FREE RADICAL DAMAGE TO CHROMATIN

BY

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DECLARATION

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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Date

ABSTRACT

Oxygen-derived free radicals are implicated in many human diseases including cancer. The mutagenic and carcinogenic properties of these species manifest themselves in lingering damage to several biomolecules including DNA. Here, efforts have been made to chemically characterize DNA base damage and DNA-protein cross-links produced in isolated chromatin and in cultured mammalian cells exposed to ionizing radiation or to H_2O_2/metal ions. Gas chromatography/mass spectrometry with selected ion-monitoring was used for this purpose.

Eleven modified DNA bases were identified and quantitated in chromatin treated with Ni(II)/H_2O_2 or Co(II)/H_2O_2. Treatment of chromatin with H_2O_2/Co(II) caused significantly greater damage to DNA bases than Ni(II)/H_2O_2. Treatment of chromatin with Ni(II)/H_2O_2 required longer incubation times and a rise in Ni(II) concentration before product yields were detected above background levels. Ascorbic acid and glutathione did not affect product yields. Chelation of Ni(II) and Co(II) ions with EDTA almost completely inhibited product formation. Hydroxyl radical involvement was inferred from the pattern of lesions and inhibition by dimethylsulphoxide.

A DNA-protein cross-link involving thymine and tyrosine was detected exclusively in chromatin exposed to γ-rays or to H_2O_2/metal ions in the presence of oxygen. Other DNA-protein cross-links which are known to occur in nucleoprotein irradiated under anoxic conditions, were not observed. This

was due to inhibition by O_2 as shown by experiments that were carried out using ionizing radiation in the presence of N_2O/O_2 and N_2O. Fe(III) and Cu(II), in the presence of H_2O_2, caused formation of the thymine-tyrosine cross-link depending on the type of metal ion employed and its chelation status. Mannitol and dimethylsulphoxide inhibited cross-link formation in the case of H_2O_2/chelated ions and afforded partial inhibition in the case of H_2O_2/unchelated ions. Superoxide dismutase afforded partial inhibition only when chelated ions were used.

DNA base products were identified and quantitated in chromatin extracted from mammalian cells exposed to H_2O_2 or ionizing radiation. Modified bases identified were typical products of $^{\cdot}OH$ attack on DNA bases. Direct ionization of DNA bases in γ-irradiated cells may also give rise to modified products. The radiation yields of several modified bases were increased significantly over their background levels at 42 Gy and reached a plateau above 420 Gy. The high resistance of chromatin to irradiation is attributed to higher order structure (supercoiling) which protects DNA from irradiation damage. This is the first comprehensive study giving qualitative and quantitative evidence of multiple DNA lesions in chromatin extracted from treated cells or exposed *in vitro* to free radical generators. It forms the basis for further analysis of the biological significance and repairability of DNA lesions in cells.

OPSOMMING

Vry radikaal derivate van suurstof word geimpliseer in verskeie siektes insluitende kanker. Mutasies en kankerwekkende eienskappe van hierdie spesies word geopenbaar deur langdurige skade aan veskeie biomolekules insluitende DNS. Geisoleerde kromatien sowel as kromatien afkomstig van gekultureerde soogdier selle is hier ionies bestraal of behandel met H_2O_2/metaal ione. Chemiese skade berokken aan DNS basisse en DNS-proteien kruisverbindings is met behulp van gas chromatografie/massa spektrometrie (insluitende geselekteerde ion-waarneming) gemonitor.

Elf gemodifiseerde DNS basisse is geidentifiseer in kromatien behandel met Ni(II)/H_2O_2 of Co/H_2O_2. Meer beskadigde DNS basisse is geidentifiseer in die geval van kromatien behandel met Co(II)/H_2O_2 as met Ni(II)/H_2O_2. Kromatien moes langer geinkubeer word in die aanwesigheid van Ni(II)/H_2O_2 en die Ni(II) konsentrasie moes verhoog word alvorens enige DNS beskadiging bo kontrole vlakke gesien kon word. Vitamien C en glutatioon het nie die hoeveelheid produkte geaffekteer nie. Die formasie van produkte is geinhibeer toe Ni(II) en Co(II) ione in die aanwesigheid van die chelaat, EDTA, geplaas is. Die patroon van beskadigde produkte en inhibisie deur dimetielsulfoksied dui op die betrekking van hidroksiel radikale.

'n DNS-proteienkruisverbinding tussen timien en tiroksien is geidentifiseer in kromatien wat ionies bestraal is of behandel is met H_2O_2/metaal ione in die teenwoordigheid van suurstof. Die ander bekende DNS-proteienkruisvebindings wat in bestraalde

nukleoproteien voorkom (in die afwesigheid van suurstof) is nie hier waargeneem nie. Die effek van suurstof is geillustreer in eksperimente waartydens ioniese strale in die aanwesigheid van N_2O/O_2 en N_2O gebruik is. Fe(III) en Cu(II), in die aanwesigheid van waterstofperoksied, veroorsaak die vorming van die timien-tiroksien kruisverbinding afhangende van die tipe metaal ioon en sy chelasie status. Die vorming van produkte is gedeeltelik geinhibeer wanneer superoksied dismutase plus gecheleerde ione gebruik is.

DNS basisse is geidentifiseer en gekwantiteer in geisoleerde kromatien afkomstig van soogdier selle wat ionies bestraal of met H2O2 behandel is. Die gemodifiseerde basisse wat so verkry is, is tiperend van hidroksiel radikaal-beskadiging van DNS basisse. Direkte ioniese bestraling van DNS basisse kan ook aanleiding gee tot hierdie gemodifiseerde produkte. Verskeie gemodifiseerde basis hoeveelhede is verhoog bo kontrole vlakke tussen 42 Gy ioniese bestraling en 'n hoogvlakte bo 420 Gy. Kromatien mag meer weerstand bied teen bestraling weens die drie-dimensionele struktuur (kronkeling) wat DNS beskerm. Dit is die eerste omvattende studie wat kwalitatiewe en kwantitatiewe bewys lewer van veelvuldige DNS letsels in kromatien ekstraksies afkomstig van selle wat met ioniese strale of ander vry radikaal genereerders behandel is. Dit vorm die grondwerk vir die verdere analise van die biologiese betekenis en herstelbaarheid van DNS letsels in selle.

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GLOSSARY OF ABBREVIATIONS

* **Unless specified , S. I. units were used for physical quantities (see Mills *et al.*, 1988).**

2-OH-Ade, 2-hydroxyadenine (isoguanine); **5,6-diHThy,** 5,6-dihydrothymine; **5,6-diOHCyt,** 5,6-dihydroxycytosine; **5,6-diOHUra,** 5,6-dihydroxyuracil; **5-OH-Cyt,** 5-hydroxycytosine; **5-OH-Hyd,** 5-hydroxyhydantoin; **5-OH-Ura,** 5-hydroxyuracil; **5-OH-5-MeHyd,** 5-hydroxy-5-methylhydantoin; **5-OH-6HCyt,** 5-hydroxy-6-hydrocytosine; **5-OH-6-HThy,** 5-hydroxy-6-hydrothymine; **5-OH-6-HUra,** 5-hydroxy-6-hydrouracil; **5-OHMe-Ura,** 5-(hydroxymethyl)uracil; **8-OH-Ade,** 8-hydroxyadenine; **8-OH-Gua,** 8-hydroxyguanine; **Ade,** Adenine; **AMPS,** ammonium persulphate; **asc,** ascorbate; **Bis,** NN'-methylene-bisacrylamide; **bp,** base pairs; **BSTFA,** bis(trimethylsilyl)trifluoroacetamide; **chr,** chromatin; **Cyt,** cytosine; **DMEM/F12,** 50:50 (v/v) mixture of Dulbecco's modification of Minimum Essential Medium plus Ham's F12 Medium; **DNA,** deoxyribonucleic acid; **DPC(s),** DNA-protein cross-link(s); **DTT,** dithiothreitol; ϵ, molar extinction coefficient; e^-, electron; e_{aq}^-, hydrated electron; **EDTA,** Ethylene-diamine-tetra-acetic acid; **FapyAde,** 4,6-diamino-5-formamidopyrimidine; **FapyGua,** 2,6-diamino-4-hydroxy-5-formamidopyrimidine; **GC/MS,** gas chromatography/mass spectrometry; **Gua,** guanine; **Gy,** Gray (J/kg; 1 Gy = 100 rad = 6.24×10^{15} eV/g); H_2O_2, hydrogen peroxide; $HO_2^{\bullet}$, hydroperoxyl radical; **HPLC,** high performance liquid chromatography; **i.d.,** internal diameter; **IgG,** Immunoglobulin G; **incub.,** incubation; **irrad.,** irradiation; **Leu,** leucine; **Lys,** lysine; **Me_2SO or DMSO,**

dimethylsulphoxide; **Me_3Si**, trimethylsilyl; **n**, sample number; **n.d.**, not detected; **NMR**, nuclear magnetic resonance; **NTA**, nitrilotriacetic acid; **O_2^-**, superoxide radical; **$^{\cdot}OH$**, hydroxyl radical; **p**, probability; **PBS**, phosphate buffered saline; **Phe-Phe**, phenylalanylphenylalanine; **PMSF**, phenylmethanesulfonyl fluoride; **RMRF(s)**, relative molar response factor(s); **RNA**, ribonucleic acid; **RPMI**, Roswell Park Memorial Institute 1640 Medium; **SD**, standard deviation; **SDS**, sodium dodecyl sulphate; **SIM**, selected-ion monitoring; **SOD**, superoxide dismutase; **TEMED**, N,N,N',N'-Tetramethylethylene-diamine; **Thy**, thymine; **Thy-Tyr**, 3-[(1,3-dihydro-2,4-dioxopyrimidine-5-yl)-methyl]-L-tyrosine; **TMCS**, trimethylchlorosilane; **Tris**, Tris(hydroxymethyl)aminomethane; **Tyr**, tyrosine; **u**, units; **UV**, ultraviolet radiation; **v**, volume; **w**, weight

1. INTRODUCTION

1.1 Motivation

Oxygen consumption is both vital and energetically advantageous to the maintenance of aerobic life. Respiration involving oxygen as the terminal oxidant and photosynthesis are two mechanisms which have evolved to harvest this rich energy source (Halliwell, 1987). However, high intracellular concentrations of oxygen are toxic (Halliwell and Gutteridge, 1990). Elevated levels of free radicals derived from oxygen can damage critical biomolecules such as DNA, proteins, lipids and thiols. A number of disease states including mutagenesis, cancer and aging have been ascribed to this type of damage (Halliwell and Gutteridge, 1990).

Mutagenesis, reproductive cell death and cancer are often correlated with DNA damage. Previous studies of DNA damage have been hampered by the chemical complexity of mammalian cells and have relied heavily on model systems using isolated DNA. Extrapolations to *in vivo* conditions are suspect, since those studies neglect the association of DNA with basic nuclear proteins (histones) which give rise to compaction and the formation of protected and exposed DNA regions.

DNA folding as chromatin within the mammalian cell is important for two reasons. First, folding levels determine the accessibility of the DNA duplex to free radicals or repair enzymes. Second, biological processes known to modulate DNA

folding, e.g., progression through the cell cycle can profoundly affect the survival of mammalian cells after exposure to free radical generators such as ionizing radiation. For example, V79 Chinese hamster lung fibroblast cell survival decreases from 42 % in S-phase to 13 % in G1-phase after exposure to (0-10 Gy) γ-rays (Travis, 1980). Knowledge of the chemical nature of DNA lesions giving rise to these differences would have applications in basic and clinical research.

At the molecular level, chemical characterization of DNA lesions in the cellular milieu will contribute to the understanding of their mechanisms of formation and to the assessment of their biological consequences.

The present study focusses on the chemical analysis of DNA base lesions and DNA-protein cross-links in chromatin produced *in vitro* and in cultured mammalian cells exposed to ionizing radiation or H_2O_2/metal ions. Gas chromatography/mass spectrometry with selected ion-monitoring was used throughout to identify and quantitate multiple DNA lesions in chromatin.

1.2 Free Radicals

Free radicals can be generated *in vitro* by normal aerobic metabolism or by a number of exogenous sources such as redox-cycling drugs, ionizing radiations, UV radiation, carcinogenic compounds etc. (Halliwell and Gutteridge, 1990).

Intracellular oxygen reduction *via* a pathway of reactive

oxygen intermediates is depicted in reaction 1. These reduction metabolites are (reaction 1): superoxide radical (O_2^-), hydrogen peroxide (H_2O_2; two-electron reduction) and hydroxyl radical ($^{\cdot}OH$; three electron reduction). The pathway culminates in the formation of water (four-electron reduction)(Halliwell and Gutteridge, 1990).

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} {}^{\cdot}OH \xrightarrow{e^-} H_2O$$

Reaction 1: Monovalent reduction of oxygen.

Neither O_2^- nor H_2O_2 appear to cause DNA strand breaks or base damage (Lesko *et al.*, 1980; Rowley *et al.*, 1983; Aruoma *et al.*, 1989a and 1989b). Thus, much of the toxicity of these two species *in vivo* is thought to arise from their metal-ion catalyzed conversion to the highly reactive hydroxyl radical:

$$M^{n+1} + O_2^- \rightarrow M^{n+} + O_2 \qquad (2)$$

$$M^{n+} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + M^{n+1} \text{ (Fenton reaction)} \qquad (3)$$

$$H_2O_2 + O_2^- \rightarrow {}^{\cdot}OH + OH^- + O_2 \text{ (Haber-Weiss reaction)} \qquad (4)$$

The catalyst, M^{n+}, is usually a transition metal ion such as iron ion or copper ion (Halliwell and Gutteridge, 1985; 1988). Copper ions can also catalyze the autoxidation of ascorbic acid *via* the Fenton reaction (Samuni *et al.*, 1983). Other

transition metal ions, such as Ni(II) and Co(II) ions, may also catalyze the reaction between $O_2^{\cdot-}$ and H_2O_2. These metal ions are known to be carcinogenic to humans and animals (Costa, 1991; Léonard and Lauwerys, 1990). However, the mechanisms involved in tumour production remain elusive. In mammalian cells, nickel compounds affect the genetic material, producing sister-chromatid exchanges and chromosomal aberrations (Sen and Costa, 1985; Conway *et al.*, 1987). There is evidence for binding of Ni(II) to cell nuclei (Ciccarelli and Wetterhahn, 1982; Kasprzak and Poirier, 1985) and for induction by Ni(II) of DNA strand breaks and DNA-protein cross-links (Ciccarelli and Wetterhahn, 1982; Ciccarelli *et al.*, 1981; Robison *et al.*, 1982; Patierno *et al.*, 1985; Kasprzak and Bare, 1989). However, Ni(II) alone causes no damage to isolated DNA (Kawanishi *et al.*, 1989), and the relatively weak interactions between Ni(II) and DNA are unlikely to be responsible for the genotoxic effects in cells exposed to Ni(II) (Kasprzak and Bare, 1989). Thus, it has been proposed that Ni(II) reacts with endogenous H_2O_2 in cells to form $^{\cdot}OH$, which causes DNA damage (Kawanishi *et al.*, 1989; Kasprzak and Hernandez, 1989). Recent *in vitro* studies have indicated the formation of $^{\cdot}OH$ in reactions of Ni(II) and Ni(II)-peptide complexes with H_2O_2 (Kawanishi *et al.*, 1989; Kasprzak and Hernandez, 1989; Inoue and Kawanishi, 1989; Torreilles and Guérin, 1990). On the other hand, studies of the effects of $^{\cdot}OH$ scavengers gave equivocal results

(Kawanishi *et al.*, 1989). When $^{\cdot}OH$ is generated by reaction of H_2O_2 with transition metal ions bound to the DNA, it is often difficult to completely protect the DNA from $^{\cdot}OH$ attack by adding $^{\cdot}OH$ scavengers because of the possible "site-specific" generation of $^{\cdot}OH$ (Halliwell and Gutteridge, 1990; Aruoma *et al.*, 1991; Dizdaroglu *et al.*, 1991a; Goldstein and Czapski, 1986; Stoewe and Prütz, 1987).

The mechanisms underlying cobalt toxicity have not been established, but oxygen-derived species may be involved (Jacobsen *et al.*, 1984). It has been proposed that oxidation of some organic compounds by cobalt complexes in the presence of oxygen or hydrogen peroxide involves oxygen-centered free radicals and proceeds via a Haber-Weiss mechanism (Saussine *et al.*, 1985; Hamilton *et al.*, 1987; Tung and Sawyer, 1990). It has been suggested that Co(II) reacts with H_2O_2 to form $^{\cdot}OH$ (Gutteridge, 1983; Hamilton *et al.*, 1987; Moorhouse *et al.*, 1985) but this view has been questioned on the basis of electron spin resonance studies (Kadiiska *et al.*, 1989).

Transition metal ion-catalyzed reactions are not the only source of oxygen radicals which can damage biomolecules such as DNA. Iron can react with alkyl peroxides, forming alkoxyl radicals (Halliwell and Gutteridge, 1985). These radicals can produce DNA lesions.

Many semiquinones react with oxygen:

$$\text{Semiquinone} + O_2 \rightarrow \text{quinone} + O_2^{\cdot-} \quad (5)$$

Semiquinones can also interact with H_2O_2 to form $^{\cdot}OH$ (Meneghini, 1988):

R = alkyl group (6)

Semiquinones occur naturally in the mammalian cell and may serve as a reservoir for continuous $^{\cdot}OH$ radical production.

External sources of hydroxyl radical production include ionizing radiations. DNA is considered to be the critical target for the biological effects of ionizing radiation (Painter, 1980). Studies done with radical scavengers have shown that the contribution of free radicals to DNA damage by ionizing radiation amounts to ≈70% in oxic cells (Roots and Okada, 1972, 1975; Chapman *et al.*, 1973).

Radiation-induced chemical modifications of DNA may occur by direct ionization of the DNA molecule, or by indirect processes involving water radiolysis species (von Sonntag, 1987). The abundance of water in the mammalian cell lends credence to the indirect mechanism. In addition, the DNA duplex is surrounded by a hydration layer of precisely bound water molecules (Saenger, 1984).

Water radiolysis has been well-documented (for a review see von Sonntag, 1987) and is summarized below.

$$H_2O \xrightarrow{\gamma\text{-, X-rays}} H_2O^{\cdot} + e^- \quad (7)$$

$$H_2O \xrightarrow{\gamma\text{-, X-rays}} H_2O^* \qquad (8)$$

The first two reactions depict the instantaneous processes of ionization and excitation accompanying ionizing radiation interaction with water. Within 10^{-16} sec after ionization, water radical cations (H_2O^+), excited water molecules (H_2O^*) and electrons (e^-) are located in clusters along the track of the ionizing particle. These clusters are called spurs.

The water radical cation (H_2O^+) can lose a proton to another water molecule:

$$H_2O^+ + H_2O \rightarrow H_3O^+ + {}^{\bullet}OH \qquad (9)$$

The electron is hydrated within less than 10^{-12} sec:

$$e^- + nH_2O \rightarrow e_{aq}^- \qquad (10)$$

The excited water molecules formed in the initial reaction give rise to H atoms and OH radicals:

$$H_2O^* \rightarrow H^{\bullet} + {}^{\bullet}OH \qquad (11)$$

H atoms are also produced by the reactions of the hydrated electron (e_{aq}^-) with protons:

$$e_{aq}^- + H^+ \rightarrow H^{\bullet} \qquad (12)$$

The products H_2 and H_2O_2 may result from the following reactions:

$$H^{\bullet} + H^{\bullet} \rightarrow H_2 \qquad (13)$$

$$e_{aq}^- + e_{aq}^- \rightarrow H_2 + 2OH^- \qquad (14)$$

$$^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2 \qquad (15)$$

H atoms and OH radicals recombine in spurs to form water and protons and hydroxide ions react to give water:

$$H^{\bullet} + {}^{\bullet}OH \rightarrow H_2O \qquad (16)$$

$$e_{aq}^{-} + {}^{\bullet}OH \rightarrow OH^{-} \qquad (17)$$

$$H^{+} + OH^{-} \rightarrow H_2O \qquad (18)$$

"Spur expansion" occurs about 10^{-7} sec after the ionization event, i.e. the radiolysis products diffuse from the spur and become evenly distributed throughout the medium.

The presence of different gases in an aqueous medium during irradiation profoundly affects the G-values[1] of these radicals and those yields are depicted in Table I:

TABLE I: Yields (μmol.J^{-1}) of radical species generated by ionizing radiation in aqueous solution under different gaseous conditions[2].

	ARGON	AIR	N_2O	N_2O/O_2
$^{\bullet}OH$	0.28	0.28	0.56	0.56
e_{aq}^{-}	0.27	-	-	-
H atom	0.057	-	0.057	-
O_2^{-}	-	0.33	-	0.057

---

[1] G values relate the yields of products in an irradiated sample to the amount of radiation energy deposited in it and are expressed in terms of μmol.J^{-1} [1 molecule (100 eV)$^{-1}$ = 0.1036 μmol.J^{-1}].

[2] Adapted from von Sonntag (1987).

In the presence of oxygen , e_{aq}^- and H atom react with oxygen and are converted to O_2^-:

$$e_{aq}^- + O_2 \rightarrow O_2^- \qquad (19)$$

$$H + O_2 \rightarrow HO_2^{\bullet} \rightleftharpoons H^+ + O_2^- \qquad (20)$$

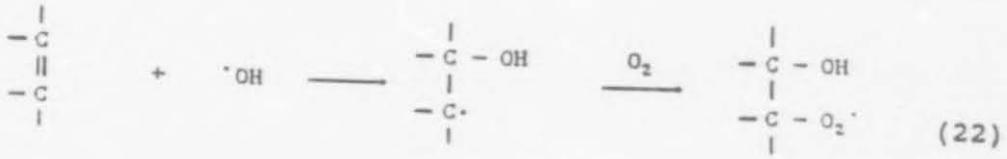
When N_2O is present, e_{aq}^- reacts with N_2O in a diffusion-controlled reaction to yield additional $^{\bullet}OH$ (von Sonntag, 1987). This reaction is written as follows:

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^{\bullet}OH + OH^- + N_2 \qquad (21)$$

Hence, when water is saturated with nitrous oxide prior to irradiation, the formation of 90% OH radicals and 10% H atoms (in terms of radical species) is promoted.

