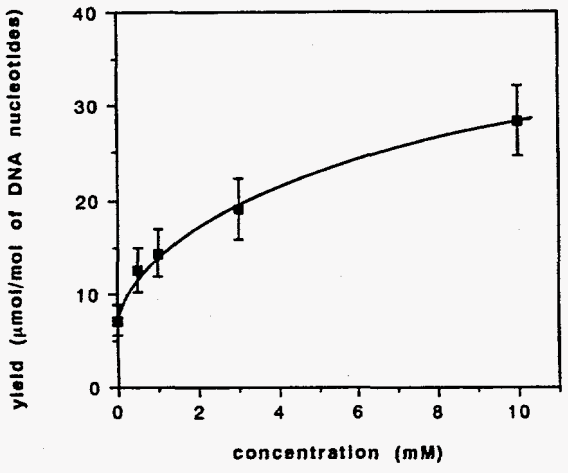


FIGURE 13: Yields of the Thy-Tyr cross-link in chromatin of γ-irradiated cultured human cells. Error bars represent standard errors of the means from five independent experiments.

was obtained in the dose range from 8.7 to 82 Gy. At higher doses (up to 400 Gy), the yield approached a plateau and did not increase significantly over the level at 82 Gy. Detectable amounts of this DNA-protein cross-link were also found in chromatin samples isolated from unirradiated cells (Figure 13). This background amount agreed well with that found in chromatin of murine hybridoma cells (Nackerdien et al. 1991b). At 82 Gy, the increase in the amount of the Thy-Tyr cross-link over its background amount was ≈4-fold. Figure 14 illustrates the dependence of the formation of the Thy-Tyr cross-link in cells on the concentration of H_2O_2. In the concentration range from 0.5 to 10 mM, the amount of the Thy-Tyr cross-link increased between ≈2-fold and ≈4-fold over the background level. At concentrations above 10 mM, the production of the Thy-Tyr cross-link approached a plateau. For further experiments, 10 mM H_2O_2 was used. The amounts of the Thy-Tyr cross-link in chromatin of cells treated under various conditions are illustrated in Figure 15.



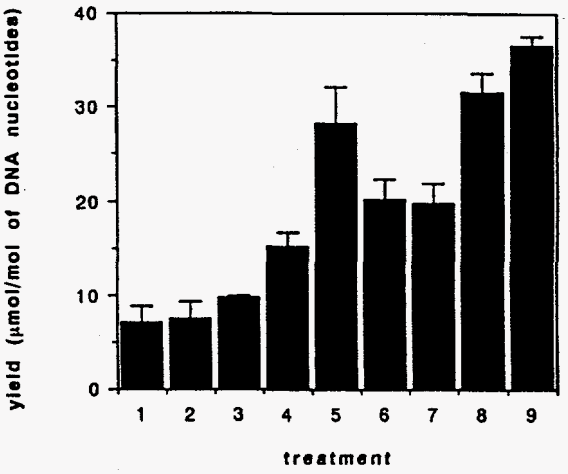
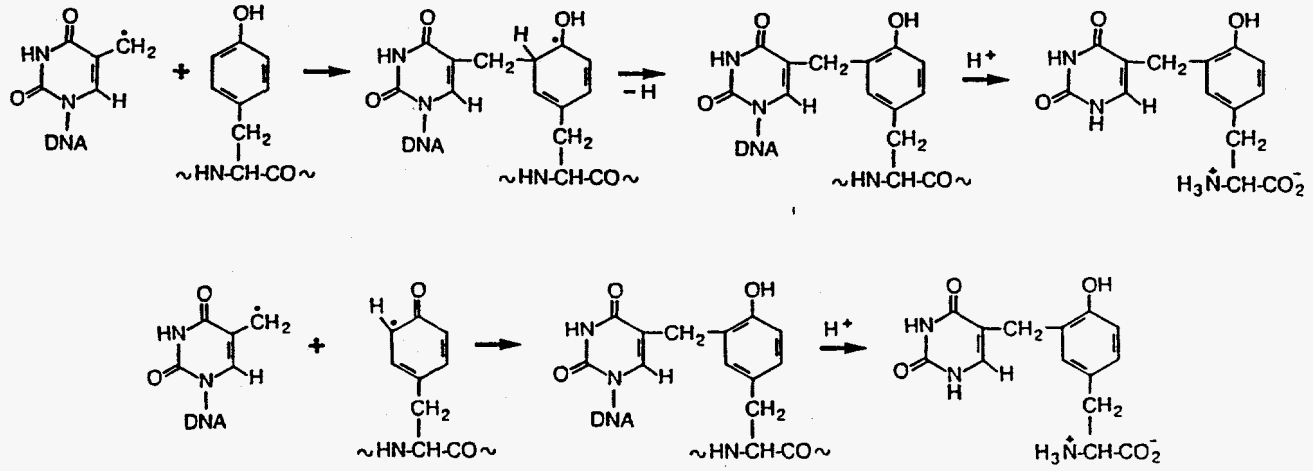


