Measurement of Oxidative Damage at Pyrimidine Bases in γ-Irradiated DNA

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Oxidized nucleobases represent one of the main classes of damage induced in DNA by ionizing radiation. Emphasis was placed in this work on the measurement of four oxidized pyrimidine bases, including 5-(hydroxymethyl)uracil (5-HMUra), 5-formyluracil (5-ForUra), 5-hydroxycytosine (5-OHCyt), and 5-hydroxyuracil (5-OHUra), in isolated DNA upon exposure to γ radiation in aerated aqueous solution. For this purpose, both high performance liquid chromatography associated with electrochemical detection (HPLC-EC) and gas chromatography coupled to mass spectrometry (GC-MS) were used. Conditions of hydrolysis of the N-glycosidic bond were carefully checked in order to achieve a quantitative release of the lesions. We showed that 60% formic acid treatment leads to the decomposition of the four lesions studied. On the other hand, hydrolysis based on the use of either 88% formic acid or 70% hydrogen fluoride in pyridine (HF/Pyr) allowed the quantitative release of the modified bases, with the exception of 5-HMUra when the latter reagent was utilized. A dose course study of the radiation-induced formation of 5-HMUra and 5-ForUra in DNA by using the GC-MS assay showed that the latter lesion was produced in a 2.1-fold higher yield than the former one. HF/Pyr and 88% formic acid hydrolysis provided similar results for 5-ForUra, indicating the reliability of both techniques for the measurement of this lesion. For 5-OHUra and 5-OHCyt, the level of modification determined by GC-MS analysis was higher after 88% formic acid treatment than upon HF/Pyr hydrolysis. When DNA was enzymatically digested and analyzed by HPLC-EC for 5-OHdCyd and 5-OHdUrd, the results were very close to those obtained by GC-MS following HF/Pyr treatment. It was concluded that additional amounts of both 5-OHUra and 5-OHCyt are produced during the 88% formic acid treatment from radiation-induced 5,6-saturated pyrimidine precursors. It is likely that cytosine and uracil diols are involved in this reaction. The radiochemical yields of formation (in $\mu mol \cdot J^{-1}$) for the products studied are in the following decreasing order: 5-ForUra (0.0083) > 5-OHCyt (0.0046) > 5-HMUra (0.0039) > 5-OHUra (0.0035).

Introduction

The indirect effect of ionizing radiation which is mostly mediated by $OH^{\bullet}$ radicals gives rise to strand breaks and base lesions as the main classes of DNA damage. The bulk of the oxidative damage to purine and pyrimidine bases has been characterized either in model compounds or in isolated DNA. However, the quantitative aspect of the formation of most of these lesions remains to be established mostly because of a lack of sensitive and reliable methods of measurement. High performance liquid chromatography (HPLC)[1] associated with electrochemical detection (EC) allowed the measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) in isolated DNA exposed to ionizing radiation *(1, 2)*. Application of HPLC-EC has been extended to the study of the formation of 8-oxo-7,8-dihydro-2′-deoxyadenosine *(2)*, 5-hydroxy-2′-deoxycytidine (5-OHdCyd), and 5-hydroxy-2′-deoxyuridine (5-OHdUrd) *(3)* in DNA exposed to γ radiation. Other approaches, based either on HPLC analysis with UV detection of enzymatically digested DNA *(4, 5)* or on detection of bases released by repair enzymes *(6, 7)* have been proposed to measure oxidized bases in irradiated DNA. However, they are either not sensitive enough or not quantitative. Gas chromatography analysis coupled to mass spectrometry is another powerful tool for the study of the formation of oxidized DNA bases. It has been applied to the measurement of radiation-induced base damage to DNA in both *in vitro (8−10)* and *in vivo* experiments *(11)*. However, recent results have shown that artifactual oxidation of normal DNA bases takes place during the silylation step aimed at preparing volatile derivatives for GC analysis *(12−14)*. It should be added that most of the early GC-MS measurements of radiation-induced oxidized DNA bases were carried out without using isotopically labeled internal standards, which are required for an accurate GC-MS analysis. Altogether, this prompts us to reconsider the data previously obtained by GC-MS analyses of $OH^{\bullet}$ mediated oxidation of pyrimidine bases. The measurement of four characteristic oxidized bases was acheived by using an improved version of the GC-MS assay. Emphasis was placed on the optimization of the conditions of DNA hydrolysis in order to obtain quantitative

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[1] Abbreviations: 5-FordUrd: 5-formyl-2′-deoxyuridine; 5-ForUra: 5-formyluracil; 5-HMdUrd: 5-(hydroxymethyl)-2′-deoxyuridine; 5-HMUra: 5-(hydroxymethyl)uracil; 5-OHCyt: 5-hydroxycytosine; 5-OHdCyd: 5-hydroxy-2′-deoxycytosine; 5-OHdUrd: 5-hydroxy-2′-deoxyuridine; 5-OHUra: 5-hydroxyuracil; 8-oxoAde: 8-oxo-7,8-dihydroadenine; 8-oxodGuo: 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGua: 8-oxo-7,8-dihydroguanine; GC-MS: gas chromatography coupled to mass spectrometry; HF/Pyr: 70% w/w solution of hydrogen fluoride in pyridine; HPLC-EC: high performance liquid chromatography with electrochemical detection.