1.3 Reactions of free radicals with DNA constituents

Hydroxyl radicals react with organic compounds by addition to double bonds and by abstraction of an H atom from C-H bonds:



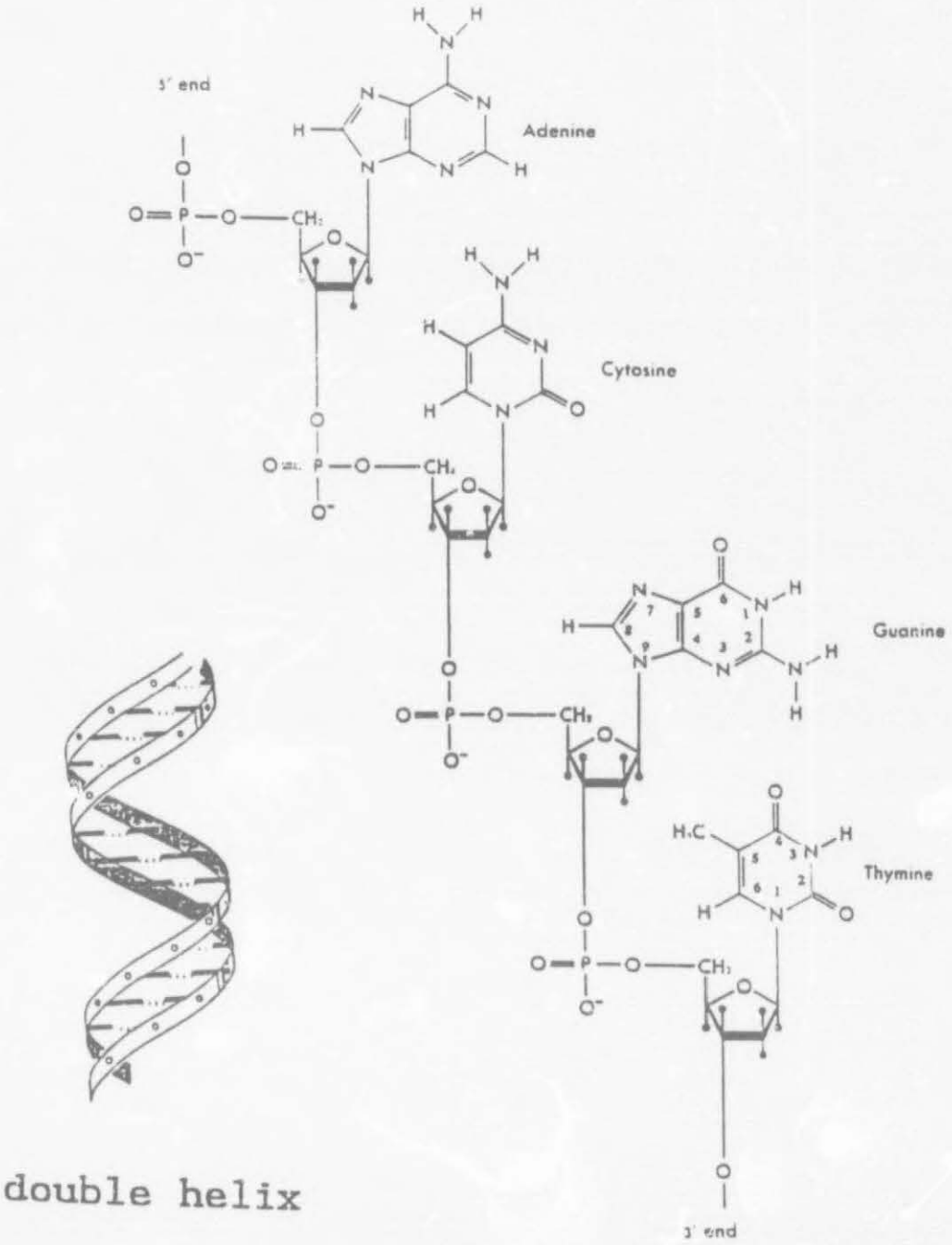
(22)

(23)

In the presence of oxygen, peroxyl radicals are formed by diffusion-controlled reactions of oxygen with C-centered radicals.

Hydroxyl radical reactions typify an important principle of free radical chemistry, namely the interaction of a free radical with a non-radical species resulting in a different free radical which may be more or less reactive than the original radical (Halliwell and Gutteridge, 1989). Hydroxyl radical damage to heterocyclic DNA bases will be investigated and therefore merits thorough discussion.

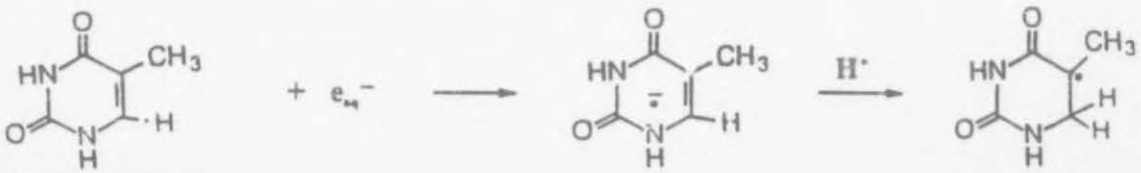
DNA consists of a sugar-phosphate backbone with purines (adenine and guanine) and pyrimidines (thymine and cytosine) attached to the sugar moiety with glycosidic bonds (Fig. 1). Hydroxyl radical reacts with DNA bases at diffusion-controlled rates, i.e., $5 \times 10^9 - 10^{10}\ M^{-1} \cdot s^{-1}$. It adds to the C5/C6 double



double helix

FIGURE 1: Structure of deoxyribonucleic acid (Watson, 1970).

bond of pyrimidines and can also abstract H atoms from the methyl group of thymine. In the case of cytosine and thymine, ·OH adds to the C5-position to the extent of ≈90% and ≈60%, respectively, and to the C6-position to the extent of ≈10% and ≈30%, respectively. Approximately 10% of ·OH reactions involve H atom abstraction from the methyl group of thymine (Fujita and Steenken, 1981). Purine sites prone to ·OH attack are the C4, C5, and C8 positions. Products formed by free radical attack have distinct properties and fates. Hydroxyl radical addition to the 5,6 double bond in thymine gives rise to the production of 5-hydroxy-6-yl and 6-hydroxy-5-yl radicals and H-abstractions from the methyl group to allyl radicals (see Fig. 2). The first two types of radicals are also formed when cytosine reacts with ·OH. The hydrated electron, e_{aq}^-, also reacts with pyrimidines at diffusion-controlled rates and electron adducts are produced in these reactions. Protonation of these adducts gives rise to 6-hydro-5-yl radicals (Novais and Steenken, 1986; Das *et al.*, 1984):



(24)

6-Hydro-5-yl radicals of thymine are also formed by addition of the H atom to the C6 of thymine.

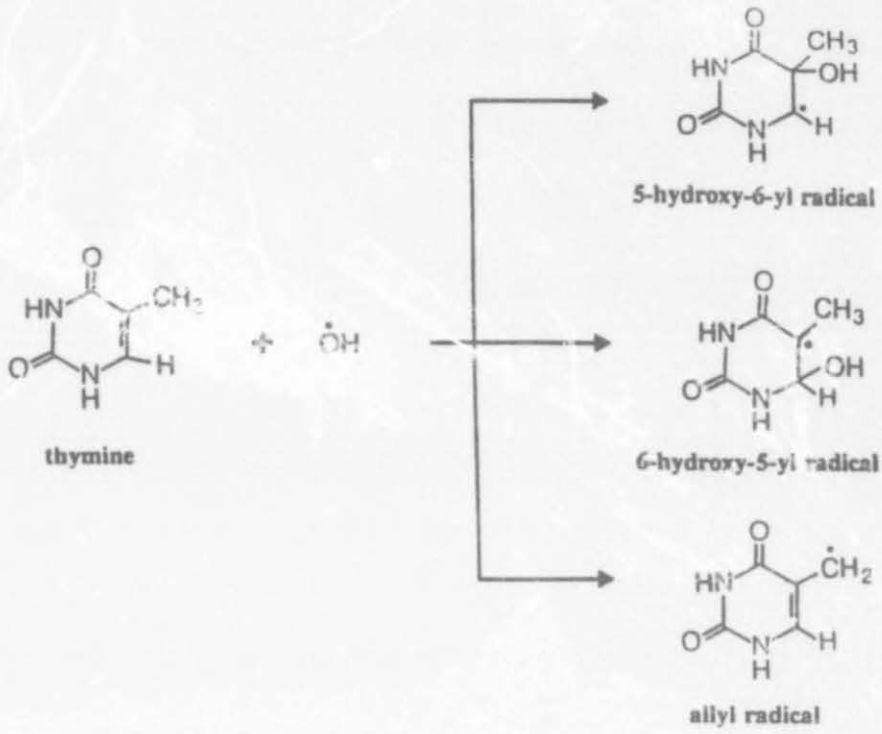
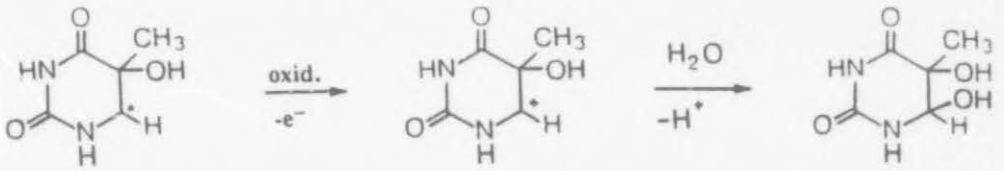


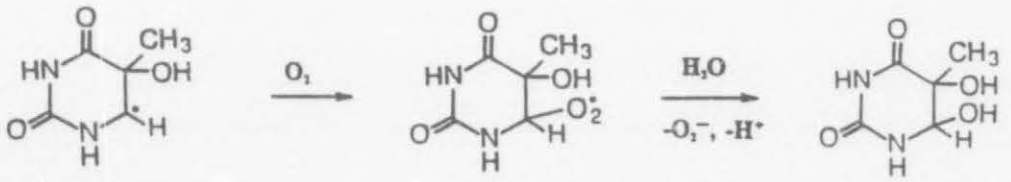
FIGURE 2: ·OH attack on thymine (von Sonntag, 1987)

Further reactions of radical adducts give rise to stable products. For example, the reduction and concomitant protonation of the 6-hydro-5-yl radical gives rise to the formation of 5,6-dihydrothymine. The oxidation of the 5-hydroxy-6-yl radical followed by addition of OH^- results in the formation of thymine glycol (Téoule, 1987; Téoule and Cadet, 1978):



5-hydroxy-6-yl radical — thymine glycol (25)

Cytosine glycol can be formed by similar reactions. Thymine glycol formation in the presence of O_2 involves the reaction of the 5-hydroxy-6-yl radical of thymine with O_2 to give the 5-hydroxy-6-peroxyl radical followed by deprotonation and elimination of O_2^-:



5-hydroxy-6-yl radical — 5-hydroxy-6-peroxyl radical — thymine glycol (26)

Reduction of the 5-hydroxy-6-yl and 6-hydroxy-5-yl radicals can give rise to the respective 5-hydroxy-6-hydro- and 6-hydroxy-5-hydropyrimidines. 5-Hydroxyhydantoins are formed as a result of C5/C6 pyrimidine bond cleavage (Téoule, 1987). Pyrimidine glycols are formed both in the presence and absence of oxygen. 5,6-Dihydrothymine, however, is formed only in the absence of oxygen because the required precursors, e_{aq}^- and H atom are scavenged by oxygen at diffusion-controlled rates.

Reactions of purines with $^{\cdot}OH$ give rise to OH-adduct radicals of purines (Fig. 3). Only base modifications involving the C8-OH adduct radical will be discussed since this is well understood. Chemical modifications of C8-OH adduct radicals of purines involve opening of the imidazole ring (for a review see Steenken, 1989). One-electron oxidation of C8-OH-adduct radicals leads to 8-hydroxypurine formation. Similarly, one-electron reduction of the ring-opened forms of C8-OH-adduct radicals gives rise to formamidopyrimidines. Reduction of C8-OH-adduct radicals without the ring opening results in the formation of 7-hydro-8-hydroxypurines which can ultimately be converted into formamidopyrimidines. Figure 4 illustrates these reactions in the case of guanine. Hydrated electrons also react with purines at diffusion-controlled rates and thus-formed electron-adducts undergo protonation reactions (Steenken, 1989).

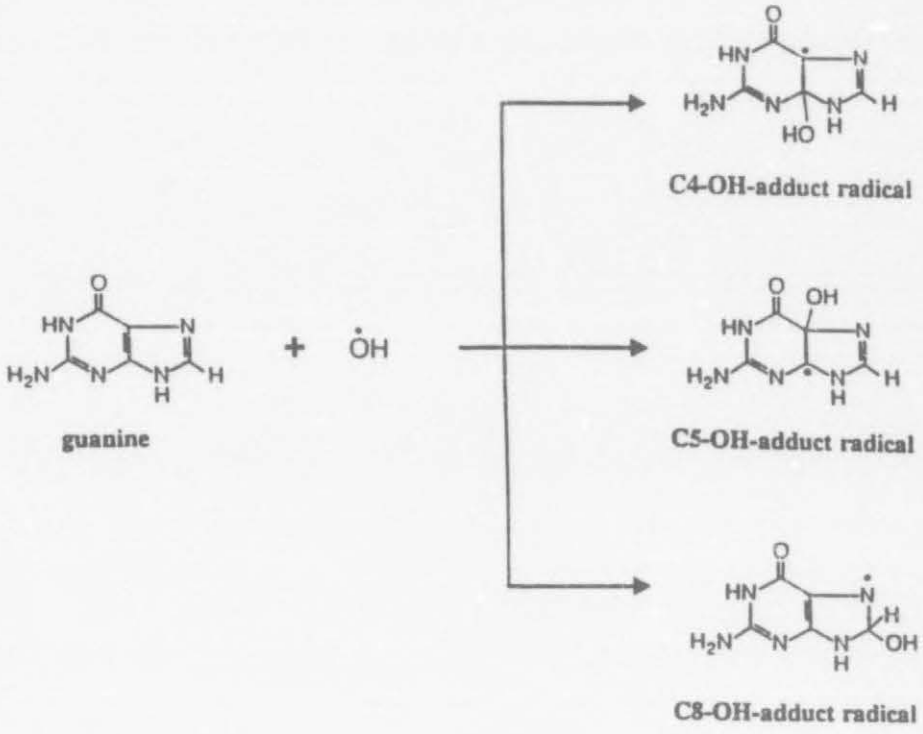


FIGURE 3: ·OH attack on guanine (O'Neill, 1983).

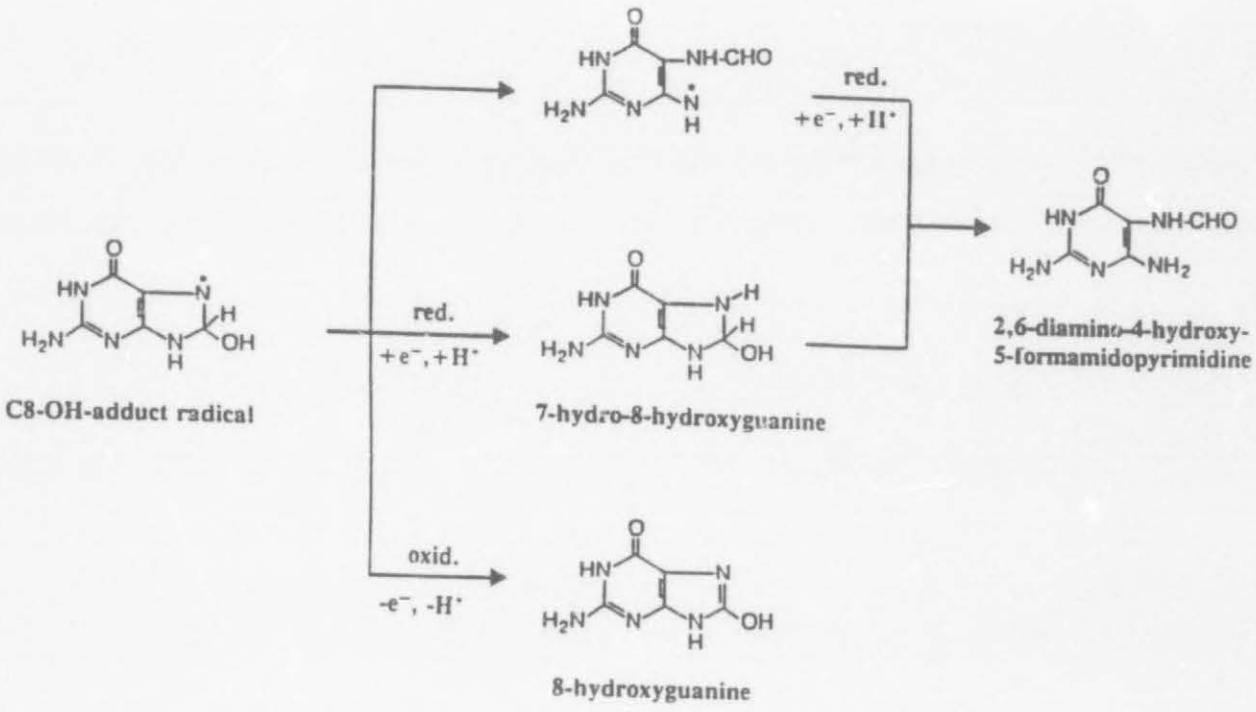
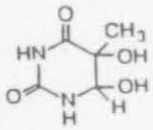


FIGURE 4: Reactions of the C8-OH-adduct radical of guanine (Steenken, 1989).

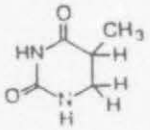
Different mesomeric structures of purine adduct radicals may have oxidizing and reducing properties (Steenken, 1987). With the known exception of $^{\cdot}$OH-adduct radicals of adenine (Vieira and Steenken, 1987), the majority of purine-derived adduct radicals do not appear to interact with oxygen (von Sonntag, 1987). 8-Hydroxypurines and formamidopyrimidines are formed both in the absence and presence of oxygen, although the formation of 8-hydroxypurines is preferred in the presence of oxygen (Fuciarelli *et al.*, 1990; Gajewski *et al.*, 1990).

Fig. 5 illustrates some of the free radical-induced DNA base modifications. The structures of 8,5'-cyclopurine-2'-deoxynucleosides and 2-hydroxyadenine are included. 8,5'-Cyclopurine-2'-deoxynucleosides are formed by addition of the C5'-centered sugar radical to the C8-position of the purine ring of the same nucleoside followed by oxidation of the thus-formed adduct radical (Fuciarelli *et al.*, 1985; Dizdaroglu, 1986; Dirksen *et al.*, 1988). Oxygen prevents the formation of 8,5'-cyclopurine-2'-deoxynucleosides by reacting with the C5'-centered radical. 2-Hydroxyadenine (see Chapter 3) is formed by attack of $^{\cdot}$OH at the C2-position of adenine in DNA followed by oxidation of the C2-OH-adduct radical, similar to the formation of 8-hydroxypurines.

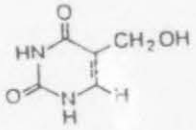
DNA base modifications have been the subject of exhaustive studies incorporating different methodologies in



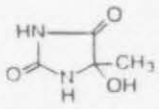
thymine glycol



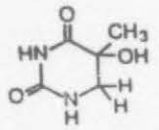
5,6-dihydrothymine



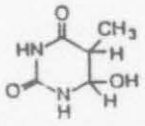
5-(hydroxymethyl)-uracil



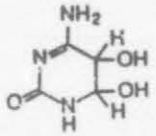
5-hydroxy-5-methyl-hydantoin



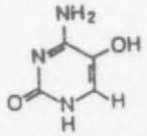
5-hydroxy-6-hydro-thymine



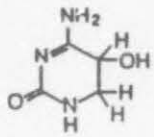
6-hydroxy-5-hydro-thymine



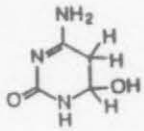
cytosine glycol



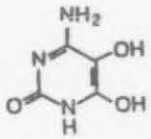
5-hydroxycytosine



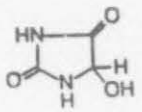
5-hydroxy-6-hydro-cytosine



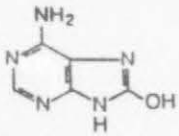
6-hydroxy-5-hydro-cytosine



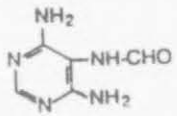
5,6-dihydroxycytosine



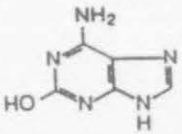
5-hydroxyhydantoin



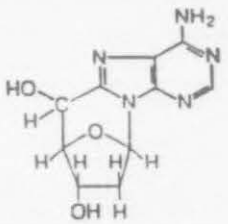
8-hydroxyadenine



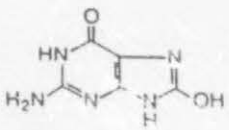
4,6-diamino-5-formamido-pyrimidine



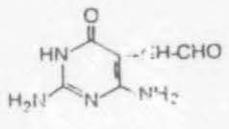
2-hydroxyadenine



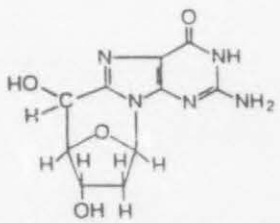
8,5'-cyclo-2'-deoxyadenosine (5'R- and 5'S-)



8-hydroxyguanine



2,6-diamino-4-hydroxy-5-formamidopyrimidine



8,5'-cyclo-2'-deoxyguanosine (5'R- and 5'S-)

FIGURE 5: Structures of some DNA base modifications.

recent years. For example, 5-hydroxyhydantoins, 5,6-dihydrothymine, 8,5'-cyclo-2'-deoxyadenosine, 5-(hydroxymethyl)uracil, 8-hydroxypurines, formamidopyrimidines and adenine N-1-oxide have been identified using techniques such as HPLC with radioactivity, absorbance and electrochemical measurements, immunochemical techniques, postlabeling assays and NMR spectroscopy (Téoule *et al.*, 1974; Téoule and Cadet, 1978; Frenkel *et al.*, 1981, 1985, 1991; Randerath *et al.*, 1981; Fuciarelli *et al.*, 1985; Kasai *et al.*, 1986). In most cases products were identified without any structural evidence. Prior radioactive labelling of DNA can complicate product analysis because of problems such as autoirradiation.

Base modifications are only one consequence of free radical damage to DNA. Other lesions to DNA and nucleoprotein include altered sugars, base-free sites, alkali-labile sites, single and double strand breaks, inter- and intra-strand DNA cross-linking and DNA-protein cross-links. The chemistry of DNA-protein cross-link formation *in vivo* has only recently been analyzed and will be discussed in Chapter 3. Hydroxyl radical is implicated in the formation of DNA-protein cross-links induced by ionizing radiation (Mee and Adelstein, 1981; Oleinick *et al.*, 1987; Lesko *et al.*, 1982).

Only precise structural identification of any of the above-mentioned lesions will contribute to the understanding of their biological consequences. The GC/MS-SIM technique

represents itself as a powerful tool to unequivocally identify and quantitate multiple lesions in DNA and nucleoprotein. Its selectivity and sensitivity have permitted the measurement of a large number of modified DNA bases, products of the sugar moiety, 8,5'-cyclopurine-2'-deoxynucleosides and DNA-protein cross-links (for a review see Dizdaroglu, 1991).

1.4 Chromatin

In living cells, DNA is not free, but complexed with histones and other biomolecules to form chromatin. This complex forms a variety of substructures such as the nucleosome, solenoid, loop, miniband and chromatid, which are present during the different phases of the cell cycle. The nucleosomal subunit consists of about 200 bp of DNA interacting with 5 histones. One function of the histones is to confer the necessary DNA compaction. Nucleosomes can be further subdivided into chromatosomes. This particle consists of 166 bp of DNA (about two turns) and the full complement of five histones. DNA is wound in a left-handed superhelix around two molecules each of histones H2A, H2B, H3 and H4. The highly conserved histones, H3 and H4 are arginine-rich, while histones, H2A and H2B are slightly lysine-rich. The $(H3/H4)_2$ tetramer may direct the supercoiling of the DNA, while the H2A/H2B dimer is proposed to associate, one on either side of the core. The two terminal 10 bp extensions on either side of the chromatosome superhelix are protected by

one molecule of histone H1. Linker DNA of variable length separates these particles. The nucleosome minus linker DNA and histone H1 is termed the "core" particle. It is described as a disc of diameter 110 Å and height 57 Å, with the DNA wound in a superhelix around the outside of the histone core. The 146 bp stretch of DNA is therefore organized in 1.75 turns around the histone core with about 80 bp per superhelical turn (Felsenfeld, 1978).