FIGURE 14: Yields of the Thy-Tyr cross-link in chromatin of H_2O_2-treated cultured human cells. Error bars represent standard errors of the means from five independent experiments.

FIGURE 15: Yields of the Thy-Tyr cross-link in chromatin of H_2O_2-treated cultured human cells under various conditions. *1*, control cells; *2*, cells treated with *o*-phenanthroline (0.1 mM); *3*, cells treated with KCN (2 mM); *4*, cells pretreated with ascorbic acid (1 mM) for 24 h; *5*, cells treated with H_2O_2 (10 mM); *6*, cells treated with *o*-phenanthroline (0.1 mM)/H_2O_2 (10 mM); *7*, cells treated with Me_2SO (100 mM)/H_2O_2 (10 mM); *8*, cells pretreated with ascorbic acid (1 mM), then treated with H_2O_2 (10 mM); *9*, cells treated with KCN (2 mM)/H_2O_2 (10 mM). Error bars represent standard errors of the means from five independent experiments.

The results show that DPCs between thymine and tyrosine are formed in chromatin of γ-irradiated or H_2O_2-treated cultured human cells. The structure of the Thy-Tyr cross-link indicates that this type of DNA-protein cross-linking occurs through a covalent bond formation between the α-carbon of thymine and the C-3 of the tyrosine ring. This means that the allyl radical of thymine [5-(2'-deoxyuridylyl)methyl radical] is the most likely precursor of the thymine moiety of the Thy-Tyr cross-link as has been suggested previously (Dizdaroglu et al. 1989). The allyl radical of thymine may be produced by deprotonation of the thymine radical cation formed by ionization of thymine (Shaw et al. 1988, Deeble

et al. 1990). The indirect effect of radiation may also account for the formation of this radical through H atom abstraction by $^{\bullet}OH$ from the methyl group of thymine (Fujita & Steenken 1981). The Thy-Tyr cross-link may be formed by addition of the allyl radical of thymine to the C-3 position of the tyrosine ring followed by oxidation of the so-formed adduct radical (Dizdaroglu et al. 1989). The combination of the allyl radical of thymine with tyrosine radicals, which result from reactions of $^{\bullet}OH$ with tyrosine (Dorfman et al. 1962, Land & Ebert 1976), would represent an alternative mechanism (Dizdaroglu et al. 1989). Some of the proposed mechanisms are:



The latter mechanism requires the formation of two radicals in close proximity. The track model of the deposition of radiation energy provides the concept of formation of several radicals in track entities such as spurs, blobs and short tracks (reviewed in Chatterjee 1987). If these track entities overlap with the chromatin structure, two hydroxyl radicals may form one thymine radical and one tyrosine radical in close proximity of each other, as has been previously suggested for the formation of DNA double strand breaks or other locally multiply damaged sites of DNA (Ward et al. 1985). These two mechanisms of formation of the Thy-Tyr cross-link are not mutually exclusive, *i.e.*, they may both take place. As for the role of oxygen in these mechanisms, we have demonstrated previously that oxygen does not inhibit the formation of the Thy-Tyr cross-link in isolated chromatin, but it inhibits DNA-protein cross-linking between thymine and aliphatic amino acids, and between cytosine and tyrosine (Nackerdien et al. 1991b). It appears that oxygen does not interfere with the formation of the Thy-Tyr cross-link in cells, either. Also similar to *in vitro* studies, thymine-aliphatic amino acid cross-links and the cytosine-tyrosine cross-link were not found in chromatin of irradiated or H_2O_2-treated cells. The production of the Thy-Tyr cross-link in γ-irradiated cells was linear with radiation dose up to 82 Gy, and then approached a plateau at doses higher than 82 Gy. This finding agrees with the previous findings that DNA-protein cross-links are produced in mammalian cells linearly with radiation dose in the range of 10 to 100 Gy (Cress & Bowden 1983, Ramakrishnan et al. 1987). In H_2O_2-treated cells, $^{\bullet}OH$ is the most likely species responsible for the formation of the Thy-Tyr cross-link. Partial inhibition by Me_2SO of the formation of the Thy-Tyr cross-link is consistent with this idea. Hydroxyl radicals are generated in cells by reactions of H_2O_2 with metal ions bound to chromatin (Haber-Weiss reaction), and are the ultimate species in causing DNA damage (Ward et al. 1985, Mello-Filho & Meneghini 1985, Meneghini 1988, Halliwell