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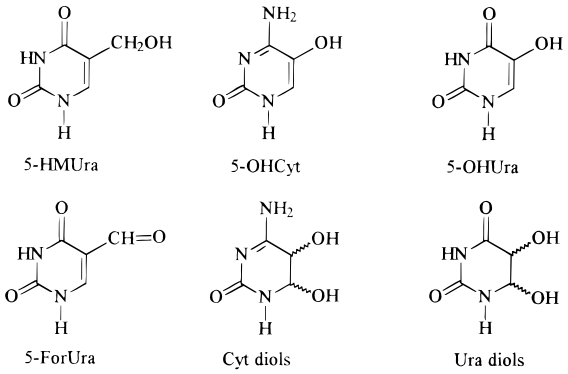

Figure 1. Chemical structure of the oxidative lesions studied.

results. The modified bases studied include 5-(hydroxymethyl)uracil (5-HMUra) and 5-formyluracil (5-ForUra) which result from the OH• mediated oxidation of the methyl group of thymine (*5*). In addition, 5-hydroxycytosine (5-OHCyt) and 5-hydroxyuracil (5-OHUra), which arise from the addition of OH• to the C5−C6 double bond of cytosine, were also measured (Figure 1). The detection of the two latter lesions was also made by HPLC-EC following enzymatic digestion. The role of cytosine and uracil diols in the detection of 5-hydroxylated cytosine derivatives was investigated through a study of the stability of 5,6-dihydroxy-5,6-dihydro-2′-deoxyuridine (dUrd diols) under various hydrolysis conditions.

Materials and Methods

Chemicals. 5-HMUra, 5-(hydroxymethyl)-2′-deoxyuridine (5-HMdUrd) and calf thymus DNA were purchased from Sigma (St Louis, MO). 5-ForUra and the 70% solution of hydrogen fluoride in pyridine (HF/Pyr) were provided by Aldrich (Steinheim, Germany). 5-OHUra (isobarbituric acid) was obtained from Fluka (Buchs, Switzerland). 5-Formyl-2′-deoxyuridine (5-FordUrd) was synthesized by menadione photosensitization of thymidine *(15)*. 5-OHCyt was prepared by using the method reported by Moshel and Behrman *(16)*. 5-OHdCyd and 5-OHdUrd were prepared by collidine treatment of 5-bromo-2′-deoxycytidine and 5-bromo-2′-deoxyuridine, respectively. The latter nucleosides were obtained by addition of bromine to a solution of 2′-deoxycytidine and 2′-deoxyuridine, respectively. Condensation of [1,3-^{15}N]uracil with [$^{2}H_2$]paraformaldehyde (MSD Isotopes, Montreal, Canada) provided [1,3-^{15}N,5-$^{2}H_2$]-5-HMUra *(15)*. An aliquot fraction of the latter product was subsequently oxidized into [1,3-^{15}N,5-^{2}H]-5-ForUra *(17)*. [2-^{13}C,1,3-^{15}N]-5-OHCyt was prepared as described for 5-OHCyt, using [2-^{13}C,1,3-^{15}N]Cyt obtained from [^{13}C,$^{15}N_2$]urea (Eurisotop, Saint Aubin, France) according to Bendich *et al. (18)*. [1,3-^{15}N,5-^{18}O]-5-OHUra was synthesized from [1,3-^{15}N]uracil by bromination followed by collidine treatment in [^{18}O]H_2O (Eurisotop, St. Aubin, France). The two *cis* diastereoisomers of dUrd diols were prepared by permanganate oxidation of an aqueous solution of 2′-deoxyuridine. The diols were purified by reverse phase HPLC and characterized by ^{1}H NMR and GC-MS analyses.