Additional information from nuclease studies led to the proposal that the nucleosome has a dyad axis of symmetry. Hence, it may be capable of unfolding symmetrically, perhaps as part of a mechanism of replication or transcription (van Holde, 1989). The second-order level of DNA packing is not as well defined as the nucleosome. It is most probably a 30 nm solenoid composed of 6-7 nucleosomes per turn and with a pitch of 110 Å (Finch *et al.*, 1977). Models from the literature do not clearly resolve the positioning of the nucleosomes and the location of histone H1 in the solenoid. However, the major consensus is that the maintenance of a solenoid structure depends on the ionic strength of the medium and the presence of histone H1. This is underlined by the disappearance of the solenoidal fibre at low ionic strength (< 60 mM NaCl) or in the absence of histone H1 (Thoma *et al.*, 1979). Cooperative binding of histone H1 to H1 molecules on adjacent nucleosomes can stabilize the 30 nm solenoid.

The chromatin loop constitutes the third level of DNA

packing in chromatin. Fig. 6 illustrates the relationship of the chromatin loop to the dimensions of chromatids. Chromatin loops are constrained both in interphase and metaphase by a protein framework termed the nuclear matrix, cage or skeleton (interphase). In the case of metaphase cells, the framework is termed a nuclear scaffold.

Chromatin loops emanate from a central axis formed by the nuclear matrix to give rise to the miniband subunit of the chromosome. Miniband subunits representing an average size of 1.1×10^6 bp align to form the chromatid, thereby increasing the DNA packing ratio from ≈ 1 to 1.2×10^4.

Mammalian chromatin is more complex and biologically more representative than pure DNA for studies of free radical-induced damage to cellular DNA. Histones that are closely associated with DNA in nucleosomes may also react with free radicals, and DNA bases may participate in formation of DNA-protein cross-links in chromatin. Furthermore, if DNA of living cells is exposed to free radical-producing systems and subsequently analyzed for DNA lesions, it would be desirable to analyze the whole chromatin rather than DNA component alone. DNA, which is covalently cross-linked to proteins, cannot be extracted efficiently (Mee and Adelstein, 1979). Thus one may lose a significant portion of modified DNA bases. For these reasons, it is important to study the free radical chemistry of mammalian chromatin under oxic and anoxic conditions of free radical production.

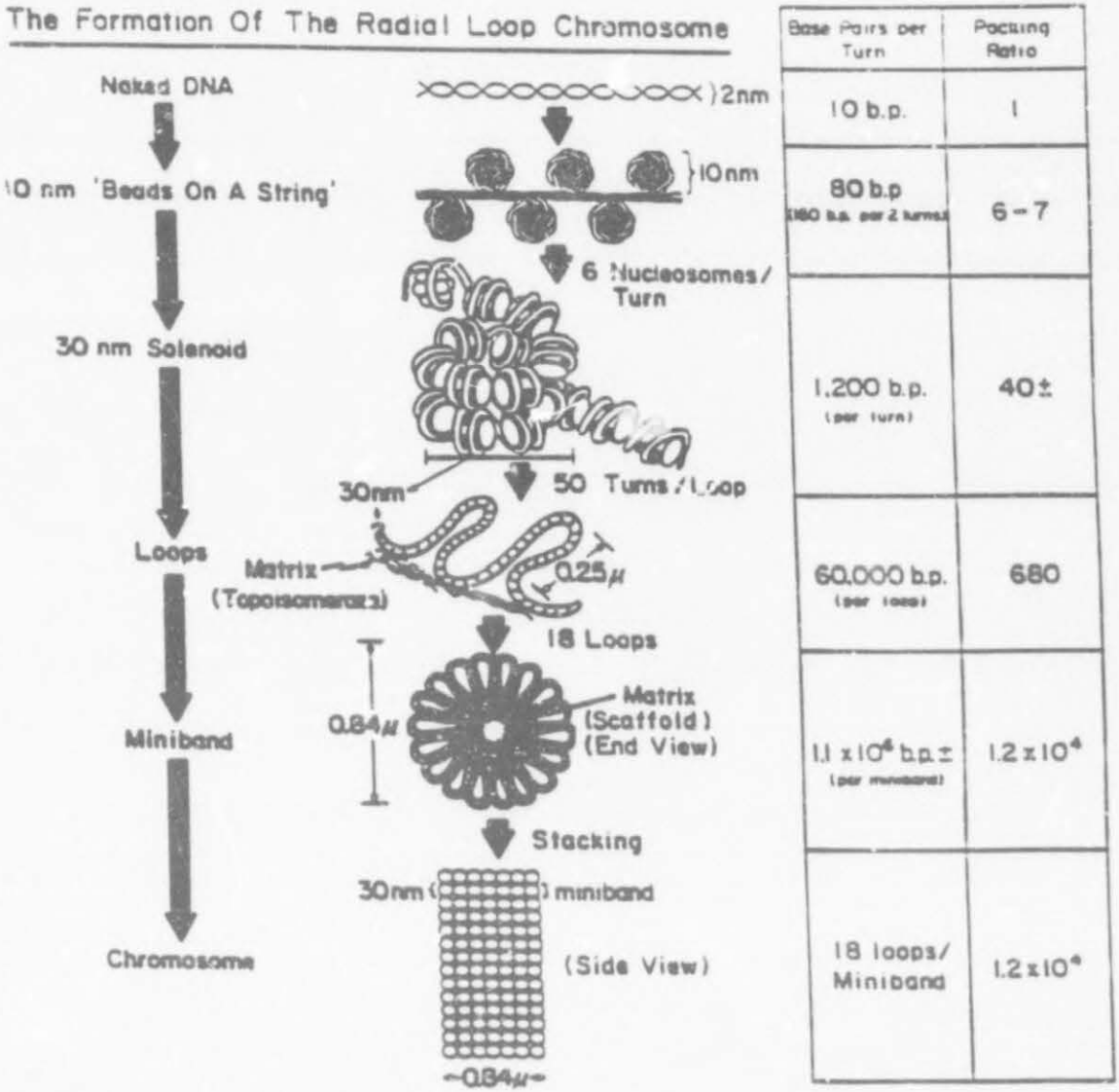


FIGURE 6: Model of DNA packing within a chromosome (Nelson et al., 1986).

2. EXPERIMENTAL PROCEDURES

2.1 Materials

Sigma Chemical Company was the source for all cell culture materials. The following chemicals were all purchased from the same co.: Phe-Phe, SOD, catalase, PMSF, PBS (Ca^{2+}, Mg^{2+} - free), sodium azide, H_2O_2, trypan blue, Me_2SO, Triton-X-100, $NiCl_2 \cdot 6H_2O$, $CoSO_4 \cdot 7H_2O$, D-mannitol, fetal bovine serum, calf thymus DNA, ascorbic acid, NTA, 6-azathymine, 8-azaadenine, glutathione, isoguanine (2-OH-Ade), thymine, cytosine, adenine, guanine. 5-OH-5-Me-Hyd was a gift from Dr. W. F. Blakely of the Armed Forces Radiobiology Research Institute, Bethesda, Maryland. Lifecell bags were from Baxter Healthcare Corp. Sterile mixtures of penicillin and streptomycin were obtained from GIBCO-BRL. Cell culture solutions made in the laboratory were either sterile-filtered using 0.22 μm filters (Nalge Co.) or autoclaved (Hirayama).

DTT was from Aldrich. The following chemicals came from Fisher Scientific: HCl, NaOH, acetic acid, H_2SO_4, $CaCl_2$, EDTA, $CuSO_4 \cdot 5H_2O$. $FeCl_3 \cdot 6H_2O$ was from Allied Chemical. Dialysis membranes with a molecular weight cutoff of 3500 were purchased from the same company. Commercial histone standards and sucrose were from Boehringer Mannheim. Methanol, ethanol, acetone, formic acid and bromophenol blue were purchased from Mallinckrodt. All the chemicals for SDS gel electrophoresis as well as Chelex 100 (200-400 mesh) came

from Bio-Rad. The protein assay kit, BSTFA, acetonitrile and 6 N HCl (constant boiling) were all obtained from Pierce.

Water purified through a Barnstead Nanopure II system was used in all experiments.

2.2 Cell culture

Two cell lines were used for these experiments. The first cell line, SP2/O-derived mouse murine hybridoma, was grown in the laboratory of Dr. G.Rao (University of Maryland, Baltimore County). The cell line has been designated HyHel-10 and produces IgG antibodies against hen egg white lysozyme (courtesy of Dr. S. J. Smith-Gill, National Cancer Institute, Bethesda, Maryland).

For *in vitro* experiments, SP2/O cells were grown at 37 °C, in an atmosphere of 95 % air and 5 % CO_2 in 750 cm^2 Lifecell bags containing 1.8 l of a 1/1 (v/v) mixture of DMEM and F-12 medium supplemented with 4 % fetal bovine serum. Cells were harvested at mid- to late-exponential growth phase at a density of approximately 7 x 10^5 cells/ml. The cells were centrifuged in 250 ml bottles at 1700 *g* for 10 min, washed once by resuspending them in 50 ml of PBS (Ca^{2+}-and Mg^{2+} - free) plus 0.01 % sodium azide, centrifuged again, and resuspended at 4 °C in 30 ml of fresh PBS containing 0.01 % sodium azide. Chromatin was isolated within 24 h.

K562 cells were first obtained as a gift from Dr. S.A. Akman (City of Hope National Medical Center, Duarte,

California) and later purchased from the American Type Culture Collection. The K562 continuous line is characterized as a human chronic myelogenous leukemia cell line. Suspension cultures of this cell line were incubated at 37 °C, in an atmosphere of 3 % CO_2 and 97% air. The growth medium consisted of RPMI medium supplemented with 10 % fetal bovine serum, L-glutamine (4 mM), penicillin (50 units/ml) and streptomycin (50 μg/ml).

2.3 Treatment of cells

2.3.1 H_2O_2

SP2/O cells were grown at 37 °C in CO_2. When cells reached a density of 8.2 x 10^5 /ml, they were split into six 50-ml parts and transferred into T-125 culture flasks. Cell viability as determined by trypan blue exclusion was ≈96 %. H_2O_2 was added to the flasks such that two contained 2 mM H_2O_2 and two contained 20 mM H_2O_2 each. One pair of T-flasks without H_2O_2 served as control. All flasks were placed in an incubator at 37 °C for 1 h, sampled for cell viability, and then simultaneously placed into an ice water bath. In all cases, cell viability was essentially unchanged. After 10 min, cells were harvested at 4 °C at 1000 *g*. The cells were washed by resuspension and subsequent centrifugation in PBS containing 0.01 % sodium azide. Chromatin was subsequently isolated as in section 2.4.

2.3.2 Irradiations

Cells in air-saturated culture medium (4 x 10^6 cells/ml) were irradiated in a ^{60}Co γ-source while their container was submerged in an ice bath. The dose range was 42 to 420 Gy and the dose rate as determined by a Fricke dosimeter was 98.4 Gy/min (Fricke and Hart, 1966). Immediately after irradiation, cells were frozen in liquid nitrogen.

2.4 Chromatin

2.4.1 Chromatin Isolation

Chromatin was isolated according to a modified procedure of Mee and Adelstein (1981). Approximately 4 x 10^8 cells in PBS were centrifuged at 300 *g*, and the PBS was removed. The cell pellet was suspended in 35 ml cold sucrose buffer (0.25 M sucrose, 3 mM $CaCl_2$, 0.1 mM PMSF, 0.1 mM DTT , 50 mM Tris-HCl at pH 7.4) and kept in an ice bath for 10 min Cells were centrifuged at 300 *g*, then lysed in 35 ml cold sucrose buffer containing 1 % Triton X-100 and kept in an ice bath for 10 min The nuclei were centrifuged at 1000 *g* for 15 min and then washed with cold sucrose buffer without Triton X-100 and recentrifuged. Sucrose buffer was removed, the nuclei were suspended in 30 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM PMSF, kept in an ice bath for 15 min, and centrifuged at 1000 *g* and the buffer was removed. This procedure was repeated with 10, 5, and 1 mM Tris-HCl buffer

each containing 0.1 mM PMSF.

Subsequently, chromatin was dialyzed extensively against 1 mM phosphate buffer (pH 7.4). All operations were carried out at 4 °C. After dialysis, chromatin was homogenized briefly with a few strokes in a glass homogenizer.

2.4.2 Irradiation of isolated chromatin

Aliquots of chromatin samples (0.12 mg of DNA/ml) dialyzed against phosphate buffer (pH 7.4) were bubbled separately with N_2O and N_2O/O_2 (4/1) for 20 min prior to and then throughout the irradiations, which were done in a ^{60}Co γ-source (dose rate 124 Gy/min). After irradiation, samples were lyophilized.

2.4.3 Treatment of isolated chromatin with H_2O_2 and transition metal ions.

For *in vitro* DPC experiments with Fe(III) and Cu(II) ions the following approach was adopted. Reaction mixtures contained the following compounds, where appropriate, in a final volume of 4 ml of 1 mM phosphate buffer (pH 7.4): chromatin dialyzed against phosphate buffer (0.12 mg of DNA/ml), H_2O_2 (2.8 mM), $FeCl_3$ (25 μM), $CuSO_4$ (25 μM), EDTA (100 μM), NTA (100 μM), ascorbic acid (100 μM), mannitol (50 mM), Me_2SO (50 mM) and SOD (200 units/ml). Where indicated, $FeCl_3$ and $CuSO_4$ were mixed with EDTA or NTA prior to addition to the reaction mixture. Chelex-treated phosphate buffer (1 mM, pH 7.4) was used for all dilutions. Mixtures were incubated

at 37 °C for 1 h. After incubation, aliquots of chromatin samples containing 0.5 mg of DNA were immediately frozen in liquid nitrogen and lyophilized.

DNA base damage experiments involving Ni(II) and Co(II) ions were approached as follows: Reaction mixtures contained the following compounds, where appropriate, in a final volume of 1.2 ml of 1 mM phosphate buffer (pH 7.4): chromatin (0.12 mg of DNA/ml); $NiCl_2$ (25 μM or 100 μM); $CoSO_4$ (25μM or 100 μM); EDTA (120 μM); H_2O_2 (2.8 mM or 10 mM); ascorbic acid (100 μM); glutathione (1 mM); mannitol (50 mM); Me_2SO (50 mM); catalase (1100 units/ml); and SOD (200 units/ml). One unit of catalase decomposes 1 μmol of H_2O_2/min at pH 7.0 at 25 °C, under the conditions given in the Sigma catalogue. Units of SOD were as defined by the cytochrome c assay (McCord and Fridovich, 1969).

In experiments with EDTA, $NiCl_2$ and $CoSO_4$ were mixed with EDTA (120 μM) prior to addition to reaction mixtures. Chelex-treated phosphate buffer (1 mM; pH 7.4) was used for all dilutions. Mixtures were incubated at 37 °C for 1 h to 24 h. After incubation, 0.5 nmol of 6-azathymine and 2 nmol of 8-azaadenine were added to aliquots of chromatin containing 0.12 mg of DNA. The samples were immediately frozen in liquid nitrogen and then lyophilized.

2.5 DNA Assay

The DNA content of chromatin was determined by UV absorption at 258 nm and Burton's assay (Burton, 1968). UV absorption at 258 nm was measured using $\epsilon = 6.6 \times 10^3\ M^{-1} \cdot cm^{-1}$. Burton's assay measures a triphenylmethyl derivative of DNA bases by spectrophotometry. The calf thymus DNA standard (Sigma; Type 1) was suspended in 5 mM NaOH (kept at 4 °C) and the concentration verified by UV absorbance at 258 nm.

The working reagent consists of two parts: Reagent A (aqueous acetaldehyde stored under N_2 and 1 ml transferred into 50 ml of water) and reagent B (1.5 g diphenylamine in 100 ml of glacial acetic acid plus 1.5 ml H_2SO_4 ; kept in the dark at 4 °C).

A DNA standard curve in the range 0 - 60 µg/ml was generated as follows: 0.75 ml of the DNA standard was mixed with 0.75 ml 10 % $HClO_4$ and the UV absorbance read at 258 nm. Various dilutions of chromatin in 0.5 ml of water were prepared. To each sample 0.5 ml 10 % $HClO_4$ was added. All samples were heated at 70 °C for 20 min.

Working reagent (2 ml) was added to each 1 ml sample. Colour development occurred after keeping tubes in the dark overnight. DNA content was determined by reading the absorbance at 600 nm.

2.6 Protein Assay

The protein content of chromatin was determined according to the protein assay reagent kit of Pierce. Three solutions are included in this kit: Reagent A (sodium bicarbonate; BCA detection reagent and tartrate in 0.1 N NaOH); Reagent B (4 % $CuSO_4 \cdot 5\ H_2O$) and albumin standard (bovine serum albumin, fraction V; 2 mg/ml in 0.9 % NaCl plus sodium azide; stored at 4 °C).

Before each assay, a fresh working reagent was prepared by adding 1 part reagent B to 50 parts of reagent A. Albumin standards were diluted in water to generate a linear standard graph in the range 0 - 1 mg/ml. Aliquots of chromatin samples were diluted in 1 ml of water. Each sample (0.1 ml) was added to 2 ml of working reagent in Kimble glass tubes. Samples were subsequently incubated in a shaking 60 °C water bath for 30 min. Samples were cooled to room temperature and the protein content determined by reading the absorbance at 562 nm.

2.7 RNA Assay

The RNA content of chromatin was determined by a modified method of Schneider (1956).

The basic procedure is described as follows: Yeast RNA Type XI (Sigma) served as a standard. The working reagent consisted of : reagent A (100 mg $FeCl_3 \cdot 6\ H_2O$ dissolved in 100 ml concentrated HCl and stored at 4 °C in the dark) and

freshly prepared reagent B (0.6 g orcinol stored under N_2 in 10 ml ethanol; kept at 4 °C in the dark). Before each experiment, working reagent was prepared by adding 3.5 ml reagent B to 100 ml of reagent A.

A standard curve was generated by diluting RNA and chromatin respectively in 1.5 ml of wat After addition of 1.5 ml of working reagent, colour deve t proceeded in a shaking 90 °C water bath for 30 min. Samples were allowed to cool and the absorbance read at 660 nm.

Based on this assay plus RNA extraction from chromatin (Dr. S. Altman, University of Maryland; unpublished observations), the RNA content of chromatin was found to be ≤ 5 % of the total nucleic acid content of chromatin.

2.8 SDS-Gel Electrophoresis

The protein components of chromatin were analyzed by gel electrophoresis essentially as described by Laemmli (1970) with a modification in the ratio of acrylamide to bis(acrylamide). Gels were cast on a Hoeffer SE 600 vertical slab gel unit (15 x 18 cm slab gel with 1.5 mm spacers). The 18 % separating gel was made by mixing appropriate volumes of the following solutions: solution 1: 18 ml of 30 % (w/v) acrylamide; 2.7 % (w/v) Bis; solution 2: 7.5 ml of 1.5 M Tris-HCl (pH 8.8) and 4.3 ml deionized water. After degassing the mixture for 5 min, the reaction was initiated by the addition of 10 µl TEMED and 150 µl 10 % (w/v) AMPS (freshly prepared

for each gel). Running gel overlay (0.375 M Tris; pH 8.8; 0.1 % SDS) was carefully applied on top of the separating gel.

The stacking gel was prepared by mixing the following solutions were together: 1.3 ml of solution 1, 2.5 ml of 0.5 M Tris-HCl (pH 6.8) and 6 ml deionized water. After degassing the mixture for 5 min, 50 µl 10 % (w/v) AMPS and 10 µl TEMED were added.

The tank buffer was prepared as follows: 12 g Tris, 57.6 g glycine and 40 ml of 10 % (w/v) SDS were dissolved in 4 l of deionized water to give 0.025 M, 0.192 M and 0.1 %. The final pH was 8.3 and the buffer was filtered prior to use.

Chromatin samples and commercial histone standard (Boehringer Mannheim) were suspended in deionized water at concentrations ranging from 0.2 - 1 mg/ml respectively and mixed 1:1 with 0.125 M Tris-HCl pH 6.8; 4 % (w/v) SDS; 20 % (v/v) glycerol and 10 % (v/v) 2-mercaptoethanol.

Electrophoresis was carried out at 40 mA for 2 h and gels were subsequently stained with 0.125 % (w/v) Coomassie Blue R-250; 50 % (v/v) methanol; 10 % (v/v) acetic acid. Gels were destained for 1 h in a shaking 37 °C water bath in a mixture of 50 % (v/v) methanol and 10 % (v/v) acetic acid. Prior to photography gels were placed in 7 % (v/v) acetic acid; 5 % (v/v) methanol.

2.9 Characterization of chromatin.

The authenticity of the protein components in isolated chromatin was determined by using SDS-polyacrylamide gel electophoresis using commercially available histones as reference compounds. The electrophoretic patterns of isolated chromatin (lane 1) and commercial histone (lane 2) are illustrated in Fig. 7. These patterns of histones are similar to those published previously (Panyim and Chalkley, 1969; Böhm *et al.*, 1973). Characterization of the non-histone proteins present in chromatin was not undertaken in this study. Figure 8 illustrates an absorption spectrum characteristic of mammalian chromatin. This is similar to those published previously (Bonner *et al.*, 1968).

The ratio of the amount of protein to that of DNA was 2 (w/w). The RNA content of chromatin was found to be ≤ 5 % of the amount of DNA. Isolated chromatin samples typically exhibited the following spectral characteristics (Gajewski *et al.*, 1991): A_{258}/A_{280} = 1.58-1.65; A_{258}/A_{230} = 1.1-1.17; A_{258}/A_{320} = 8.5-14.2; A(maximum)/A(minimum) = 1.26-1.43.