& Gutteridge 1990). Because of its high reactivity toward organic molecules, $^{\bullet}OH$ has a short diffusion distance of on average 1.5 nm in cells before reacting with DNA (Ward et al. 1985). Therefore, $^{\bullet}OH$ must be generated in close proximity to DNA by H_2O_2 and bound metal ions in a site-specific manner to cause any DNA damage (Samuni et al. 1983, Ward et al. 1985, Goldstein & Czapski 1986). Partial inhibition by Me_2SO of the formation of the Thy-Tyr cross-link may indicate the site-specific nature of cross-linking. The addition of *o*-phenanthroline to the growth medium prior to H_2O_2-treatment resulted in a partial inhibition of the Thy-Tyr cross-link formation. This is in agreement with the previous findings that *o*-phenanthroline prevents DNA strand breaks in cells caused by H_2O_2-treatment (Mello-Filho & Meneghini 1985). The addition of the catalase inhibitor KCN in the growth medium caused a $\approx$30% increase in the yield of the Thy-Tyr cross-link over the level observed with H_2O_2-treatment. This may result from the inhibition of intracellular catalase by KCN. This result is in accord with the finding that DNA single-strand breaks in H_2O_2-treated cells are enhanced by inhibition of intracellular catalase (Mello-Filho & Meneghini 1985). The formation of the Thy-Tyr cross-link by the pretreatment of cells with nontoxic levels of ascorbic acid may be due to the ability of ascorbic acid to produce H_2O_2 through the reduction of molecular oxygen (Samuni et al. 1983). With respect to the mechanisms of formation of the Thy-Tyr cross-link, the two mechanisms described above for γ-irradiated cells, which involve a radical addition reaction or a radical-radical combination, may be operative in H_2O_2-treated cells as well. In this case, however, the allyl radical of thymine should result from H abstraction by $^{\bullet}OH$, since the thymine radical cation is not expected to be formed. The site-specific formation of two radicals, *i.e.*, one thymine radical and one tyrosine radical, in close proximity of each other is likely to result from a mechanism different from that described above for irradiated cells. As has been previously proposed, a cyclic redox reaction may occur involving reduction of chromatin-bound metal ions by $O_2^{\bullet-}$ or another reductant, and their subsequent reoxidation by H_2O_2. This reaction may give rise to multiple production of $^{\bullet}OH$, and thus to multiple hits (Samuni et al. 1983, Ward et al. 1985). Again, oxygen may not be able to interfere with these mechanisms because of the site-specific nature of DNA-protein cross-linking. A manuscript describing the results on DNA-protein cross-linking in γ-irradiated or H_2O_2-treated cultured human cells has been submitted to *Journal of Biological Chemistry* (Olinski et al. 1991).