Irradiation and Hydrolysis of Isolated DNA. A 0.7 mg·mL^{-1} solution (3 mL) of calf thymus DNA was exposed under constant air bubbling to the γ rays of a ^{60}Co source placed in a pool. The dose rate was 45 Gy·min^{-1} as determined by poly(methyl methacrylate) dosimetry. An aliquot fraction (100 μL) was incubated with 10 μL of nuclease P1 buffer (300 mM sodium acetate, 1 mM $ZnSO_4$, pH 5.3) and 10 U of nuclease P1 (Boerhinger, Mannheim, Germany). The samples were held at 37 °C for 2 h. Then, dephosphorylation of the resulting nucleotides was achieved by addition of 12 μL of alkaline phosphatase buffer (500 mM Tris-HCl, 1 mM EDTA, pH 8) and 1 U of alkaline phosphatase (Boerhinger, Mannheim, Germany). After incubation for 1 h at 37 °C, proteins were precipitated by addition of 50 μL of chloroform. The samples were centrifuged and the aqueous layers collected. Another aliquot fraction (200 μL) of the irradiated DNA solution was freeze-dried and the resulting residue suspended in 1 mL of 88% v/v formic acid to which isotopically labeled internal standards (300 pmol) were added. The resulting solutions were placed in sealed glass vials and held at 140 °C for 45 min in an aluminum heating block. They were subsequently dried under vacuum. An additional aliquot fraction (300 μL) of the DNA solution was dried under vacuum in polypropylene vial. HF/Pyr (100 μL) was added and the sample was held at 37 °C for 45 min. Then, the hydrolysis mixture was poured down in a suspension of 300 mg of calcium carbonate in 1 mL of water. The mixture was shaken until the pH reached 7. The resulting suspension was centrifuged and the aqueous phase collected. The solid fraction was washed with 1 mL of a [1:1] mixture of water and ethanol. The liquid phase obtained was combined with the first one, and the resulting solution was freeze-dried. The whole irradiation and hydrolysis procedure was done in triplicate for each dose. Time course studies of the hydrolysis were performed using DNA samples which were exposed to 75 Gy. Hydrolysis experiments using 60% formic acid were carried out in a similar way as with 88% formic acid.

The samples were then injected on a HPLC apparatus (Gilson, Middletown, WI) consisting of two pumps (Model 306) controlled by a third one (Model 305). Samples dissolved in 1 mL of water were injected using a 231 XL autoinjector, and fractions of interest were collected on a FC204 fraction collector. The elution was monitored by a 111B UV spectrometer set at 280 nm connected to a D2500 integrator (Hitachi, Tokyo, Japan). The system was equipped with a home-packed semipreparative (250 × 7 mm i.d., particle size: 10 μm) Nucleosil 100-10 C_{18} octadecylsilyl silica gel column (Macherey Nagel, Düren, Germany). The elution started with a 25 mM solution of ammonium formate in water for 10 min. Methanol was linearly added over 15 min until its proportion reaches 20%. The latter composition was then kept for 5 min. The flow rate was 3 mL·min^{-1}. The two fractions eluting between 4 and 5 min on one hand, and 5 and 8 min on the other hand, were collected and dried under vaccum. Water was added (350 μL) and the resulting solution transferred into silylation vials. They were then freeze-dried overnight. Samples were subsequently derivatized for 20 min in 100 μL of a [50:50] v/v mixture of acetonitrile (silylation grade, Pierce, Rockford, IL) and N-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (Fluka, Buchs, Switzerland) at 110 °C. GC-MS analyses were performed on a HP 5890 Serie II gas chromatograph (Hewlett-Packard, Les Ulis, France) equipped with a capillary column (0.25 mm, 30 m) coated with a 0.25 μm film of methylsiloxane substituted by 5% phenylsiloxane (HP5-MS, Hewlett-Packard). The constant flow rate was 1.6 mL·min^{-1}. The injection (injection volume: 1 μL) was performed in the splitless mode with the temperature of the injection port set at 250 °C. The temperature of the GC oven was maintained at 70 °C for 1 min and then raised to 300 °C at a rate of 20 °C·min^{-1}, and finally left at the latter temperature for 3.5 min. Detection of positive ions was provided by a HP 5972 mass detector using electron impact ionization (Hewlett-Packard, Les Ulis, France). Three (*tert*-butyldimethylsilyl)-*N*-methyl- (tBDMS) groups were added to 5-OHUra, 5-OHCyt, and 5-HMUra while 5-ForUra was derivatized into its bisilylated derivative. The ion at $m/z = M - 57$ (M − butyl) was the base peak in all mass spectra and was thus used for the quantitative analysis, performed in the ion collection mode. The retention times were 9.9, 10.9, 11.1, and 11.3 min for the silylation products of 5-ForUra, 5-OHUra, 5-HMUra, and 5-OHCyt, respectively.