2.10 Gas Chromatography-Mass spectrometry (GC/MS)

2.10.1 Hydrolysis

To 1 ml samples of chromatin (in 1 mM Tris; pH 7.4) containing 0.1 mg of DNA or to 1 ml of calf thymus DNA samples (0.1 mg), 0.5 nmol of 6-azathymine and 2 nmol of 8-azaadenine

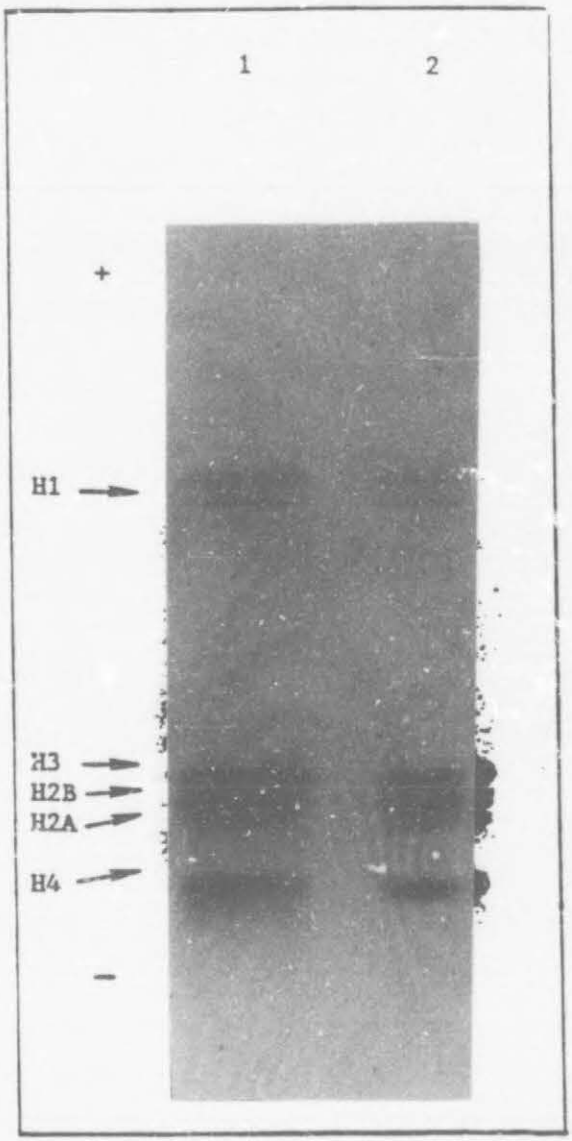


FIGURE 7: Gel electrophoresis of chromatin suspended in 1 mM phosphate buffer, pH 7.4 (lane 1) and of commercial histones H1, H3, H2B, H2A and H4 (lane 2).

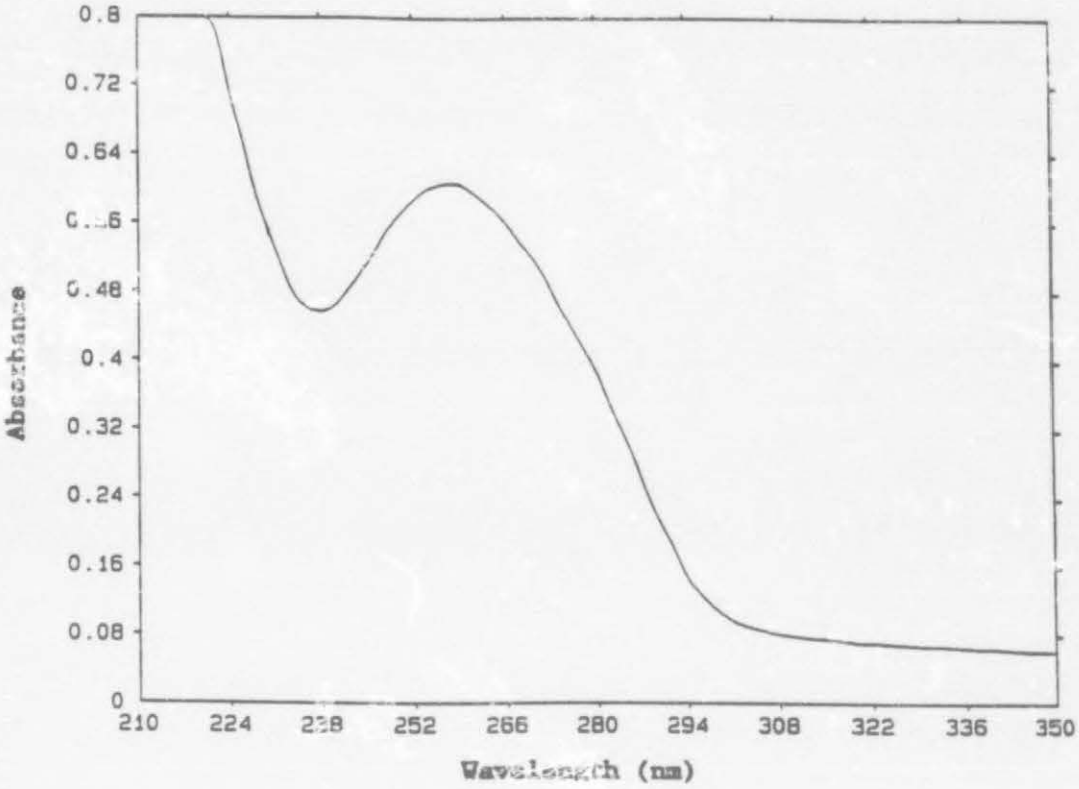


FIGURE 8: Absorption spectrum of chromatin suspended in 1 mM phosphate buffer, pH 7.4.

were added as internal standards. Samples were then lyophilized. For *in vitro* DNA base damage experiments, lyophilized aliquots of chromatin were hydrolyzed with 0.5 ml of 88 % formic acid (Fisher Scientific) in evacuated and sealed tubes for 40 min at 150 °C. Acidic hydrolysis permits the complete release of modified and intact bases by cleaving the glycosidic bonds between base and sugar moieties in DNA.

For DNA-protein cross-link experiments, lyophilized chromatin samples (0.4 - 0.5 mg of DNA) were hydrolyzed with 1 ml 6 N HCl in evacuated and sealed tubes for 6 h at 120 °C. After cooling to room temperature, an aliquot of Phe-Phe was added as an internal standard. Following the addition of the internal standard, samples were frozen immediately in liquid nitrogen and then lyophilized.

2.10.2 Derivatization

Trimethylsilylation of the modified DNA bases and DNA base-amino acid cross-links is essential in order to render the substrates volatile enough for gas chromatography.

Trimethylsilylation is a chemical reaction in which an active hydrogen (from a carboxylic acid, hydroxyl group or amino group) is substituted by a trimethylsilyl (Me_3Si) group (Schram, 1990). Chromatin samples (0.1 mg/ml with respect to DNA) were trimethylsilylated with 0.1-0.15 ml of a BSTFA/acetonitrile (2/1-4/1; v/v) mixture in poly(tetrafluoroethylene)-capped hypovials at 130 °C for 30

min. Acetonitrile served as a solvent and BSTFA served as the actual silylating agent.

2.10.3 Gas Chromatography/Mass Spectrometry with Selected Ion-Monitoring

Analysis of derivatized samples was performed by using a mass-selective detector interfaced to a gas chromatograph (both from Hewlett-Packard) equipped with an automatic sampler and a computer work station. Separations were carried out by using a fused silica capillary column [12.5 m x 0.20 mm i.d. (internal diameter)] coated with cross-linked 5 % phenylmethylsilicone gum phase (film thickness, 0.33 μm; all from Hewlett Packard). In the case of DPC experiments, separations were carried out on a 8 m (length) x 0.2 mm (i.d.) column (film thickness of 5% phenylmethylsilicone gum phase = 0.11 μm). Fused silica capillary columns provide high inertness, separation efficiency and permit accurate measurements. Helium (ultra high purity) was used as the carrier gas at an inlet pressure of 40 kPa. The injection port, the ion source and the interface were maintained at 250 °C. The glass liner in the injection port was filled with silanized glass wool. This allows the homogeneous vaporization of injected samples in the injection port and avoids peak tailing.

The selected-ion monitoring (SIM) mode increases the sensitivity and selectivity of GC/MS and enables unequivocal identification and quantitation of organic compounds (e.g. low

electron-ionization mode at 70 eV. An aliquot (0.4-4 µl of each derivatized sample) was injected without any further treatment into the injection port of the gas chromatograph using the split mode of injection (split ratio 1:20). The amount of DNA injected onto the column for each analysis was approximately 0.2-0.4 µg.

The chemical structures of modified DNA bases and DPCs were identified by comparison to the published electron-ionization mass spectra of their Me_3Si derivatives (Dizdaroglu, 1984; 1985; 1991). These mass spectra provide considerable structural information that could be used for unequivocal identification of corresponding compounds. In the case of intact DNA bases and modified DNA bases, the mass spectra are characterized by prominent ions, including the molecular ion [$M^{+\bullet}$ ion], an intense [$M-CH_3$]$^+$ ion resulting from loss of a methyl radical from the $M^{+\bullet}$ ion and, in some instances, a [M-H]$^+$ ion resulting from loss of an H atom from the $M^{+\bullet}$ ion (Dizdaroglu, 1984; 1985; 1991).

Mass spectra in the case of DPCs contain $M^{+\bullet}$ and [M-15]$^+$ ions as well as ions typifying fragmentation patterns of base and amino acid moieties (Dizdaroglu, 1984; 1990; Gajewski *et al.*, 1988).

Identification of analytes in a mixture necessitate the monitoring of a number of characteristic ions of a compound in the time that it is expected to elute from the GC column. Accurate identification is ensured when the simultaneous

Accurate identification is ensured when the simultaneous signals of the ions with the correct abundances all line up at their respective retention times (for reviews see Watson, 1985; Dizdaroglu, 1991).

Quantitative measurements depend on the calibration of the mass spectrometer with respect to the signals generated by an internal standard and the compounds of interest. Fuciarelli *et al.* (1989) established the use of 6-azathymine and 8-azaadenine as suitable internal standards for quantitation of DNA base lesions because stable isotope-containing analogues of modified DNA bases were not available. The Me_3Si derivatives of these compounds gave intense $M^{+\cdot}$ and $[M-CH_3]^+$ ions (m/z[3] 271 and 256 respectively, and m/z 280 and 265, respectively). RMRFs[3] of modified bases were determined as described by Fuciarelli *et al.* (1989). In the case of DPCs, the information obtained from using model systems (Gajewski *et al.*, 1988) was used to identify and quantitate cross-links in chromatin.

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[3] m/z, the ratio of the mass number (m) of a given particle to the number (z) of electrostatic charge units (e) carried by the particle; **RMRFs**, relative molar response factors: The ratio of the peak areas of an analyte ion and an ion of the internal standard (A/A_i) is measured and plotted as a function of the ratio of the molar amounts of the analyte and the internal standard (m/m_i). The slope of such a plot is defined as the RMRF ($A/A_i \times m_i/m$).

3. RESULTS

3.1 Studies of DNA damage in isolated chromatin

DNA base modifications have been previously identified in γ-irradiated chromatin in the presence of various free radicals (Gajewski *et al.*, 1990). As an extension of these studies, the effect of free radicals on chromatin was investigated using the H_2O_2/metal ion system as a free radical generator.

3.1.1 Base damage produced by H_2O_2/metal ions.

Modified DNA bases formed in isolated chromatin by H_2O_2 in the presence of Fe(III) or Cu(II) have been characterized in a previous investigation (Dizdaroglu *et al.*, 1991b).

In the present work, the effects of Co(II) and Ni(II) have been studied. Figure 9 illustrates a representative chromatogram with selected ion-current profiles, which were obtained during the GC/MS-SIM analysis of trimethylsilylated hydrolysate of a Ni(II)/H_2O_2-treated chromatin sample. Peak identification is given in the figure legend. In addition to the base products found in chromatin in previous publications (Dizdaroglu *et al.*, 1991b; Gajewski *et al.*, 1990) 2-hydroxyadenine (isoguanine) was also observed in the present work.

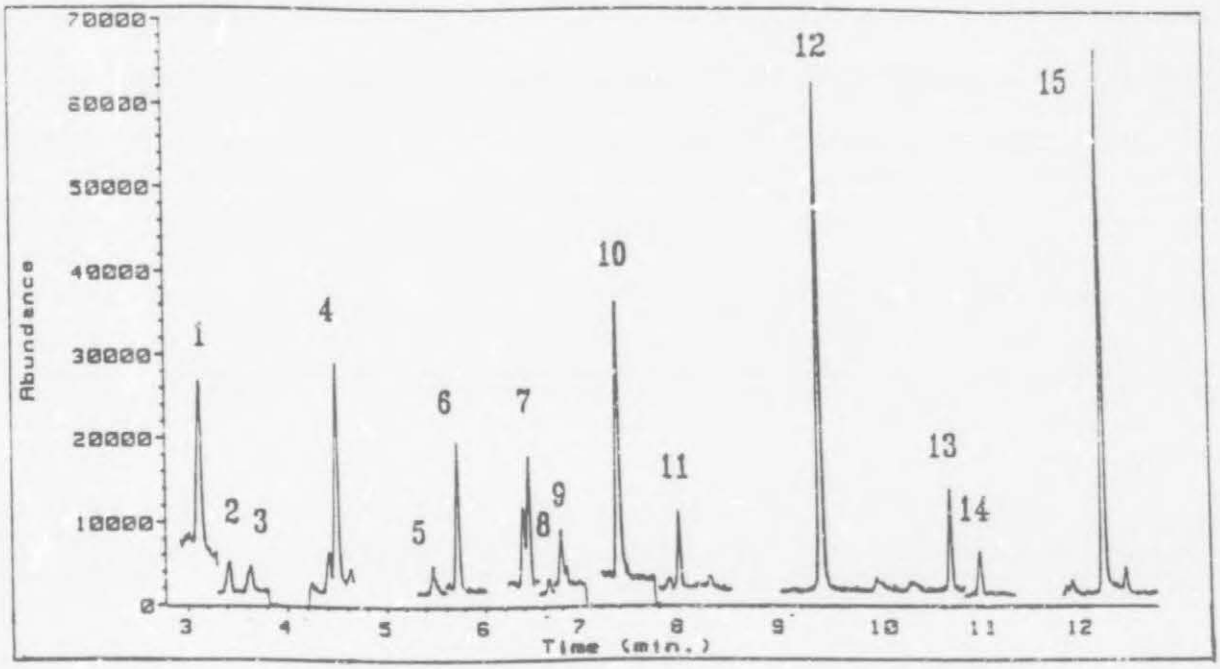

FIGURE 9: Selected ion-current profiles obtained during the GC/MS-SIM analysis of chromatin treated with Ni(II) (100 μM)/H_2O_2 (2.8 mM) for 24 h. Experimental details are in Chapter 2. Peaks (ions): 1, 6-azathymine (*m/z* 256) (internal standard); 2, 5-hydroxy-5-methylhydantoin (*m/z* 331); 3, 5-hydroxyhydantoin (*m/z* 317); 4, 5-hydroxyuracil (*m/z* 329); 5, 5-(hydroxymethyl)uracil (*m/z* 358; 6, 5-hydroxycytosine (*m/z* 343); 7, cis-Thymine glycol (*m/z* 259); 8, 5,6-dihydroxyuracil (*m/z* 417); 9, trans-Thymine-glycol (*m/z* 259); 10, 8-azaadenine (*m/z* 265) (internal standard); 11, 4,6-diamino-5-formamidopyrimidine (*m/z* 354); 12, 8-hydroxyadenine (*m/z* 352); 13, 2-hydroxyadenine (*m/z* 352); 14, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (*m/z* 442); 15, 8-hydroxyguanine (*m/z* 441).

I Effect of Co(II) ions

The yields of DNA base products formed in isolated chromatin by treatment with the Co(II)/H_2O_2 system are given in Table II. The major products were cytosine glycol, formamidopyrimidines and 8-hydroxypurines. Yield to background ratios varied between ≈2 (5-OH-Hyd) and ≈18 (Thy glycol). Addition of ascorbic acid (100 μM) had no effect on product yields. The $^{\cdot}OH$ scavengers, mannitol and Me_2SO at high concentrations (50 mM) provided significant inhibition of product formation. However, yields of products were still higher than background levels. Glutathione at a physiologically relevant concentration (1 mM) caused only a moderate decrease in some of the products. On the other hand, the yields of Cyt glycol and FapyGua were doubled by glutathione. Addition of SOD to Co(II)/H_2O_2 did not reduce product yields. On the contrary, a ≈2-fold increase in product yields was observed. Chelation of Co(II) (25 μM) with EDTA (120 μM) substantially inhibited product formation. For example, the yields of 8-OH-Gua, 8-OH-Ade, and FapyAde decreased from 5.71 ± 1.99, 1.02 ± 0.29, and 0.89 ± 0.26 to 0.59 ± 0.06, 0.24 ± 0.04, and 0.17 ± 0.005 nmol/mg of DNA, respectively (also compare these values with those of column 1 in Table II).

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

Product	Treatment					
	1[c]	2	3	4	5	6
5-OH-5-Me-Hyd	0.074±0.043	0.505±0.081*	0.390±0.035‡	0.218±0.011‡	0.278±0.046‡	1.38±0.31‡
5-OH-Hyd	0.250±0.025	0.548±0.062*	0.652±0.069	0.380±0.092‡	0.319±0.061‡	1.03±0.06‡
5-OHMe-Ura	0.024±0.003	0.227±0.041*	0.080±0.010‡	0.081±0.007‡	0.170±0.044‡	0.485±0.134‡
Cyt glycol	0.082±0.013	1.87±0.45*	0.484±0.204‡	0.478±0.145‡	3.75±0.24‡	3.01±0.48‡
Thy glycol	0.017±0.002	0.289±0.002*	0.238±0.120	0.088±0.039‡	0.225±0.	.409±0.156
5,6-diOH-Cyt	0.030±0.003	0.344±0.044*	0.077±0.038‡	0.095±0.014‡	0.302±0.0	0.636±0.076‡
FapyAde	0.115±0.028	0.895±0.255*	0.380±0.113‡	0.318±0.055‡	0.735±0.108	1.47±0.044‡
8-OH-Ade	0.113±0.035	1.02±0.29*	0.290±0.042‡	0.293±0.050‡	0.513±0.031‡	2.20±0.066‡
2-OH-Ade	0.078±0.016	0.177±0.056*	0.189±0.030	0.100±0.009‡	0.108±0.017	0.297±0.067‡
FapyGua	0.471±0.073	1.86±0.53*	-	1.09±0.23	3.76±0.374‡	2.99±0.31‡
8-OH-Gua	0.526±0.165	5.71±1.99*	1.73±0.59‡	2.40±0.57‡	4.78±0.145	12.20±2.62‡

[a]All values represent the mean ± SD (n = 3). [b]Co(II) (25 μM), H_2O_2 (2.8 mM), 1 h.

[c]**1:** chr; **2:** chr/Co(II)/H_2O_2; **3:** chr/Co(II)/H_2O_2/mannitol (50 mM); **4:** chr/Co(II)/H_2O_2/DMSO (50 mM); **5:** chr/Co(II)/H_2O_2/glutathione (1 mM); **6:** chr/Co(II)/H_2O_2/SOD (200 u/ml).

*Values significantly different from column 1 ($p < 0.05$).

‡Values significantly different from column 2 ($p < 0.05$).

II Effect of Ni(II) ions

Unlike the case of Co(II), treatment of chromatin with Ni(II) (25 μM)/H_2O_2 (2.8 mM and up to 10 mM) produced no increase in the amounts of the modified bases. Therefore, the amount of Ni(II) in the reaction mixtures was increased to 100 μM, and a time course study was undertaken. Again, the treatment of chromatin with Ni(II) (100 μM) and H_2O_2 (2.8 mM) for 1 h produced no significant rises in the amounts of modified bases over background levels. However, treatment for 5 to 24 h increased the amounts of all products. As representative examples, Fig. 10 illustrates the dependence of the yields of two modified bases on the treatment time. DNA base damage caused by Ni(II)/H_2O_2 increased steadily up to 24 h of treatment. For the rest of the experiments with Ni(II) a treatment time of 24 h was used. Results obtained with Ni(II) (100 μM) are shown in Table III. Ni(II) alone caused significant increases in the amounts of some, but not all of the products (Table III). Addition of catalase (100 units/ml) with the Ni(II) caused no change in the product yields shown. Treatment with H_2O_2 alone for 24 h also raised the background amounts of several products. Since H_2O_2 does not react with DNA (Aruoma *et al.*, 1989a; Blakely *et al.*, 1990), this may indicate the presence of some remaining metal ions which were not completely removed by the experimental procedures used. Ni(II)/H_2O_2 markedly increased product yields over the levels observed with Ni(II) alone or H_2O_2 alone. The highest

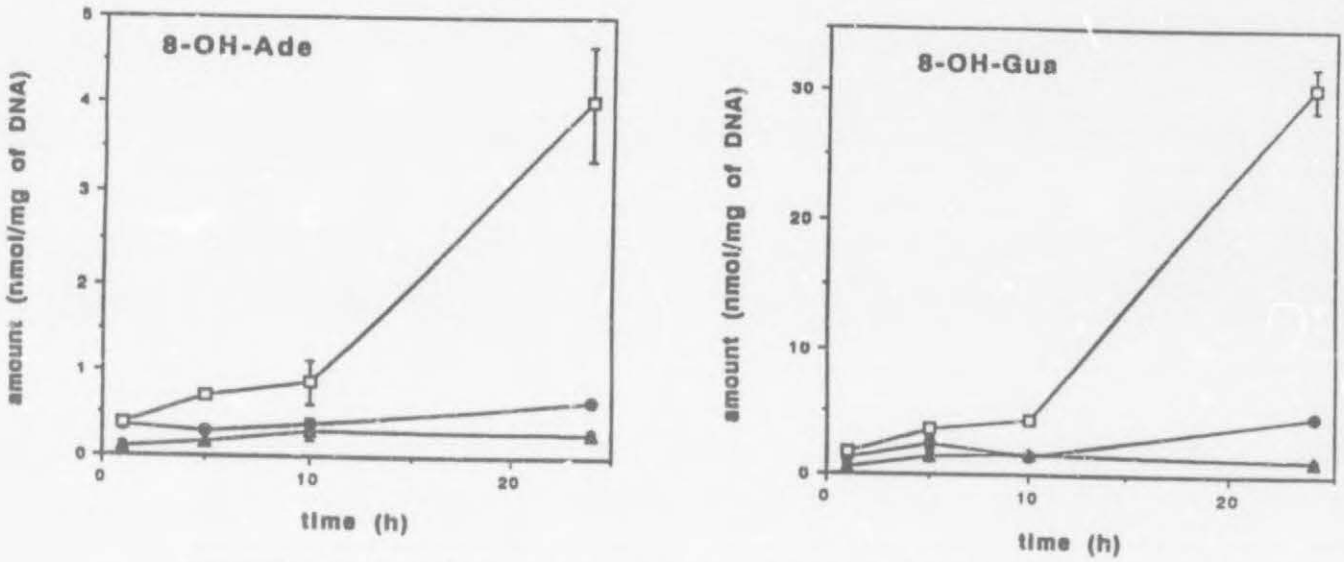

FIGURE 10: Dependence of the yields of 8-OH-Ade and 8-OH-Gua on the treatment time. ▲, chromatin; ●, chromatin/H_2O_2 ; □, chromatin/Ni(II)/H_2O_2. *Points*, mean ; *bars*, SD.

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

Product	Treatment						
	1[c]	2	3	4	5	6	7
5-OH-5-Me-Hyd	0.176±0.052	0.407±0.062*	0.538±0.074*	1.97±1.09ǂ	0.796±0.153#	1.14±0.310#	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#	1.74±0.126
5-OHMe-Ura	0.097±0.022	0.144±0.015*	0.204±0.097*	0.632±0.127ǂ	0.631±0.211	0.378±0.049#	1.53±0.212#
Cyt glycol	0.153±0.006	0.146±0.042	1.04±0.133*	3.37±0.716ǂ	2.99±0.396	2.00±0.629	5.70±0.637#
Thy glycol	0.031±0.004	0.084±0.049	0.578±0.095*	1.02±0.178ǂ	0.603±0.209	0.544±0.152#	1.14±0.036
5,6-diOH-Cyt	0.079±0.016	0.185±0.028*	0.151±0.053	0.597±0.250ǂ	0.178±0.074#	0.669±0.054	0.550±0.007
FapyAde	0.340±0.049	0.308±0.056	0.728±0.009*	2.[illegible]±0.169ǂ	1.45±0.432	1.44±0.217#	3.41±0.403#
8-OH-Ade	0.277±0.037	0.591±0.152*	0.646±0.024*	4.02±0.649ǂ	3.29±0.10	0.508±0.052#	9.28±1.06#
2-OH-Ade	0.140±0.005	0.419±0.047*	0.374±0.009*	0.567±0.057	0.653±0.120	0.193±0.029#	0.971±0.132#
FapyGua	1.88±0.074	2.11±0.030	2.55±0.315	6.39±0.789ǂ	3.14±0.658#	14.7±3.89#	5.05±0.628
8-OH-Gua	1.14±0.108	2.73±0.406*	4.62±0.131*	30.2±1.77ǂ	13.8±1.34#	18.9±2.67#	30.3±3.10

[a]All values represent the mean ± SD (n = 3). [b]Ni(II) (100 μM), H_2O_2 (2.8 mM), 24 h.