IV. STUDIES OF DNA REPAIR

In our original proposal (page 12, Section III, 2.d.), we proposed to study the cellular repair of DNA lesions observed in γ-irradiated cells. We have just completed the first set of measurements of DNA lesions in cells (see above). Because of time limitation, the DNA repair in cells could not be studied until now, and will be pursued in the next stage of the proposal. However, we have studied the excision by a DNA repair enzyme of radiation-modified bases in DNA *in vitro*. This work was done in collaboration with Dr. S. Boiteux and Dr. J. Laval of the Institut Gustave Roussy, Villejuif, France. The enzyme used was the Fpg protein, which is a DNA glycosylase from *Escherichia coli* [the formamidopyrimidine (Fapy)—DNA glycosylase]. This enzyme also exists in mammalian cells (Margison & Pegg 1981), and has been shown to recognize purines with an opened imidazole ring (Chetsanga & Lindahl 1979, Breimer 1984, Boiteux et al. 1984). The *Escherichia coli fpg*$^+$ gene coding for the Fapy-DNA glycosylase has been cloned recently and the Fpg protein has been purified to homogeneity (Boiteux et al. 1987, 1990). The excision of 8-OH-Gua by the Fpg protein from small duplex oligonucleotides containing a single 8-OH-Gua residue has been reported (Tchou et al. 1991). However, the excision by

the Fpg protein of this lesion or any other radiation-induced (or free radical-induced) products of pyrimidines and purines in DNA has not been demonstrated. We have investigated the ability of the Fpg protein to excise modified pyrimidine and purine bases from γ-irradiated DNA. The products excised (or non-excised) from DNA by the Fpg protein were unambiguously characterized by GC/MS-SIM. DNA was γ-irradiated in N_2O-saturated aqueous solution. Irradiated DNA was incubated with either the native enzyme or the boiled enzyme and subsequently ethanol-precipitated. The precipitates were analyzed by GC/MS-SIM after acidic hydrolysis and trimethylsilylation. The supernatant fractions of the samples were analyzed after lyophilization and trimethylsilylation without acidic hydrolysis. Irradiated DNA and unirradiated DNA without incubation with the enzyme but after precipitation in the incubation buffer were also analyzed as controls. Thirteen modified DNA bases were identified and quantitatively measured (Table VIII). The boiled Fpg protein excised none of the pyrimidine- and purine-derived

TABLE VIII: Yields of Products in Pellets of DNA and in Supernatant Fractions

	treatment[b]				
product (nmol/mg of DNA)[a]	1	2	3	4	5
5,6-diHThy	nd	0.116 ± 0.015	0.107 ± 0.032	0.096 ± 0.016	nd
5-OH-5-Me-Hyd	0.056 ± 0.010	0.686 ± 0.165	0.495 ± 0.092	0.692 ± 0.150	nd
5-OH-Hyd	0.014 ± 0.020	0.207 ± 0.040	0.163 ± 0.019	0.161 ± 0.019	nd
5-OH-6-HThy	nd	0.204 ± 0.013	0.177 ± 0.011	0.192 ± 0.008	nd
5-OH-6-HCyt	nd	0.159 ± 0.020	0.176 ± 0.018	0.155 ± 0.016	nd
5-OHMe-Ura	nd	0.135 ± 0.050	0.135 ± 0.015	0.115 ± 0.026	nd
Cyt glycol	0.061 ± 0.011	0.645 ± 0.021	0.665 ± 0.059	0.650 ± 0.080	nd
Thy glycol	0.045 ± 0.013	0.675 ± 0.017	0.540 ± 0.070	0.575 ± 0.082	nd
5,6-diOH-Cyt	nd	0.078 ± 0.010	0.064 ± 0.009	0.060 ± 0.015	nd
FapyAde	0.085 ± 0.013	1.22 ± 0.024	1.13 ± 0.168	0.678 ± 0.102	0.630 ± 0.240
8-OH-Ade	0.204 ± 0.023	1.12 ± 0.056	1.11 ± 0.079	0.977 ± 0.059	0.166 ± 0.010
FapyGua	0.136 ± 0.003	1.76 ± 0.333	1.63 ± 0.244	0.761 ± 0.056	1.39 ± 0.510
8-OH-Gua	0.347 ± 0.059	2.67 ± 0.084	2.86 ± 0.155	1.17 ± 0.217	2.33 ± 0.470

[a] Values represent the mean ± standard deviation from three independent measurements. [b] Treatment: 1, unirradiated DNA (pellet); 2, γ-irradiated DNA (pellet); 3, pellet from γ-irradiated DNA after incubation with the boiled Fpg protein; 4, pellet from γ-irradiated DNA after incubation with the native Fpg protein; 5, supernatant fraction from γ-irradiated DNA after incubation with the native Fpg protein.