[c]**1:** chr; **2:** chr/Ni(II); **3:** chr/H_2O_2; **4:** chr/Ni(II)/H_2O_2; **5:** chr/Ni(II)/H_2O_2/DMSO (50 mM); **6:** chr/Ni(II)/H_2O_2/glutathione (1 mM); **7:** chr/Ni(II)/H_2O_2/SOD (200 u/ml).

*Values significantly different from column 1 ($p < 0.05$). ǂValues significantly different from columns 2 and 3 ($p < 0.05$). #Values significantly different from column 4 ($p < 0.05$).

proportional increase (≈6-fold) was in the presence of 8-hydroxypurines. The major products were Cyt glycol, formamidopyrimidines, and 8-hydroxypurines (as in the case with Co(II)/H_2O_2). As for Co(II), the addition of ascorbic acid (100 μM) to Ni(II)/H_2O_2 had no significant effect on product yields. Dimethylsulphoxide partially decreased the yields of some products up to 60 % (Table III). Nevertheless, product yields were still much higher than background levels. Mannitol had a similar effect (data not shown). Glutathione at 1 mM had an inhibitory effect on some of the products , although the yield of FapyGua was doubled (Table III), in analogy to the results obtained with Co(II). The presence of SOD in the reaction mixture did not inhibit product formation. On the contrary, a ≈2-fold increase in the yields of 5-OHMe-Ura, Cyt glycol, FapyAde, 8-OH-Ade, and 2-OH-Ade was observed (Table III). Chelation of Ni(II) (100 μM) with EDTA (120 μM) caused a marked decrease in the yields of all products. For example, the yields of 8-OH-Gua, 8-OH-Ade, and Thymine glycol given in Table III were decreased from 30.2 ± 1.77, 4.02 ± 0.65, and 1.02 ± 0.18 to 0.80 ± 0.08, 0.53 ± 0.18 and 0.31 ± 0.09 respectively (also compare these values with those elsewhere in Table III).

III Effect on calf thymus DNA

Table IV illustrates the results obtained with calf thymus DNA under the same conditions using the same amount of DNA as in chromatin. Treatment of calf thymus DNA with Co(II) (25 μM)/H_2O_2 for 1 h produced significant increases in the amounts of modified bases (Table IV). Except for the yields of formamidopyrimidines, the product yields in calf thymus DNA were higher than those in chromatin (compare Table II with Table IV). Treatment of calf thymus DNA with Ni(II)(100 μM)/H_2O_2 for 24 h significantly increased the amounts of products (Table IV). The product yields obtained in calf thymus DNA with Ni(II)/H_2O_2 treatment were lower (except for the yields of Thy glycol) than those in chromatin (compare Table III with Table IV).

3.1.2 DNA-protein cross-links produced by ionizing radiation or H_2O_2/metal ions.

In this study, chromatin was treated with H_2O_2 in the presence of Fe(III) or Cu(II) ions. The GC/MS technique was used in conjunction with chemical knowledge of cross-links described in the literature (Margolis *et al.*, 1988; Gajewski *et al.*, 1988; Dizdaroglu *et al.*, 1989; Dizdaroglu and Gajewski, 1989; Gajewski and Dizdaroglu, 1990) to search for DPCs in trimethylsilylated hydrolysates of chromatin.

Exposure of calf thymus nucleohistone to other free radical generators such as ionizing radiation (under anoxic conditions), generates a number of DPCs (see Gajewski *et al.*,

TABLE IV: *Yields[a] (nmol/mg of DNA) of base products formed in calf thymus DNA by treatment with Co(II)/H_2O_2 and Ni(II)/H_2O_2 systems*

Product	Treatment			
	1[b]	2	3	4
5-OH-5-Me-Hyd	0.129±0.017	0.217±0.019	1.66±0.12*	1.42±0.30*
5-OH-Hyd	0.079±0.012	0.104±0.021	0.702±0.076*	1.24±0.47*
5-OHMe-Ura	0.032±0.006	0.061±0.008	0.568±0.062*	0.296±0.015*
Cyt glycol	0.209±0.030	0.898±0.059	3.69±0.25*	1.29±0.097
Thy glycol	0.556±0.179	0.932±0.099	3.34±0.03*	5.01±1.09*
5,6-diOH-Cyt	0.006±0.002	0.012±0.002	0.580±0.091*	0.211±0.033*
FapyAde	0.164±0.025	0.235±0.039	0.667±0.042*	0.613±0.059*
8-OH-Ade	0.088±0.010	0.166±0.013	2.15±0.286*	3.08±0.55*
2-OH-Ade	0.039±0.007	0.039±0.007	0.162±0.01*	0.148±0.021*
FapyGua	0.242±0.053	0.352±0.074	0.789±0.138*	0.717±0.080*
8-OH-Gua	0.389±0.047	0.996±0.183	7.37±0.87*	7.23±0.827*

[a]All values represent the mean ± SD (n = 3).

[b]**1:** DNA (24 h); **2:** DNA/H_2O_2 (24 h); **3:** DNA/Co(II) (25 μM)/H_2O_2 (1 h); **4:** DNA/Ni(II) (100 μM)/H_2O_2 (24 h).

*Values significantly different from column 2 ($p < 0.05$).

1988). In the present study, however, only 3-[(1,3-dihydro-2,4-dioxopyrimidine-5-yl)-methyl]-L-tyrosine (Thy-Tyr cross-link) was detected in the treated chromatin samples. A typical example is given in Fig. 11 showing the identification of the Thy-Tyr cross-link in chromatin after exposure to H_2O_2 and Cu(II) ions. Parts A and B of Fig. 11 illustrate the ion-current profiles of the *m/z* 218, 448, 548 and 650 ions respectively. A number of other characteristic ions from the known mass spectrum of the trimethylsilyl derivative of the Thy-Tyr cross-link (Margolis *et al.*, 1988; Dizdaroglu, 1990) were also monitored in the expected retention time region. For practical reasons, profiles of only four ions are plotted in Fig. 11. Signals of the monitored ions occurred at the expected retention time of the Me_3Si derivative of the Thy-Tyr cross-link. Subsequently, a mass spectrum (Fig. 12A) was obtained on the basis of monitored ions in Fig. 11. This partial spectrum was identical with the mass spectrum of the Me_3Si derivative of the Thy-Tyr cross-link depicted in Fig. 12B. It is therefore concluded that exposure of isolated chromatin to Cu(II)/H_2O_2 generates the Thy-Tyr cross-link. The insert in Fig. 12B illustrates the chemical structure of the derivatized Thy-Tyr cross-link. Quantitative measurements of the Thy-Tyr cross-link in chromatin samples were performed as described previously (Dizdaroglu *et al.*, 1989).

Since only the Thy-Tyr cross-link was found and no other other DPCs , efforts were made to find out whether formation

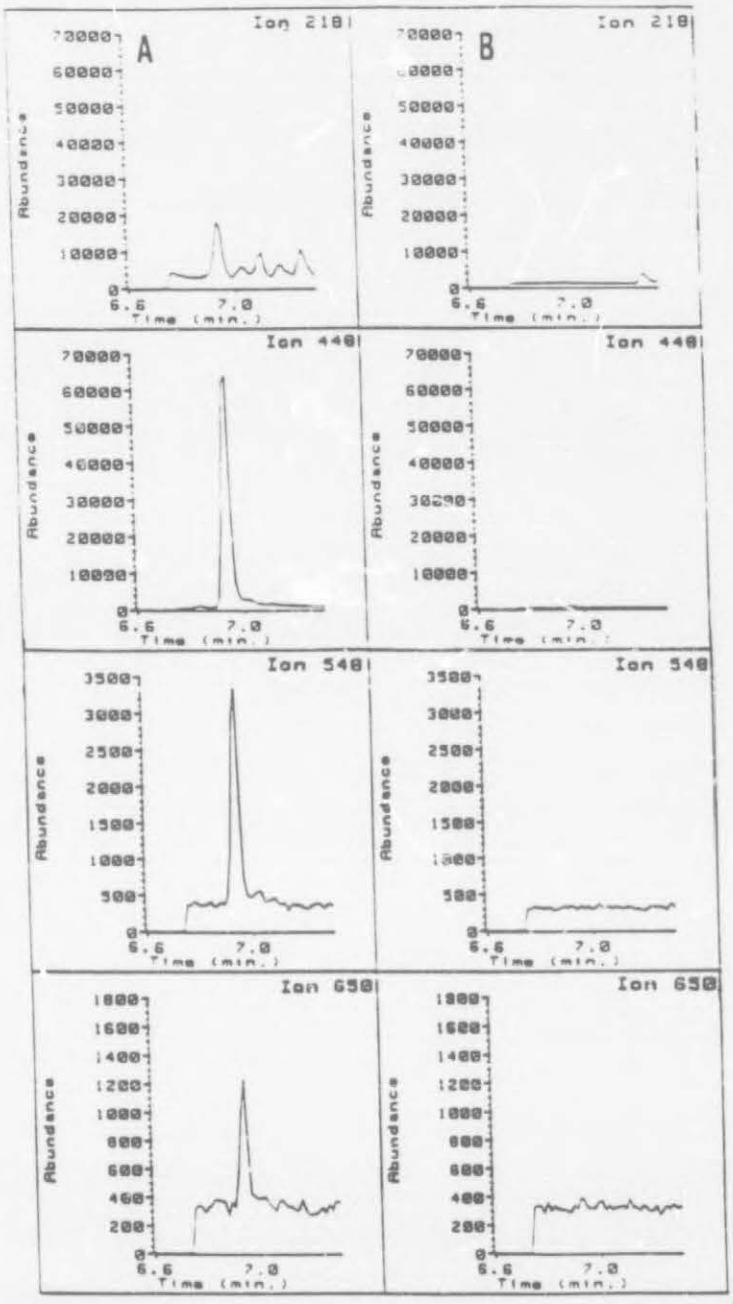

FIGURE 11: Ion-current profiles of the ions at *m/z* 218, 448, 548, and 650 obtained during GC/MS-SIM analysis of trimethylsilylated hydrolysates of chromatin. (A) Chromatin treated with H_2O_2/Cu(II) ; (B) untreated chromatin. The GC column was programmed from 190 to 270 °C at 10 °C/min. after 1 min. at 190 °C. See Chapter 2 for further experimental details.

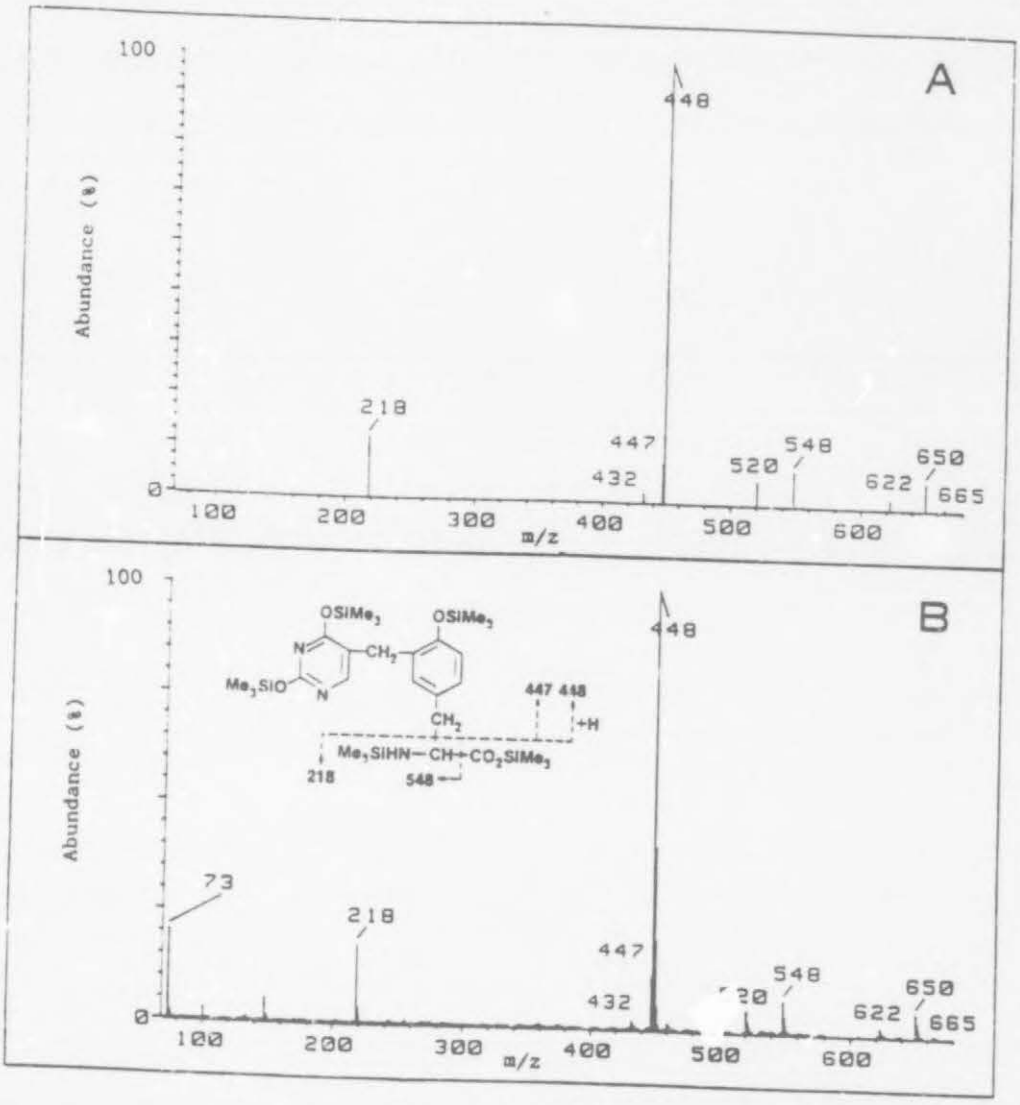

FIGURE 12: (A) Partial mass spectrum of the Thy-Tyr cross-link obtained on the basis of the ions and their abundances in Fig. 11. (B) Mass spectrum of the Me,Si derivative of the Thy-Tyr cross-link. The insert (B) illustrates the structure of this compound and its fragmentation patterns leading to the characteristic ions.

of other DPCs was inhibited by oxygen. For this purpose, parallel experiments were carried out using ionizing radiation instead of using H_2O_2 and metal ions. Chromatin samples were γ-irradiated separately under oxic conditions, i.e., with N_2O/O_2 (4/1) bubbling, and under anoxic conditions, i.e., with N_2O bubbling. Under these conditions, $^{\cdot}OH$ is formed almost exclusively as a radical species with a yield of 0.56 μmol/J. The merits of the N_2O/O_2 system have been reviewed by von Sonntag (1987). Analysis of irradiated samples by GC/MS-SIM showed that DPCs identified previously in calf thymus nucleohistone upon exposure to γ-radiation under anoxic conditions (Gajewski *et al.*, 1988; Dizdaroglu *et al.*, 1989; Dizdaroglu and Gajewski, 1989; Gajewski and Dizdaroglu, 1990), were also formed when chromatin was subjected to γ-radiation under anoxic conditions (with N_2O bubbling). However, only the Thy-Tyr cross-link was detected in chromatin γ-irradiated under oxic conditions (N_2O/O_2 bubbling). The profiles of ions representing the Thy-Tyr cross-link and three other DPCs (Thy-Leu, Thy-Lys and Cyt-Tyr) in Fig. 13 clearly show that only the Thy-Tyr cross-link was formed under oxic conditions.

Fig. 14 illustrates the yields of the Thy-Tyr cross-link in mammalian chromatin under various conditions of H_2O_2 treatment in the presence of unchelated Fe(III) ions. The yields of the Thy-Tyr cross-link in irradiated chromatin were found to be 54 ± 7.5 (N_2O bubbling) and 63 ± 2.5 μmol/mol of nucleotides (N_2O/O_2 bubbling), respectively. Treatment of

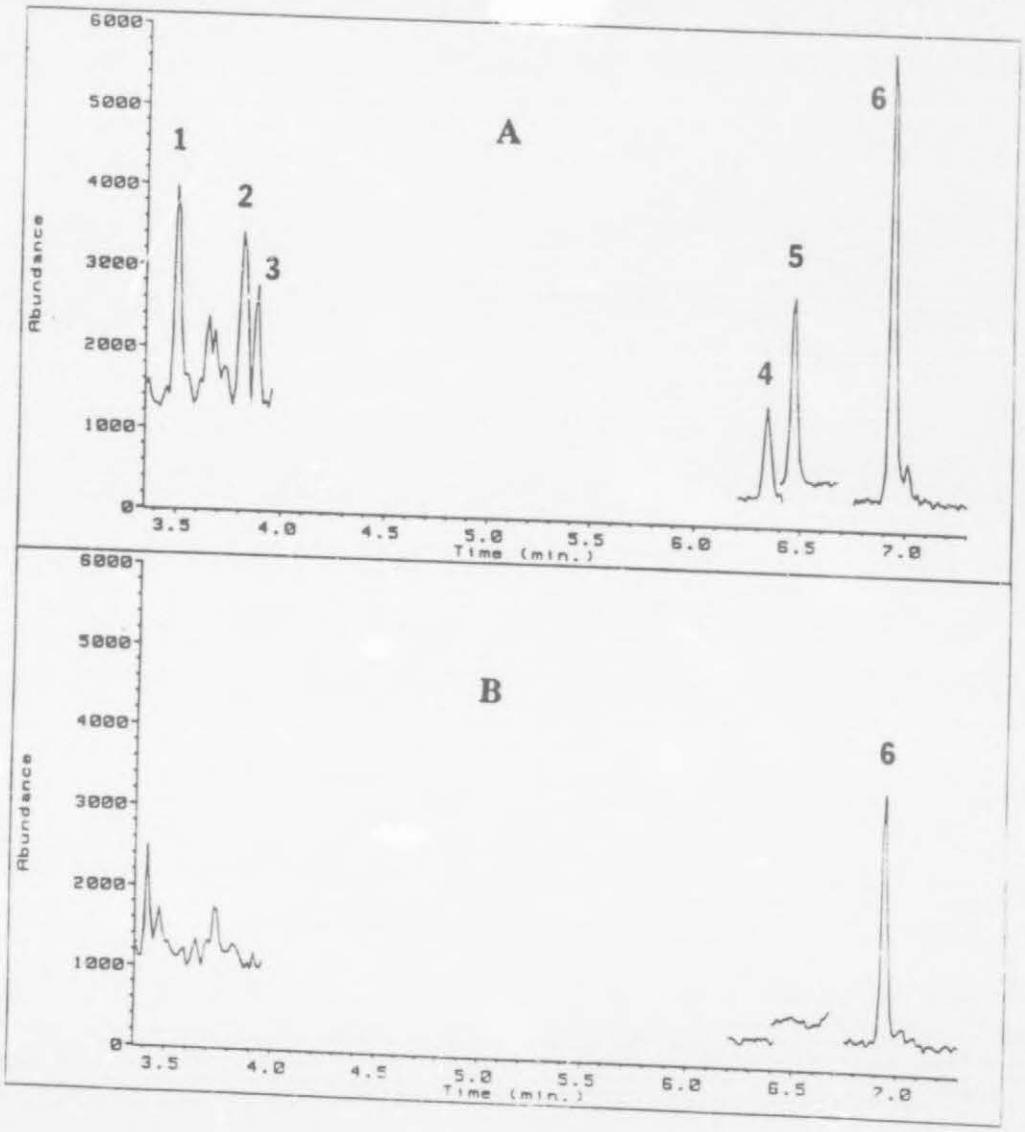

FIGURE 13: Ion-current profiles of ions representing (1, 2, 3) Thy-Leu, (4) Thy-Lys, (5) Cyt-Tyr and (6) Thy-Tyr respectively. Data were obtained during GC/MS-SIM analysis of trimethylsilylated hydrolysates of chromatin. (A) Chromatin γ-irradiated under anoxic conditions (N_2O bubbling); (B) Chromatin γ-irradiated under oxic conditions (N_2O/O_2 bubbling).

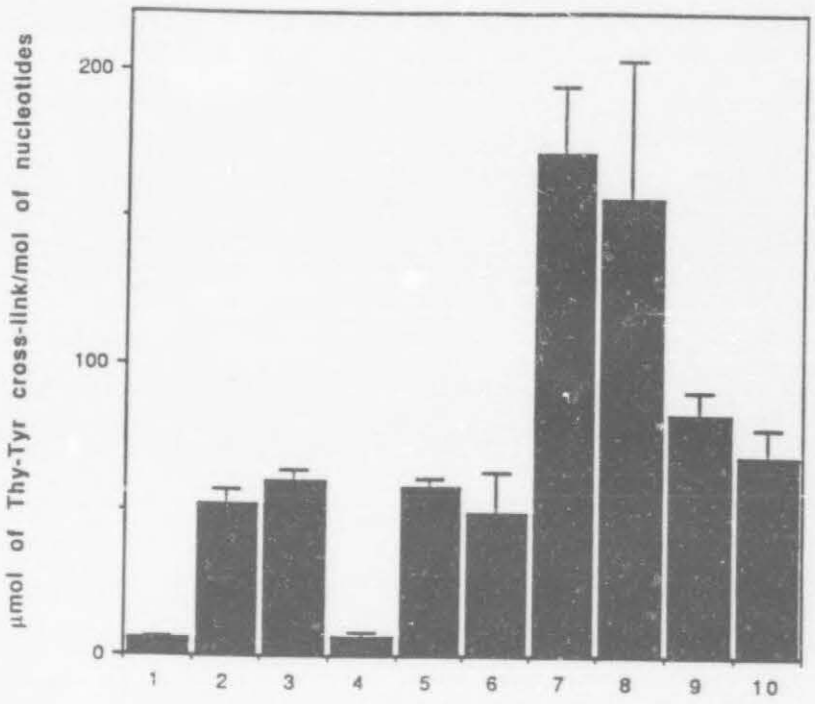

FIGURE 14: Yields of the Thy-Tyr cross-link in chromatin under various conditions (unchelated Fe(III)): 1, chr; 2, chr, γ- irradiated with N_2O bubbling (dose 200 Gy); 3, chr, γ-irradiated with N_2O/O_2 bubbling (200 Gy); 4, chr/Fe(III); 5, chr/H_2O_2; 6, chr/H_2O_2/Fe(III); 7, chr/H_2O_2/Fe(III)/asc; 8, chr/H_2O_2/Fe(III)/asc/SOD; 9, chr/H_2O_2/Fe(III)/asc/mannitol; 10, chr/H_2O_2/Fe(III)/asc/Me_2SO. Graphs represent the mean ± SD from triplicate measurements.

chromatin with Fe(III) alone did not increase the amount of the Thy-Tyr cross-link over the control level, although treatment with H_2O_2 alone caused formation of the Thy-Tyr cross-link. H_2O_2/Fe(III) produced the same level of Thy-Tyr cross-link as H_2O_2 alone. Addition of ascorbic acid to H_2O_2/Fe(III) markedly increased the yield of the cross-link. It was also observed that SOD addition to chromatin in the presence of H_2O_2/Fe(III)/ascorbic acid did not inhibit cross-link formation. However, the hydroxyl radical scavengers, mannitol and Me_2SO, at concentrations of 50 mM provided partial inhibition of cross-linking and produced 50-60 % reduction in Thy-Tyr cross-link yield.