lesions from γ-irradiated DNA (compare columns 2 and 3 in Table VIII). None of these modified bases was detectable in the supernatant fraction. In contrast, ≈42% of FapyAde, ≈55% of FapyGua, and ≈ 58% of 8-OH-Gua were excised by the native enzyme under the conditions used (Table VIII, column 4). The excision of these compounds was confirmed by their presence in the supernatant fraction (Table VIII, column 5). The amounts found in the supernatant fraction roughly corresponded to the amounts excised (compare columns 3, 4 and 5). A small amount (≈10-15%) of 8-OH-Ade also appeared to be removed by the native enzyme. This was confirmed by a small but detectable amount of this compound found in the supernatant fraction (Table VIII, column 5). We did not observe any excision of the other modified bases in significant amounts (compare columns 2, 3 and 4). This was also confirmed by the absence of those modified bases in the supernatant fraction. The excision of modified bases from DNA irradiated at a higher dose than 42 Gy was also investigated. In DNA irradiated at 150 Gy, the amounts of 8-OH-Gua (≈3.2 molecules/10^3 DNA bases) and of other modified bases were ≈2- to 3-fold higher than those in DNA irradiated at 42 Gy. Similar to the results discussed above, FapyAde and FapyGua were excised by the Fpg protein to an extent of ≈37% and ≈40%, respectively, from DNA irradiated at 150 Gy. In contrast, the excision of 8-OH-Gua and 8-OH-Ade by the Fpg protein was not observed from DNA irradiated at 150 Gy.

The results show that FapyGua in γ-irradiated DNA is recognized and excised by the Fpg protein. This is the first demonstration of the excision of FapyGua by the Fpg protein. In addition, the present work demonstrates the ability of the Fpg protein to recognize and excise FapyAde not only from homopolymers of 2'-deoxyadenosine (dA) (Breimer, 1984), but also from DNA. While the present work was in progress, Tchou et al. (1991) reported the excision of 8-OH-Gua by the Fpg protein from a small synthetic oligonucleotide containing a single 8-OH-Gua residue. Our results demonstrate the ability of the Fpg protein to excise 8-OH-Gua from DNA as well. In addition to the excision of significant amounts of formamidopyrimidines and 8-OH-Gua, the native Fpg protein excised a small but detectable amount of 8-OH-Ade from γ-irradiated DNA. By inference to 8-OH-Gua, the excision of 8-OH-Ade by the Fpg protein would be expected. However, the small amount excised appears to be insignificant in order to draw any conclusion on the substrate specificity of the Fpg protein concerning this lesion. The excision of 8-hydroxypurines by the Fpg protein was inhibited from DNA irradiated at a higher dose, although formamidopyrimidines were excised. These results imply that the structural requirement for the recognition of formamidopyrimidines by the Fpg protein is somewhat different from that for the recognition of 8-hydroxypurines. The rational for this difference remains unclear. In addition to γ-irradiated DNA, visible light/methylene blue-treated DNA was also used as a substrate for the Fpg protein. Fapy and 8-OH-Gua, which were the only products detected in this DNA, were excised by the Fpg protein (for details see Boiteux et al. 1991). The results support the view that the biological role of the Fpg protein is the repair of DNA damage caused by ionizing radiation or other free radical-producing systems or other oxygen-derived species. This enzyme appears to be specific for purine-derived bases in DNA. The excision of 8-OH-Gua implies that imidazole ring opening is not an absolute requirement for the recognition of modified purines by the Fpg protein. As was suggested previously (Dizdaroglu, 1985), the analytical approach used in this work might be useful for the unequivocal characterization of various pyrimidine- and purine-derived lesions recognized (or not recognized) by DNA repair enzymes, and thus for the elucidation of mechanisms underlying the repair of DNA damage. This work may serve as an example for studies of specificity of DNA repair enzymes because the specificity of an enzyme on numerous purine- and pyrimidine-derived DNA lesions was tested for the first time because of the capability of the GC/MS-SIM technique to measure those lesions. A paper describing the findings in this work is in press in *Biochemistry* (Boiteux et al. 1991).

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VI. SUMMARY

Since the beginning of DOE's support of our work in July 1989, we have met a large portion of the stated objectives in the original proposal. The first year's support was minimal ($15,000). The second year's and the third year's supports were higher ($60,000 and $65,000, respectively). The third year of the proposal began in July 1991. As the present progress report indicates, the proposed *in vitro* studies have been completed except for the proposed studies on the sugar damage in chromatin DNA. First, a cell culture laboratory was established and a methodology for a routine isolation of chromatin from mammalian cells was developed. Subsequently, radiation-induced DNA base damage in chromatin *in vitro* was investigated. Results showed a pronounced effect of various radical environments and oxygen on the types and the yields of DNA base products in chromatin. The same type of DNA base products were also found in chromatin treated with the radiomimetic agent hydrogen peroxide in the presence of metal ions. Various metal ions and their chelation status were shown to have a substantial effect on the yields of products. Results also indicated the protective effect of proteins in chromatin on product formation. A number of DNA-protein cross-links were identified in chromatin γ-irradiated under anoxic conditions. However, oxygen inhibited the formation of those DNA-protein cross-links with the exception of the DNA-protein cross-link involving thymine and tyrosine. The thymine-tyrosine cross-link was also detected and quantitated in chromatin treated with hydrogen peroxide in the presence of metal ions. As in the case of DNA base products, various metal ions and their chelation status had a pronounced effect on the formation of this DNA-protein cross-link. In the second phase of the studies, DNA damage in cultured human cells was investigated. A number of DNA base products were identified and quantitated in chromatin of γ-irradiated cultured human cells. Linear dose-yield relationships were obtained for most of the products at relatively low radiation doses. We also showed the formation of DNA-protein cross-links involving thymine and tyrosine in chromatin of γ-irradiated cultured human cells. Treatment of cells with hydrogen peroxide yielded similar DNA base products and the thymine-tyrosine cross-link in cellular chromatin. Because of the limitation of time, the repair of DNA in cells could not be studied. So far, we studied the specificity of a DNA glycosylase, *i.e.*, Fpg protein, on a large number of radiation-induced base products in DNA. The Fpg protein was capable of excising the purine-derived lesions, *i.e.*, formamidopyrimidines and 8-hydroxypurines from DNA, whereas none of the nine pyrimidine-derived lesions detected in γ-irradiated DNA was a substrate for this enzyme. If the DOE's support continues, the repair of DNA damage in cells and other objectives of the proposal will be studied.

As a result of the studies reported in this progress report, eleven papers have been published or are in press in peer-reviewed journals. Three of these papers are review articles that I have prepared at invitation of the editors of the corresponding journals. These papers have also been peer-reviewed. Two other manuscripts have been submitted for publication.

In March 1990, I was awarded *the Hillebrand Prize* of the Washington DC Section of the American Chemical Society for my work done in the past five years. This award, which was established in 1925, is the oldest and highest honor given by the Washington DC Section of the American Chemical Society.

VII. PUBLICATIONS ACKNOWLEDGING THE SUPPORT FROM THE DEPARTMENT OF ENERGY

1. Gajewski, E., Rao, G., Nackerdien, Z., and Dizdaroglu, M., "Modification of DNA Bases in Mammalian Chromatin by Radiation-Generated Free Radicals," *Biochemistry* 29, 7876-7882 (1990).

2. Dizdaroglu, M., (**invited paper**), "Measurement of DNA Base Damage and Protein Cross-links in Mammalian Chromatin," in *Ionizing Radiation Damage to DNA: Molecular Aspects, UCLA Symposia on Molecular and Cellular Biology*, New Series, Vol. 136, pp. 17-30, 1990.

3. Dizdaroglu, M., Rao, G., Halliwell, B., and Gajewski, E., "Damage to the Bases in Mammalian Chromatin by Hydrogen Peroxide in the Presence of Ferric and Cupric Ions," *Arch. Biochem. Biophys.* 285, 317-324 (1991).

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10. Boiteux, S., Gajewski, E., Laval, J., and Dizdaroglu, M., "Substrate Specificity of the *Escherichia Coli* Fpg Protein (Formamidopyrimidine—DNA Glycosylase): Excision of Purine Lesions in DNA Produced by Ionizing Radiation or Photosensitization," *Biochemistry* 61, 106-110 (1992).

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13. Olinski, R., Nackerdien, Z., and Dizdaroglu, M., "DNA-Protein Cross-linking Between Thymine and Tyrosine in Chromatin of γ-Irradiated or H_2O_2-Treated Cultured Human Cells," *Archiv. Biochem. Biophys.* 297, 139-143 (1992).