When chelated Fe(III) ions were used instead of unchelated Fe(III) ions, higher yields of the Thy-Tyr cross-link were obtained (Fig. 15). The yield of the Thy-Tyr cross-link obtained by treatment of the chromatin with H_2O_2/Fe(III)-EDTA was approximately 3 times that obtained with H_2O_2/Fe(III). Addition of ascorbic acid to H_2O_2/Fe(III)-EDTA caused no increase in Thy-Tyr cross-link yield (Fig. 15).

H_2O_2/Fe(III)-NTA generated considerably more cross-linking than H_2O_2/Fe(III)-EDTA. The yield obtained with H_2O_2/Fe(III)-NTA was 400 ± 2 μmol of Thy-Tyr cross-link/mol of nucleotides and this was approximately 2 times that obtained with H_2O_2/Fe(III)-EDTA. Addition of ascorbic acid to H_2O_2/Fe(III)-NTA caused only a slight increase in the yield of Thy-Tyr cross-link. Superoxide dismutase provided partial inhibition

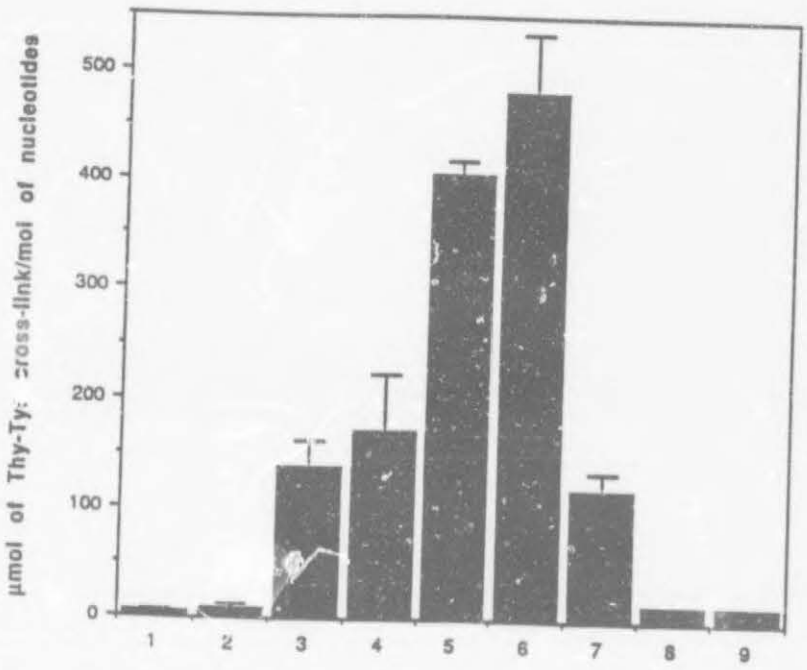

FIGURE 15: Yields of the Thy-Tyr cross-link in chromatin under various conditions (chelated Fe(III)): 1, chr; 2, chr/Fe(III)-NTA; 3, chr/H_2O_2/Fe(III)-EDTA; 4, chr/H_2O_2/Fe(III)-EDTA/asc; 5, chr/H_2O_2/Fe(III)-NTA; 6, chr/H_2O_2/Fe(III)-NTA/asc; 7, chr/H_2O_2/Fe(III)-NTA/asc/SOD; 8, chr/H_2O_2/Fe(III)-NTA/asc/mannitol; 9, chr/H_2O_2/Fe(III)-NTA/Me_2SO. Graphs represent the mean ± SD from triplicate measurements.

of cross-linking by reducing the yield by approximately 75%. Hydroxyl radical scavengers mannitol and Me_2SO at concentrations of 50 mM almost completely inhibited cross-link formation.

Exposure of chromatin to Cu(II) ions alone doubled the formation of the Thy-Tyr cross-link above the background level (Fig. 16). The H_2O_2/Cu(II) system increased the yield of the Thy-Tyr cross-link ≈7-fold (compare 2 and 3 in Fig. 16). Addition of ascorbic acid to H_2O_2/Cu(II) did not cause any further increase in the yield over the level caused by H_2O_2/Cu(II) alone. Superoxide dismutase had no inhibitory effect, but increased the yield of the Thy-Tyr cross-link by about 30% (compare 4 and 5 in Fig. 16). Mannitol and Me_2SO provided partial inhibition of cross-link formation. Chelation of Cu(II) ions with NTA completely inhibited the formation of the Thy-Tyr cross-link. Cu(II)-EDTA had a similar effect (data not shown). However, the addition of ascorbic acid to the H_2O_2/Cu(II)-NTA system caused the formation of the Thy-Tyr cross-link. In contrast to the H_2O_2/Cu(II) system, SOD provided a partial inhibition of cross-link formation. An almost complete inhibition of cross-link formation was obtained by Me_2SO.

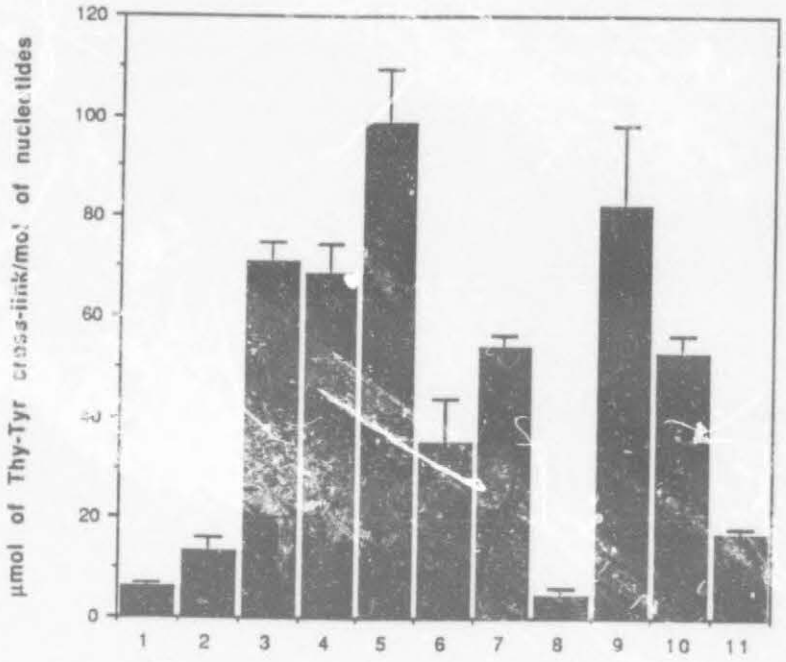

FIGURE 16: Yields of the Thy-Tyr cross-link in chromatin under various conditions 1, chr; 2, chr/Cu(II); 3, chr/Cu(II)/H_2O_2; 4, chr/H_2O_2/Cu(II)/asc; 5, chr/H_2O_2/Cu(II)/asc/SOD; 6, chr/H_2O_2/Cu(II)/asc/mannitol; 7, chr/H_2O_2/Cu(II)/asc/Me_2SO; 8, chr/H_2O_2/Cu(II)-NTA; 9, chr/H_2O_2/Cu(II)-NTA/asc; 10, chr/H_2O_2/Cu(II)-NTA/asc/SOD; 11, chr/H_2O_2/Cu(II)-NTA/asc/Me_2SO. Graphs represent the mean ± SD from triplicate measurements.

3.2 DNA base damage in chromatin of γ-irradiated cultured mammalian cells.

A further objective of this work was to identify and quantitate modified DNA bases generated in chromatin of γ-irradiated cultured mammalian cells. Chromatin was isolated from γ-irradiated cells and hydrolyzed in formic acid. A recent study (Fuciarelli *et al.*, 1989) has evaluated the stability of modified DNA bases in formic acid. In order to further optimize hydrolysis conditions, the stability of modified bases and their release from DNA at different concentrations of formic acid were examined. In the first step , the relative molar response factor (RMRF) of each modified base was determined (Fuciarelli *et al.*, 1989) with and without the use of formic acid at various concentrations. Table V shows the measured values. 5-OHMe-Ura and Thy glycol were destroyed to an extent of approximately 20-25 % by treatment with 60 % (v/v) formic acid as indicated by significantly greater RMRFs (compare column 2 to column 1 of Table V). The RMRFs of other modified bases showed no significant difference with the use of 60 % formic acid and without it. This indicates that these compounds underwent no significant destruction by treatment with 60 % formic acid. At higher formic acid concentrations, 5-OH-5-Me-Hyd, 5,6-diOH-Ura and FapyGua were also significantly destroyed. The use of lower formic acid concentrations (50 %) resulted in RMRFs similar to those obtained with 60 % formic acid (not shown).

TABLE V: *Dependence on Formic Acid Concentration of Relative Molar Response Factors of Modified Bases*

Base (ion used)		Formic Acid Concentration			
		no acid	60%	70%	88%
5,6-diHThy	(m/z 257)	0.530±0.012*	0.514±0.017	0.526±0.018	0.581±0.006
5-OH-5-Me-Hyd	(m/z 331)	0.463±0.050	0.627±0.060	0.857±0.032	1.34±0.01*‡
5-OHMe-Ura	(m/z 358)	0.431±0.016	0.604±0.034*	1.05±0.03*	1.95±0.07*‡
5-OH-Ura	(m/z 329)	0.361±0.020	0.293±0.013	0.317±0.007	0.357±0.028
Thy glycol	(m/z 259)	0.204±0.013	0.312±0.024*	0.345±0.006*	0.413±0.005*‡
5,6-diOH-Ura	(m/z 417)	3.12±0.35	3.84±0.26	4.98±0.96	5.28±0.48
FapyAde	(m/z 354)	1.10±0.09	0.960±0.090	1.08±0.04	1.10±0.12
8-OH-Ade	(m/z 352)	0.296±0.024	0.276±0.013	0.315±0.028	0.347±0.015
2-OH-Ade	(m/z 352)	0.188±0.013	0.164±0.012	0.197±0.008	0.205±0.015
FapyGua	(m/z 442)	0.637±0.059	1.03±0.17	1.60±0.12*	1.41±0.15*
8-OH-Gua	(m/z 440)	0.415±0.030	0.398±0.046	0.395±0.049	0.452±0.008

*Each value represents the mean ± standard error from three independent experiments.

*Significantly different from the value in column 1 ($p \leq 0.05$).

‡Significantly different from the value in column 2 ($p \leq 0.05$).

The marked stability of 6-azathymine and 8-azaadenine at the 98 % formic acid concentration is in agreement with the observations of Fuciarelli *et al.*, (1989).

Having determined the extent and stability of modified bases, the release of modified bases from control and irradiated DNA was examined (Calf thymus DNA in N_2O-saturated aqueous solution was exposed to 240 Gy of γ-radiation). Results are shown in Table VI. Fifteen modified bases were identified. 5-OH-Ura and 5-OH-Cyt result from acid-induced modification of Cyt glycol, the former by deamination and dehydration, and the latter by dehydration; 5,6-diOH-Ura is formed by deamination of 5,6-diOH-Cyt (Dizdaroglu, 1984; Dizdaroglu *et al.*, 1986). Similarly , 5-OH-6-HUra is thought to result from acid-induced deamination of 5-OH-6-HCyt. The RMRFs given in Table V were used for calculation of the amounts shown in Table VI. There were no authentic compounds available for 5-OH-Hyd, 5-OH-6-HThy, 5-OH-6-HUra and 5-OH-Cyt. Their gas chromatographic retention times and their mass spectra were obtained using trimethylsilylated samples of γ-irradiated thymine (for 5-OH-6-HThy) and cytosine (for 5-OH-Hyd, 5-OH-6-HUra and 5-OH-Cyt after treatment with formic acid). The RMRF of the trimethylsilylated derivative (Me_3Si) of 5-OH-Hyd was assumed to be the same as that of the Me_3Si derivative of 5-OH-Cyt, which has a mass spectrum similar to that of the Me_3Si derivative of 5-OH-Ura (Dizdaroglu, 1985; Dizdaroglu and Bergtold, 1986). The RMRFs of Me_3Si

Table VI: *Dependence on Formic Acid Concentration of the Release of Modified Bases (molecules/10^7 DNA bases) from DNA*

Base	Formic Acid Concentration							
	50%		60%		70%		88%	
	control (1)	irrad[a]. (2)	control (3)	irrad[a]. (4)	control (5)	irrad[a]. (6)	control (7)	irrad[a]. (8)
5,6-diHThy	n.d.[b]	36.8±1.7[c]	n.d.	31.8±3.5	n.d.	34.2±1.0	n.d.	35.8±3.4
5-OH-5-Me-Hyd	2.14±0.16	19.9±0.9	2.24±0.13	19.5±0.9	2.82±0.22	23.4±1.1	3.84±0.54	20.9±0.5
5-OH-Hyd	1.60±0.19	9.06±0.74	1.57±0.19	8.93±0.32	1.38±0.35	10.7±1.1	1.76±0.16	10.3±0.80
5-OH-6-HThy	n.d.	88.0±1.7	n.d.	87.0±1.8	n.d.	85.1±2.3	n.d.	84.5±3.1
5-OH-6-HUra	n.d.	19.4±0.5	n.d.	19.8±0.7	n.d.	19.0±0.9	n.d.	18.4±0.7
5-OHMe-Ura	0.93±0.06	18.6±1.0	2.43±0.45	17.5±1.5	2.69±0.26	23.7±3.3	2.69±0.51	24.5±0.5*|
5-OH-Ura	n.d.	29.1±0.7	n.d.	30.1±1.8	n.d.	30.1±3.6	n.d.	30.9±2.7
5-OH-Cyt	6.11±1.15	40.3±0.9	7.20±0.86	45.0±1.6	8.26±0.16	46.4±5.3	8.90±1.54	48.5±4.8
Thy glycol	0.19±0.02	22.4±1.5	0.74±0.10	27.5±2.1	0.68±0.06	33.0±4.5	1.85±0.16	45.1±1.0*|
5,6-diOH-Ura	0.14±0.02	10.6±1.3	0.14±0.02	9.98±0.42	0.18±0.03	12.0±0.7	0.18±0.02	12.6±0.4
FapyAde	0.67±0.10	55.7±6.1	0.90±0.03	60.2±7.3	0.67±0.10	66.2±7.5	1.06±0.19	81.6±6.1
8-OH-Ade	11.7±1.1	86.7±4.6	14.2±2.9	93.4±3.9	18.0±0.6	90.6±5.7	19.4±2.1	61.3±2.2
2-OH-Ade	0.65±0.07	4.00±0.19	0.70±0.06	4.83±0.16	0.64±0.06	5.98±0.13*|	0.74±0.10	8.38±1.31*|
FapyGua	0.67±0.06	38.4±1.6	1.12±0.10	42.6±2.6	0.86±0.13	49.6±7.7	0.61±0.08	57.6±1.5*
8-OH-Gua	48.9±7.7	152.6±12.6	50.9±9.9	214.4±18.8	52.5±3.2	241.9±22.8*	55.0±11.8	251.2±4.2*

[b]Not detected. [c]Each value represents the mean ± standard error from three independent experiments. [a] One dose of 240 Gy.

*Significantly different from the value in column 2 ($p \leq 0.05$).

|Significantly different from the value in column 4 ($p \leq 0.05$).

derivatives of 5-OH-6-HThy and 5-OH-6-HUra were estimated from their mass spectra (Dizdaroglu, 1984, 1985; Fuciarelli *et al.*, 1989) and therefore may have an error of 10% associated with them.

Results in Table VI indicate that, under all four hydrolysis conditions, similar yields of modified bases were obtained in irradiated DNA with a few exceptions. This means that a compensation for losses of labile modified bases such as 5-OHMe-Ura, Thy glycol and FapyGua even at formic acid concentrations higher than 60 % can be achieved by the use of corresponding RMRFs in Table V. Background amounts for each modified base were similar and were obtained in unirradiated DNA for various formic acid concentrations (Table VI).

Table VI indicates that some modified bases were already present in unirradiated DNA. Occurrence of these modified bases in untreated DNA and chromatin has been shown previously using the GC/MS technique (for a review see Dizdaroglu 1991). There is a possibility that these modified bases may be formed in DNA by acid treatment. This possibility was investigated by analyzing individual DNA bases under the conditions used for hydrolysis of DNA. Equimolar amounts of commercial thymine, cytosine, adenine and guanine were mixed, aliquots of the mixture were lyophilized and then subjected to formic acid at various concentrations. Additional aliquots treated with water instead of formic acid (under similar hydrolysis conditions) served as controls.

The samples were then lyophilized, trimethylsilylated and analyzed by GC/MS-SIM for modified bases given in Table VI. The results in Table VII show that the amounts of modified DNA bases in commercial thymine, cytosine, adenine and guanine were not increased by the acidic treatment under the conditions used for DNA hydrolysis.

From the results presented in Tables V, VI and VII, it was concluded that hydrolysis with 60 % formic acid was optimal for DNA hydrolysis.

Having established the optimal conditions for hydrolysis, DNA base damage was investigated in the chromatin of irradiated cells. DNA cannot be extracted efficiently from irradiated cells because of the formation of DPCs and DNA fragmentation (Mee and Adelstein, 1979). It is also possible that the unextracted DNA may contain significant portions of modified bases. For this reason the chromatin was isolated from irradiated cells and directly subjected to acid hydrolysis and derivatization. The derivatized hydrolysates were analyzed by GC/MS-SIM.

Table VIII shows that twelve modified bases were identified at 42, 116, 214 and 420 Gy doses of γ-radiation. Of the modified bases identified in calf thymus DNA irradiated in aqueous solution, 5,6-diHThy and 5-OH-6HUra were not detected in chromatin hydrolysates.

An example of the GC/MS-SIM identification of modified bases in chromatin is given in Fig. 17 using selected-ion

TABLE VII: *Dependence on Various Treatments of the Amounts[a] (molecules/10^7 DNA bases) of Modified Bases in Individual DNA Bases*

Base	Treatment					
	Water		Formic Acid			
	no incub. (1)	with incub. (2)	50% (3)	60% (4)	70% (5)	88% (6)
5,6-diHThy	n.d.[b]	n.d.	n.d.	n.d.	n.d.	n.d.
5-OH-5-Me-Hyd	3.93±0.33	3.87±0.63	3.83±0.53	3.75±0.79	3.08±0.39	3.35±0.47
5-OH-Hyd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-OH-6-HThy	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-OH-6-HUra	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-OHMe-Ura	5.80±0.58	6.46±1.17	5.43±0.85	4.92±1.15	4.26±0.94	4.09±0.58
5-OH-Ura	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5-OH-Cyt	10.27±0.41	11.89±3.17	13.54±2.35	10.73±2.72	14.98±2.44	14.89±2.59
Thy glycol	1.61±0.46	1.40±0.36	1.80±0.23	1.28±0.28	1.67±0.28	1.84±0.24
5,6-diOH-Ura	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
FapyAde	24.63±4.82	34.97±3.43	34.20±7.20	33.75±7.16	36.41±2.31	27.21±3.34
8-OH-Ade	43.98±6.38	44.65±8.69	40. 7.30	46.03±7.50	43.67±9.52	47.20±7.30
2-OH-Ade	2.05±0.24	2.30±0.50	3.06±0.82	3.19±0.79	3.30±0.71	2.60±0.27
FapyGua	6.27±1.10	7.44±1.69	7.28±1.11	8.22±0.68	7.12±1.52	6.93±0.96
8-OH-Gua	76.40±11.28	80.64±14.11	82.55±5.15	74.85±11.27	68.67±5.81	78.00±25.90

[a]Each value represents the mean ± standard error from five independent experiments.

[b]Not detected.

TABLE VIII: *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±2.13	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 ($p \leq 0.05$).

current profiles of three characteristic ions. Signals of the three monitored ions are seen at the expected retention time (indicated by arrow) of the Me$_3$SO derivative of FapyGua in both Fig. 17A and Fig. 17B. On the basis of the signals of the monitored ions and their relative abundances a partial mass spectrum was obtained. This mass spectrum was then compared with that of the authentic material [for a detailed description see Dizdaroglu and Gajewski (1990)].

The measured amounts of modified bases in control chromatin and in chromatin from irradiated cells are given in Table VIII. Dose-yield relationships of some modified bases are illustrated in Figures 18-20. In the case of 5-OHMe-Ura, 5-OH-Ura and FapyAde, base yields increased up to 214 Gy and then levelled off. At 420 Gy, no further significant increase in the yields of these modified bases was observed. This is confirmed by the 822 Gy dose which produced virtually identical yields. The amounts of 5-OH-Cyt and 8-OH-Ade were not significantly increased over the control levels by increasing doses up to 214 Gy (Table VIII). Significant increases in the amounts of modified bases were only observed at doses ≥420 Gy. The amount of 5-OH-5-Me-Hyd observed in control chromatin was not increased significantly after irradiation of cells. The yields of products were increased over the background levels in different ratios. For example at 214 Gy, the highest ratios of increase were ≈8-9 fold for 5,6-diOH-Cyt and FapyGua and ≈4-fold for 5-OH-Ura and Thy glycol.

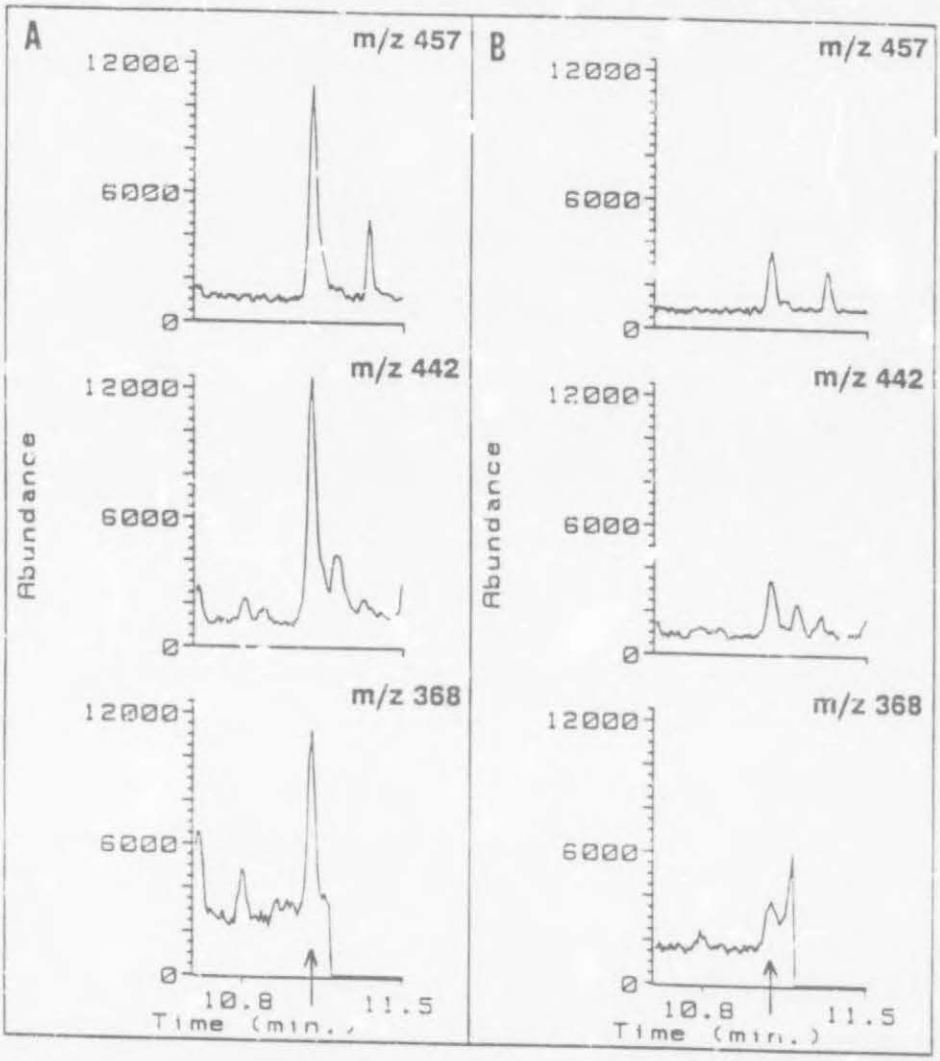

FIGURE 17: Selected ion-current profiles of the ions at *m/z* 368, 442, and 457 obtained during GC/MS-SIM analysis of trimethylsilylated hydrolysates of chromatin. (A) Chromatin isolated from γ-irradiated cells (dose, 116 Gy); (B) chromatin isolated from unirradiated cells. The temperature of the GC column was programmed from 150 to 260 °C at 8 °C/min. after 2 min. at 150 °C. For other details see Experimental Procedures.

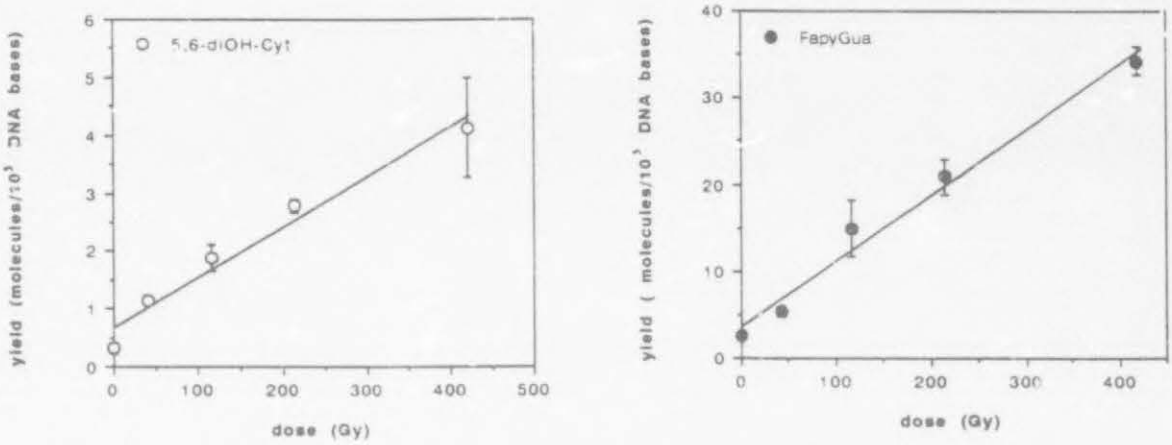

FIGURE 18: Dose-yields plots of 5,6-diOH-Cyt and FapyGua in chromatin of γ-irradiated cells. Error bars represent standard errors of the means from five independent experiments. Lines were drawn by linear regression analysis.

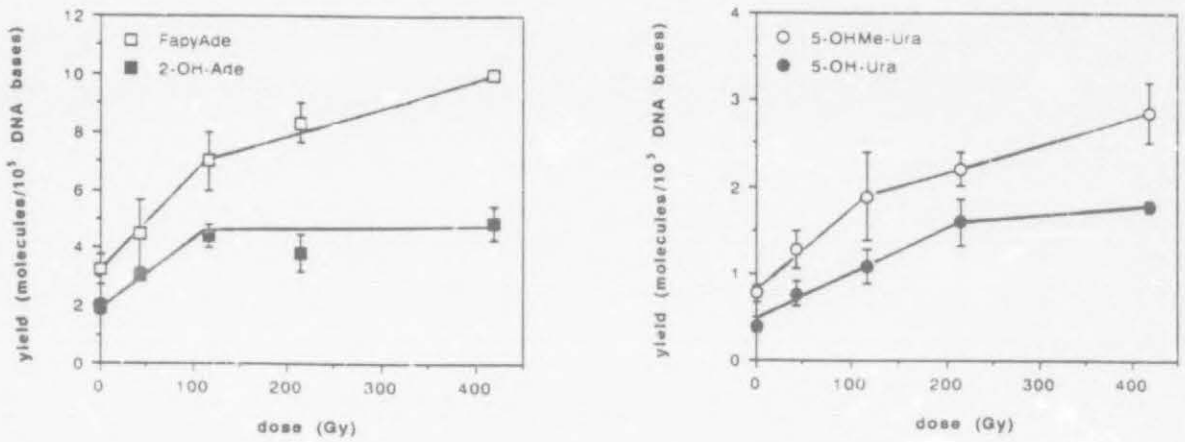

FIGURE 19: Dose-yield plots of Fapy Ade, 2-OH-Ade, 5-OHMe-Ura and 5-OH-Ura in chromatin of γ-irradiated cells. Error bars represent standard errors of the means from 5 independent experiments.

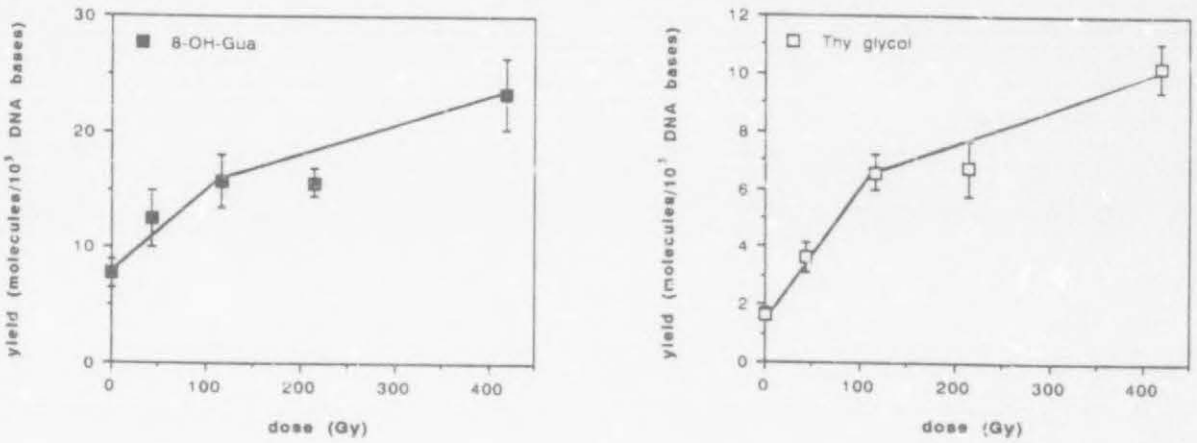

FIGURE 20: Dose-yield plots of 8-OH-Gua and Thy Glycol in chromatin of γ-irradiated cells. Error bars represent standard errors of the means from 5 independent experiments.

3.3 DNA base damage in chromatin of H_2O_2-treated cells

DNA base products produced by treatment of cells with H_2O_2 were identified by GC/MS-SIM. Ten DNA base products identified and quantitated were 5-hydroxy-5-methylhydantoin, 5-hydroxyhydantoin, 5-hydroxymethyluracil, 5-hydroxycytosine, *cis*-thymine glycol, 5,6-dihydroxyuracil, 4,6-diamino-5-formamidopyrimidine, 8-hydroxyadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-hydroxyguanine (Fig. 21). The yields and their dependence on H_2O_2 concentration are given in Fig. 22. Modified DNA bases were also present in untreated cells.

The yield of cytosine glycol was taken as the sum of the amounts of 5-hydroxyuracil and 5-hydroxyc[illegible] (Fuciarelli *et al.*, 1989). In the case of 5-hydroxy-5-methylhydantoin and 5-hydroxyhydantoin, 2 mM H_2O_2 produced no increase over the background. The lowest amount of a modified DNA base i.e. 5,6-dihydroxycytosine, detectable in untreated control cells was approximately 3 µmol/mol of DNA nucleotides or 1 in 10^6 DNA nucleotides (Fig. 22). 8-Hydroxyadenine and 8-hydroxyguanine had the highest background amounts. At 20 mM H_2O_2 the yields of 5,6-dihydroxycytosine, 4,6-diamino-5-formamidopyrimidine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine were approximately 10-fold above background, whereas the other products showed yields of between 2.5 - 5-fold above background.

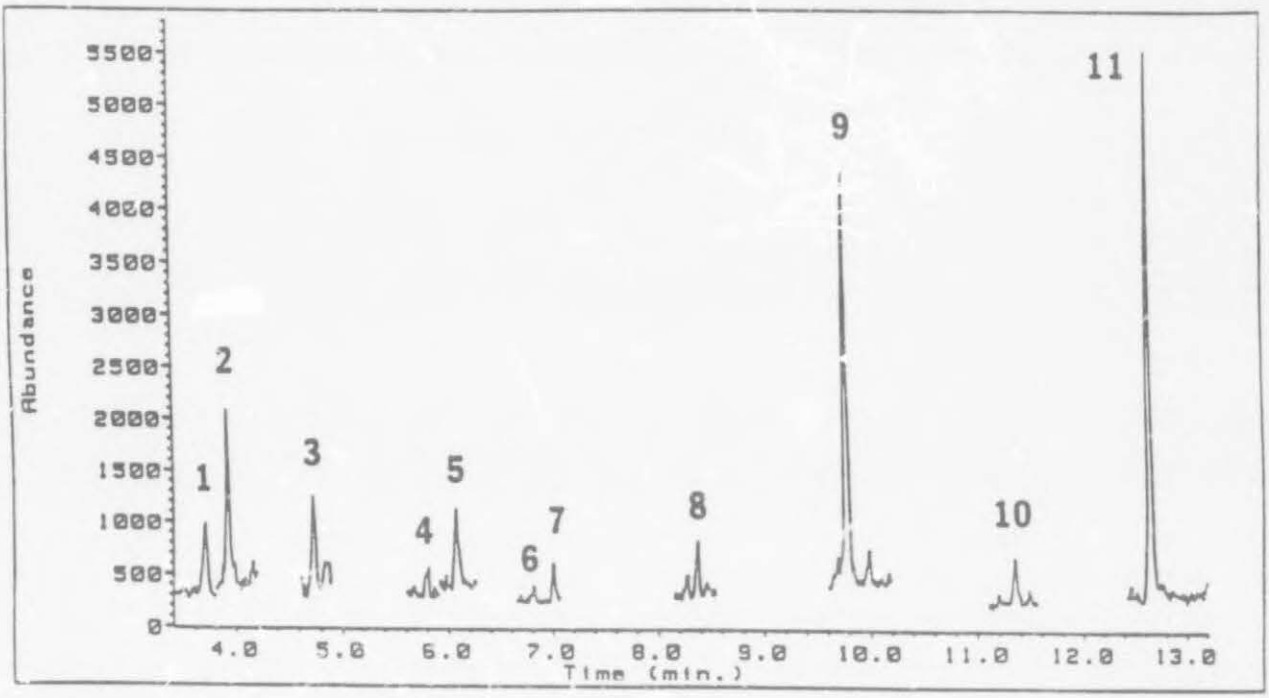

FIGURE 21: Ion-current profiles obtained during the GC/MS-SIM analysis of a trimethylsilylated hydrolysate isolated from mammalian cells treated with 20 mM H_2O_2. Analysis conditions were as in (Experimental details in Chapter 2). Peaks: 1, 5-hydroxy-5-methylhydantoin (*m/z* 331); 2, 5-hydroxyhydantoin (*m/z* 317); 3, 5-hydroxyuracil (*m/z* 329); 4, 5-hydroxymethyluracil (*m/z* 358); 5, 5-hydroxycytosine (*m/z* 343); 6, cis-thymine glycol (*m/z* 259); 7, 5,6-dihydroxyuracil (*m/z* 417); 8, 4,6-diamino-5-formamidopyrimidine (*m/z* 354); 9, 8-hydroxyadenine (*m/z* 352); 10, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (*m/z* 442); 11, 8-hydroxyguanine (*m/z* 440).

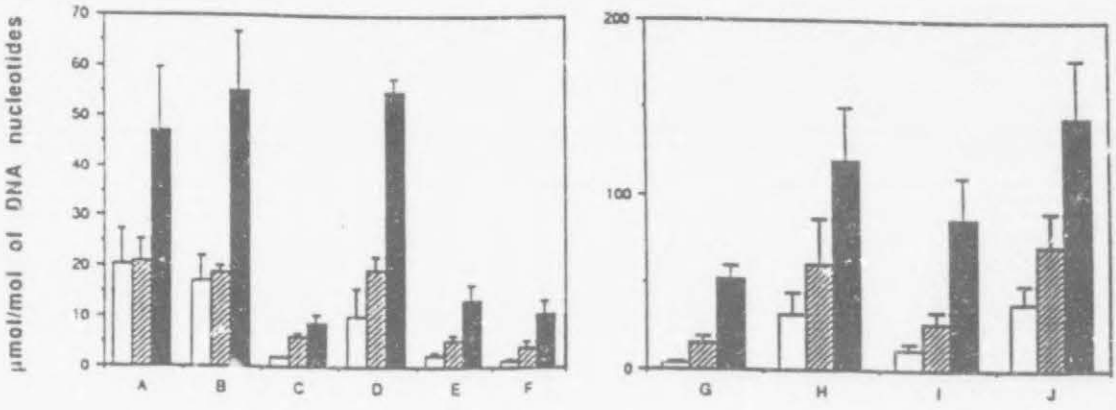

FIGURE 22: Yields of DNA base products in chromatin isolated from cells, as measured by GC/MS-SIM. : □, untreated; ▨, treated with 2 mM H_2O_2; ■, treated with 20 mM H_2O_2. A, 5-hydroxy-5- methylhydantoin; B, 5-hydroxyhydantoin; C, 5-hydroxymethyluracil; D, cytosine glycol; E, thymine glycol; F, 5,6-dihydroxycytosine; G, 4,6-diamino-5-formamidopyrimidine; H, 8-hydroxyadenine; I, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; J, 8-hydroxyguanine. Graphs represent the means ± SD from 4 independent experiments.

4. DISCUSSION

4.1 Studies of DNA damage in isolated chromatin.

4.1.1 Base damage produced by H_2O_2/metal ions.

The DNA base products generated by H_2O_2 in the presence of Ni(II) or Co(II) are typical products of $^{\cdot}OH$ reactions with DNA (for reviews see von Sonntag, 1987; Dizdaroglu, 1991; Téoule and Cadet, 1978). This pattern of products suggests that reaction of Co(II) or Ni(II) with H_2O_2 produces $^{\cdot}OH$, which attacks the DNA bases. Partial inhibition of modified DNA bases by typical $^{\cdot}OH$ scavengers is consistent with this view. The failure of scavengers to prevent DNA damage altogether might be due to "site-specific" formation of $^{\cdot}OH$ (Halliwell and Gutteridge, 1990; Goldstein and Czapski, 1986; Stoewe and Prütz, 1987). Metal ions bound to DNA or to other chromatin constituents could cause formation of $^{\cdot}OH$ in close proximity to DNA. Thus, $^{\cdot}OH$ produced *in situ* may react with DNA to a greater extent than with scavengers or be relatively inaccessible to scavengers.

In contrast to Co(II), Ni(II) on its own 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) to bind with certain peptide sequences of the nuclear proteins to generate free radicals in the presence of oxygen, as described previously (Kasprzak and Bare, 1989; Bossu *et al.*, 1978; Nieboer *et al.*, 1986). The fact that the

yields of products generated by Ni(II)/H_2O_2 were higher in chromatin than in calf thymus DNA may indicate the enhancement of free radical production by Ni(II)-peptide (or protein) complexes and is of special interest in relation to the established carcinogenicity of this metal. On the other hand, Co(II)/H_2O_2 caused more damage in both chromatin and calf thymus DNA than did 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 backgr und amounts of base products in chromatin.

Addition of ascorbic acid had little effect on product yields. By contrast, ascorbic acid greatly stimulates DNA base modifications produced by Cu(II)/H_2O_2 or Fe(III)/H_2O_2 (Dizdaroglu *et al.*, 1991b). Thus, contamination of chromatin or of the added Co(II) or Ni(II) with Cu(II) or Fe(III) can be ruled out. The SH-containing compound, glutathione, was used exclusively in this study. Glutathione at physiological concentrations caused no marked inhibition of DNA base damage. This may be due to the inability of glutathione (possesses a net negative charge) to approach DNA. Alternatively, the net increase in the yield of FapyGua observed for both Co(II) and Ni(II) could result from increased reduction by glutathione of the C-8-OH-adduct radical that is formed from addition of $^{\cdot}OH$ to the C-8 position of guanine. The site specificity of $^{\cdot}OH$ generation may account for the inability of glutathione to markedly inhibit DNA damage. Glutathione thus may not be able

to prevent DNA damage mediated by metal ions and this is consistent with previous findings (Kasprzak *et al.*, 1990a and 1990b).

The inability of SOD to inhibit product formation suggests that O_2^- was not required in generation of $^{\cdot}OH$ by Co(II)/H_2O_2 or Ni(II)/H_2O_2. It must be noted that the metal ion content of SOD was not measured. The increase in product yields seen after addition of SOD to Co(II)/H_2O_2 or to Ni(II)/H_2O_2 may indicate generation of additional $^{\cdot}OH$ in these systems by an unknown mechanism. Stimulation of DNA damage in isolated chromatin by the presence of SOD in mixtures containing Cu(II)/H_2O_2 or Fe(III)/H_2O_2 has also been observed previously (Dizdaroglu *et al.*, 1991b; Nackerdien *et al.*, 1991a).

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 (Dizdaroglu *et al.*, 1991; Nackerdien *et al.*, 1991). The inability of Co(II)/EDTA to produce DNA base modification is in agreement with the results reported by Kadiiska *et al.* (1989).

4.1.2 DNA-protein cross-links produced by ionizing radiation or H_2O_2/metal ions.

The results presented here show that cross-linking between Thy and Tyr occurs in chromatin exposed to H_2O_2 and various forms of Fe(III) and Cu(II) ions. The Thy-Tyr cross-

link is formed despite the presence of oxygen in the reaction mixtures. Generally, oxygen reacts with carbon-centered radicals at diffusion-controlled rates (for a review see 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 and Adelstein, 1981). In mammalian cells, formation of DPCs is also reduced by the presence of oxygen (Oleinick *et al.*, 1987). Results based on ionizing radiation instead of H_2O_2 and metal ions show that DPCs involving Thy and aliphatic amino acids, and Cyt and Tyr, were not formed in chromatin under oxic conditions. This is consistent with the inhibition of DPCs by oxygen. These DPCs were also inhibited in chromatin exposed to H_2O_2 and metal ions. 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 formation of the Thy-Tyr cross-link.

Possible mechanisms of the formation of Thy-Tyr cross-links are given in Fig. 23. DNA-protein cross-linking probably occurs through covalent bonds between the α-carbon and the C-3 of the tyrosine ring. 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 suggested previously (Dizdaroglu *et al.*, 1989). The allyl radical of thymine may be formed by deprotonation of the

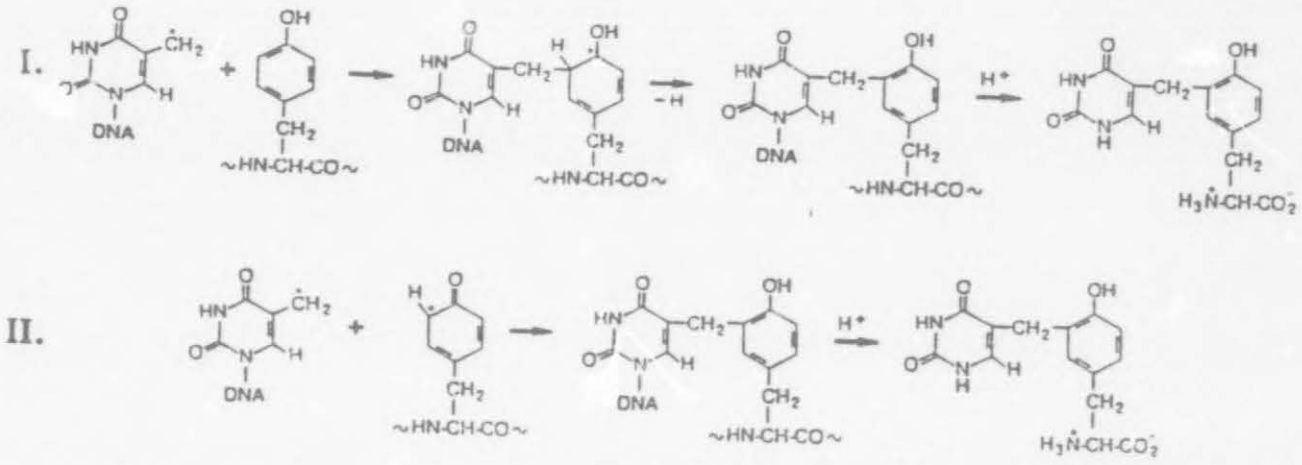

FIGURE 23: Possible mechanisms of Thy-Tyr cross-link formation.

thymine radical cation formed by thymine ionization (Shaw *et al.*, 1988; Deeble *et al.*, 1990). Alternatively, the indirect effect of radiation may play a role through H atom abstraction by $^{\cdot}OH$ from the methyl group of thymine (Fujita and Steenken, 1981). One mechanism for Thy-Tyr cross-link formation involves the addition of the allyl radical of thymine to the C-3 position of the tyrosine ring followed by oxidation of the adduct radical (Dizdaroglu *et al.*, 1989). Another mechanism involves the combination of the allyl radical of thymine with tyrosine radicals, which result from reactions of $^{\cdot}OH$ with tyrosine (Dorfman *et al.*, 1962; Land and Ebert, 1976).

Mechanism II in Fig. 23 requires the formation of two radicals in close proximity. This is feasible when one considers the track model of the deposition of radiation energy in which several radicals are formed in track entities such as spurs, blobs and short tracks (see Chatterjee, 1987). When these entities overlap with chromatin, two $^{\cdot}OH$ radicals may form one thymine an one tyrosine radical in close proximity of each other. This is similar to the mechanism proposed for DNA double strand breaks or other locally multiply damaged sites of DNA (Ward *et al.*, 1985). Both mechanisms depicted in Fig. 23 may contribute to the Thy-Tyr cross-link formation. Oxygen may not interfere with formation of the Thy-Tyr cross-link because of the site-specific nature of this DNA -protein cross-linking.

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. It was found that 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. The extensive formation of DPCs by H_2O_2/Fe(III)-NTA might contribute to the carcinogenicity and nephrotoxicity associated with NTA (Goyer *et al.*, 1981; Hiasa *et al.*, 1984; Ebina *et al.*, 1986). Unchelated Cu(II) ions were also very effective in producing DPCs. The patterns of the yield of Thy-Tyr cross-link produced by H_2O_2 and Fe(III) or Cu(II) ions are analogous to those of the yields of modified DNA bases in mammalian chromatin which was treated with H_2O_2 and Fe(III) or Cu(II) ions under similar conditions (Dizdaroglu *et al.*, 1991b). Since H_2O_2 alone does not cause any DNA damage (Aruoma *et al.*, 1989a,b; Dizdaroglu *et al.*, 1991a), DPCs generated by H_2O_2 may arise because of metal ions naturally present in chromatin. Removal of naturally bound metal ions from chromatin was not undertaken in this study, because metal ions, e.g., copper ions, play an important role in chromatin structure (Lebkowski and Laemmli, 1982; Lewis and Laemmli, 1982; George *et al.*, 1987).

The presence of ascorbic acid in reaction mixtures generally caused an increase in the yield of the Thy-Tyr cross-link (Figs. 14 - 16). Ascorbic acid is an antioxidant, but it also acts as a prooxidant in the presence of metal ions, most likely by reducing them (Samuni *et al.*, 1983;

Davison *et al.*, 1986; Buettner, 1986; Stoewe and Prütz, 1987; Halliwell, 1990), in the present case by reducing Fe(III) and Cu(II) ions to Fe(II) and Cu(I) ions, respectively.

Partial inhibition of DPCs by mannitol and Me_2SO in the case of H_2O_2 and unchelated Cu(II) or Fe(III) ions might be due to "site-specific" generation of $^{\cdot}OH$ (Sagripanti and Kraemer, 1989; Halliwell and Gutteridge, 1988; Samuni *et al.*, 1983; Stoewe and Prütz, 1987; Goldstein and Czapski, 1986). This means that $^{\cdot}OH$ produced *in situ* will immediately attack nearby chromatin constituents before it can be scavenged. The inability of mannitol and Me_2SO to completely inhibit the formation of Thy-Tyr cross-link is attributed to generation of $^{\cdot}OH$ near Thy and Tyr cross-links in chromatin. On the other hand, the involvement (or release) of $^{\cdot}OH$ has been questioned in reactions of bound metal ions with H_2O_2 (Sutton and Winterbourn, 1989). It has been hypothesized that, perhaps, metal ions in higher oxidation states are involved in such reactions to cause damage to biomolecules. However, the Thy-Tyr cross-link identified in the present work is a typical $^{\cdot}OH$-induced product as shown previously (Margolis *et al.*, 1988; Dizdaroglu *et al.*, 1989) and by results reported here. This fact strongly implicates the involvement of $^{\cdot}OH$ in production of this DPC by bound metal ions and H_2O_2.

Significant inhibition of H_2O_2/metal ion-induced cross-linking by SOD implicates the involvement of $O_2^{\cdot-}$ in generation of $^{\cdot}OH$. By contrast, $O_2^{\cdot-}$ might not be involved in production

of $^{\cdot}OH$ in reactions with H_2O_2 with unchelated Fe(III) or Cu(II) ions because of the failure of SOD to inhibit cross-linking in these cases (Figures 14 and 16). Alternatively, the site specificity of the reaction of H_2O_2 with bound metal ions might prevent scavenging of $O_2^{\cdot-}$ by SOD. The Thy-Tyr cross-link has also been identified in γ-irradiated and H_2O_2-treated cultured human cells (Olinski *et al.*, 1992).

4.2 DNA base damage in chromatin of γ-irradiated cultured mammalian cells.

The results of the present work show the formation of a number of modified DNA bases in chromatin isolated from γ-irradiated cells. 5-OH-5-Me-Hyd, 5-OHMe-Ura, Thy glycol and 8-OH-Gua have been identified previously in DNA of irradiated cells (Hariharan & Cerutti, 1972; Mattern *et al.*, 1975; Frenkel *et al.*, 1981; Leadon & Hanawalt, 1983; Teebor *et al.*, 1984; Frenkel *et al.*, 1985; Breimer & Lindahl, 1985; Patil *et al.*, 1985; Kasai *et al.*, 1986; Furlong *et al.*, 1986; Leadon, 1990). As for the remaining modified DNA bases, the present work represents the first demonstration of their formation in chromatin of irradiated cells. Except for 2-OH-Ade, modified bases identified here have also been shown to be formed when isolated chromatin instead of intact cells are exposed to γ-irradiation (Gajewski *et al.*, 1990). 2-OH-Ade has recently been identified in chromatin treated *in vitro* with H_2O_2 in the presence of metal ions (Nackerdien *et al.*, 1991b). The yields

of modified bases measured here were much lower than their yields measured in isolated chromatin, which was γ-irradiated in air-saturated aqeuous suspension (Gajewski *et al.*, 1990). In the previous study, chromatin fully expanded in a low ionic strength buffer was 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 is highly condensed. In fact, the susceptibility of isolated chromatin to damage by ionizing radiation has been found to be greater than that of chromatin in intact cells when measured as base damage, DNA-protein cross-links, and strand breaks (Chiu *et al.*, 1992; Roti Roti *et al.*, 1974; Mee *et al.*, 1978; Mee and Adelstein, 1979; Heussen *et al.*, 1987; Warters and Lyons, 1990; Ljungman, 1991). The results in the present study are consistent with the findings in the above-mentioned literature.

The formation of modified bases in chromatin of irradiated cells is most likely due to reactions of $^{\cdot}OH$ produced from cellular water with DNA bases. Approximately 70% of radiation-induced lethality and DNA damage in oxic cells is caused by $^{\cdot}OH$ (Roots and Okada, 1972, 1975; Chapman *et al.*, 1973). The hydroxyl radical is highly reactive towards organic molecules and has a short diffusion distance in cells. Hence, $^{\cdot}OH$ has to be generated in close proximity to DNA in order to cause any DNA damage (Roots and 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). Modified products can also be formed by direct ionization of DNA bases (reviewed in Steenken, 1989).

Three modified bases namely 5,6-diHThy, 5-OH-6-HThy and 5-OH-6-HCyt identified in DNA γ-irradiated in N_2O-saturated solution were not detected in chromatin of irradiated cells. The absence of 5,6-diHThy, which is induced by e_{aq}^- and/or H atom, is likely due to scavenging of e_{aq}^- and H atom by oxygen. Hydroxyl radical-induced 5-OH-6-HThy and 5-OH-6-HCyt are known to be formed only in deoxygenated systems (reviewed in von Sonntag, 1987; Téoule and Cadet, 1978). The absence of these three modified bases has also been shown in chromatin γ-irradiated in air-saturated aqueous suspension (Gajewski *et al.*, 1990).

Modified DNA bases found in chromatin of irradiated cells were also found in chromatin of unirradiated cells (Table VIII). Intrinsic DNA damage may occur as a result of production of free radicals by cellular metabolic processes (Halliwell and Gutteridge, 1989). Various steps in the procedure of chromatin isolation may also contribute to the formation of modified bases in chromatin. However, the isolation procedure used in this study employs mild treatment of cells, isolated nuclei and chromatin, and does not expose

cells to phenol. Lysis of nuclei in detergents of low ionic strength is bound to be milder and less destructive than the procedures generally used for DNA isolation. Formic acid treatment may also contribute to background levels of modified bases in chromatin. However, the results presented above indicate that modified bases were not significantly formed from cytosine, adenine, guanine and thymine by treatment with formic acid. The background levels of modified bases in chromatin, and not the sensitivity of the technique, represent a limiting factor for the measurement of modified bases in irradiated cells at lower doses. The GC/MS-SIM technique is capable of measuring modified bases at levels of ≈1-3 molecules per 10^6 DNA bases. For example, the lowest amount of a modified base detected in unirradiated DNA [i.e. 5,6-diOH-Ura (Table VI)] corresponded to ≈1.4 molecules per 10^6 DNA bases. Significant increases in the yields of a number of modified bases in chromatin were observed at a dose of as low as 42 Gy and this appears to be the threshold dose for these modified bases.

Calculated from the yields at 116 Gy after subtraction of background levels, guanine-derived bases accounted for ≈45 % of the net yield of modified bases. Adenine-, thymine-, and cytosine-derived bases constituted the rest of the net total yield in almost equal proportions. These results are similar to those previously obtained when isolated chromatin was irradiated in aqueous suspension (Gajewski *et al.*, 1990).

The high net yield of guanine derivatives could be due to the high reactivity of guanine residues with 'OH. The guanine-derived bases, FapyGua and 8-OH-Gua can also result from reactions of the guanine radical cation formed by direct ionization of guanine residues. These conclusions are consistent with the well-known property of guanine as the most easily oxidized DNA base by the direct ionization with photons and 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). These base products result from one-electron reduction and oxidation of C-8-OH-adduct radicals, respectively (for a review, see Steenken, 1989). The yields of these compounds resemble that obtained with isolated chromatin γ-irradiated in deoxygenated aqueous suspensions (Gajewski *et al.*, 1990). The lack of sensitivity of the compounds to inhibition by oxygen may be due to the inability of oxygen to counteract or compete with reactions of C8-OH-adduct radicals of purines. The hypoxic nature of the environment of the cell nucleus (reviewed in Joenje, 1989) is another possibility.

4.3 DNA base damage in chromatin of H_2O_2-treated cells.

The pattern of DNA base modifications observed in H_2O_2-treated cells resembles that of γ-irradiated cells. In both cases, low levels of modified DNA bases were also detected in chromatin from untreated control cells.

The products identified here are known to be typical $^{\cdot}OH$ radical-induced products of DNA bases (reviewed in von Sonntag, 1987). They have recently been identified when isolated DNA and isolated chromatin were exposed to various free radical-producing systems (Dizdaroglu *et al.*, 1991b; Gajewski *et al.*, 1990; Aruoma *et al.*, 1989a,b; Blakely *et al.*, 1990; Dizdaroglu *et al.*, 1990; Aruoma *et al.*, 1991). The pattern of DNA base modifications generated by H_2O_2 indicate that the reactive species responsible for product formation *in vivo* is unlikely to be any other than the hydroxyl radical. This species might be generated *in vivo* by a Fenton-type reaction initiated by transition metal ions (Halliwell and Gutteridge, 1988; 1990).

Because of its high reactivity, the hydroxyl radical must be generated in close proximity to DNA in a site-specific manner, in order to cause any damage *in vivo* (Ward *et al.*, 1985; Goldstein and Czapski, 1986). The necessary metal ions might be bound to chromatin *in vivo*, or liberated by oxidative stress from their storage sites (Halliwell and Gutteridge, 1989; Halliwell and Gutteridge, 1990; Halliwell, 1987). Evidence exists for the role of hydroxyl radicals in biological effects of H_2O_2 *in vivo* involving naturally occurring metal ions (Halliwell, 1987; Mello-Filho *et al.*, 1984; Imlay and Linn, 1988). The critical role of the ions is also evident from the fact that hydrogen peroxide alone does not cause formation of the any of the DNA products identified

above (Dizdaroglu *et al.*, 1991b; Aruoma *et al.*, 1989b; Blakely *et al.*, 1990; Aruoma *et al.*, 1989b).

4.4 Conclusions

I. Co(II) and Ni(II) ions in the presence of H_2O_2 cause formation of typical $^{\cdot}OH$-induced products of DNA bases in isolated human chromatin. Partial product inhibition by typical scavengers of $^{\cdot}OH$ supports the idea of involvement of $^{\cdot}OH$ in product formation. Substantial quantitative differences exist between the effects of Co(II) and Ni(II). DNA base damage in chromatin mediated by Co(II) and Ni(II) may contribute to the established carcinogenicity and genotoxicity of these metal ions.

II. A DNA-protein cross-link produced in mammalian chromatin by H_2O_2 and metal ions has been chemically characterized. It consists of Thy and Tyr. Hydroxyl radical generated in a "site-specific" manner is implicated in the formation of this cross-link.

Substantial differences exist between the effects of various chelation states of the same metal ion, and between the effects of Fe(III) and Cu(II) ions on formation of DPCs. Chromatin-bound Cu(II) ions, but not Fe(III) ions appear to be likely candidates in mediating production of DPCs *in vivo*, unless Fe(III) ions are reduced by a reducing agent.

III. Numerous modified bases were detected in chromatin of H_2O_2-treated and γ-irradiated cultured mammalian cells.

These bases are known to be the typical ·OH-induced products of DNA bases. The variety of modified DNA bases detected in chromatin treated with radical generators makes it difficult to assess their role in biological end-points such as mutagenesis, carcinogenesis and cell death. The contribution of the modified bases, which have been identified in chromatin of treated cells, to the biological effects of ionizing radiation is as yet unknown. The measurement of modified DNA bases provides a sound basis for further studies addressing the toxicity of free radicals.

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6. APPENDIX

6.1 GC/MS of modified DNA bases

Free radical interactions with heterocyclic bases in DNA result in formation of a large number of base products. Modified DNA bases remain attached to the polynucleotide chain and must be released prior to GC/MS analysis. Acidic hydrolysis facilitates the release of both intact and modified bases *via* cleavage of the glycosidic bonds between the bases and the sugar moiety. GC/MS is only applicable to compounds that are volatile. Thus, hydrolyzed DNA samples must be derivatized before injection onto the GC column. DNA bases are not sufficiently volatile and hence are converted into volatile derivatives prior to GC/MS analysis. Trimethylsilylation can be used for this purpose. This is a chemical reaction in which an active hydrogen (from a carboxyl, amino or hydroxyl group) is replaced by a trimethylsilyl [$Si(CH_3)_3$]group. The derivatized hydrolysates of DNA are then injected onto a fused silica capillary column. Mass spectra of the products eluting from the GC column are then recorded in the electron ionization mode.

6.2 Characterization of modified DNA bases.

As an example, Figure 1A shows a typical GC-separation of a trimethylsilylated acid hydrolysate of DNA that had been exposed to $^{\cdot}OH$ and H atom generated by ionizing radiation in aqueous solution (Dizdaroglu, 1985). Peak identification is given in the

legend to Fig. 1A. Peaks I, II, III, IV and V represent the Me_3Si derivatives of phosphoric acid, thymine, cytosine, adenine, and guanine, respectively. Peaks IIIa, IVa, and Va also represent the last 3 bases, but with an additional Me_3Si group attached to their amino groups as revealed by comparison with authentic material and by mass spectrometry. Peaks a to g were also present in control DNA samples treated under similar hydrolysis conditions and, with the exception of peak d, could not be identified. Peak d corresponds to the Me_3Si derivative of 5-methylcytosine. Radiation-induced DNA base modifications are represented by peaks 1-13.

Electron-ionization mass spectra of the DNA base products are characterized by an intense molecular ion ($M^{+\cdot}$ ion) and an intense $[M-CH_3]^+$ ion, which results from loss of a methyl radical from the $M^{+\cdot}$ ion. Examples of mass spectra are given in Figs. 2A and 3A. The mass spectrum of 5-hydroxyuracil (peak 4; Fig. 1A) is depicted in Fig. 2A. The $[M-CH_3]^+$ ion appears as the base peak at *m/z* 329, while the $M^{+\cdot}$ ion and a characteristic $[M-H]^+$ ion are observed at *m/z* 344 and 343, respectively. The ions at *m/z* 147 and 73 are commonly observed with Me_3Si derivatives and serve no diagnostic purpose.

Figure 3A depicts the mass spectrum derived from peak 13 in Fig. 1A, i.e., the Me_3Si derivative of 8-hydroxyguanine. It is characterized by intense $M^{+\cdot}$ and $[M-CH_3]^+$ ions at *m/z* 455 and *m/z* 440 (base peak), respectively.

6.3 Detection of modified DNA bases at low concentrations.

For complex mixtures such as chromatin, precise quantitation of DNA lesions at low concentrations is carried out using GC/MS with selected-ion monitoring (SIM). The mass spectrometer is programmed to monitor several characteristic ions of a product during the time it elutes from the GC column. The product is identified unequivocally when the signals of the monitored ions with the correct abundances all line up at its retention time. Therefore prior knowledge of the mass spectrum and the retention time of the product under investigation is required. Retention times can be measured with great accuracy and therefore play an important role in reliable identification, in addition to the simultaneous measurement of masses and abundances of typical ions of a product.

6.4 Quantitation of DNA lesions.

An internal standard, usually a stable isotope-labeled analog of an analyte, is added to chromatin or DNA samples prior to GC/MS-SIM analysis to permit accurate quantitation of the analyte. First, a calibration plot is obtained for the response of the mass spectrometer to known amounts of both the analyte and the internal standard. The slope of such a plot is termed the relative molar response factor (see footnote in section 2.10.3). For these studies, isotope-labeled analogues were not available and structurally similar compounds were used instead as internal standards. After GC/MS-SIM analysis, peak areas of the

corresponding ions are integrated, and the amount of an analyte in a sample is calculated using these peak areas, its RMRF and the known amount of internal standard. More details of the detection and quantitation of modified bases can be found elsewhere (Watson, 1985; Dizdaroglu, 1991).

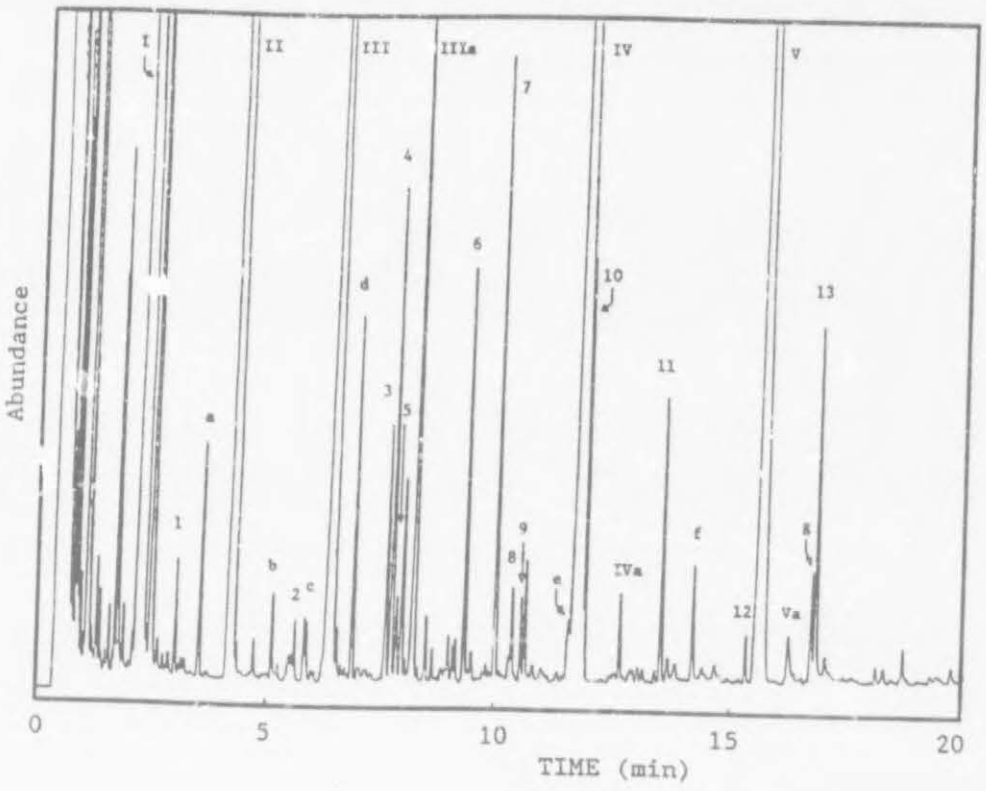

Figure 1A: Gas chromatogram obtained with formic acid hydrolysate of DNA after trimethylsilylation (Dizdaroglu, 1985). Peaks: **I**, phosphoric acid; **1**, uracil; **II**, thymine; **2**, 5,6-dihydrothymine; **III**, cytosine; **d**, 5-methylcytosine; **3**, 5-hydroxy-5,6-dihydrothymine; **4**, 5-hydroxyuracil; **5**, 5-hydroxy-5,6-dihydrouracil; **IIIa**, cytosine; **6**, 5-hydroxycytosine; **7** and **8**, *cis*- and *trans*-thymine glycol; **IV**, adenine; **10**, 4,6-diamino-5-formamidopyrimidine; **IVa**, adenine; **11**, 8-hydroxyadenine; **12**, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; **V**, guanine; **Va**, guanine; **13**, 8-hydroxyguanine.

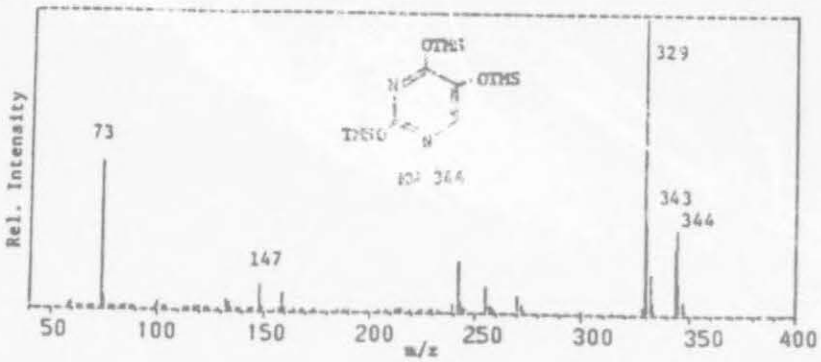

Figure 2A: Mass spectrum taken from peak 4 (5-hydroxyuracil) in Fig. 1A (Dizdaroglu, 1985).

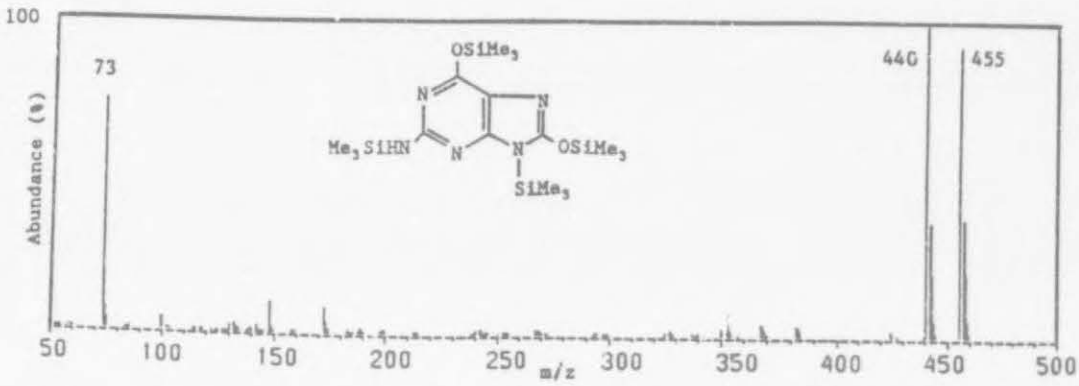

Figure 3A: Mass spectrum taken from peak 13 (trimethylsilyl derivative of 8-hydroxyguanine) in Fig. 1A (Dizdaroglu, 1985).