Hydrolysis of the Nucleosides. A solution containing 5-HMdUrd, 5-FordUrd, 5-OHdCyd, and 5-OHdUrd (1 nmol of each) was freeze-dried in polypropylene tubes. HF/Pyr (100 μL) was added and the sample held at 37 °C for increasing periods of time. The solution was poured down into a suspension of 300 μg of calcium carbonate in 1 mL of water. Mixture of the isotopically labeled bases (300 pmol of 5-OHUra, 5-OHCyt,

5-HMUra, and 5-ForUra) was added. The mixture was then shaken until pH reached 7. The suspension was centrifuged and the liquid phase collected. The solid fraction was washed with 500 μL of a [1:1] mixture of ethanol and water. The two liquid phases were mixed and freeze-dried. The resulting samples were then prepurified by HPLC and analyzed by GC-MS as reported above for DNA. Other aliquot fractions of the solution of the four nucleosides were freeze-dried in glass vials, and 500 μL of either 60% or 88% formic acid was added. The vials were sealed and held at 140 °C for increasing periods of time. Formic acid was removed under vacuum, and 300 pmol of the isotopically labeled bases dissolved in 1 mL of water were added. The samples were then prepurified by HPLC and analyzed by GC-MS as reported above for DNA. HF/Pyr and 88% formic acid hydrolysis of the dUrd diols (250 μg) was performed for 45 min in a similar way, without adding isotopically labeled internal standard. A third sample of dUrd diols was incubated for 2 h in the nuclease P1 buffer and then 1 h in the alkaline phosphatase buffer used in the enzymatic digestion (*vide supra*). The hydrolyzed dUrd diol samples were analyzed by reverse phase HPLC using a Interchrom HC18-25F octadecylsilyl silica gel column (250 × 4.6 mm i.d.) (Interchim, Montluçon, France) and a 25 mM ammonium formate aqueous solution as the isocratic eluent. The detection was provided by a Waters 990 diode array UV spectrometer. Under these conditions, the retention time of the two diastereoisomers of dUrd diols was 4.12 and 4.68 min. Their UV absorption spectra only exhibited a maximum at 210 nm. Pure 5-OHUra (t_R: 4.3 min) and 5-OHdUrd (t_R: 13.1 min) were injected on the HPLC system in order to determine their retention times. Their UV spectra exhibited an additional absorption maximum at 280 nm. In HF/Pyr hydrolyzed samples, a compound exhibiting a retention time of 3.4 min was identified as *cis*-5,6-dihydroxy-5,6-dihydrouracil (Ura diol). Hydrolyzed dUrd diols samples were also analyzed by GC-MS in the SCAN mode following trimethylsilylation. The respective retention times of the two hexasilylated diastereoisomers of dUrd diols and trisilylated 5-OHUra were 10.99, 11.40, and 7.97 min. The mass spectra of the two silylated diastereoisomers of dUrd diols exhibited pseudomolecular ions at $m/z = 622$. The tetrasilylated Ura diol exhibited a retention time of 7.97 min. A pseudomolecular ion at $m/z = 434$ was observed in its EI mass spectrum. In all mass spectra the ion at m/z = M − 15 (M − methyl) was observed together with characteristic fragments.

HPLC-EC Analysis. The HPLC-EC system consisted of a model 2150 LKB pump (Pharmacia LKB Biotechnology, Uppsala, Sweden) connected to a SIL-9A autosampler (Shimadzu, Kyoto, Japan) equipped with a Interchrom HC18-25F octadecylsilyl silica gel column (250 × 4.6 mm i.d.) (Interchim, Montluçon, France). The isocratic eluent was a mixture of 50 mM sodium citrate (pH 5) containing 1.5% of methanol. The coulometric detection was provided by a Coulochem II detector (Esa, Chelmsford, MA). The detection potentials were set at +200 and +450 mV for electrodes 1 and 2, respectively. The retention times of 5-OHdCyd and 5-OHdUrd were 10.5 and 12.9 min, respectively. Unmodified nucleosides were monitored by a Waters 484 UV detector set at 280 nm. Both signals were collected on a D7500 Hitachi (Tokyo, Japan) integrator. The amount of DNA analyzed was inferred from the area of the peak of 2′-deoxyguanosine (t_R: 45 min) after appropriate calibration.

Results

Study of the Hydrolysis Step. A preliminary study was made to assess the quantitative aspects of the acidic hydrolysis step prior to GC-MS analysis for the four lesions studied. A first experiment was carried out with 1 nmol of each of the corresponding nucleosides (5-HMdUrd, 5-FordUrd, 5-OHdCyd, and 5-OHdUrd) that were hydrolyzed for increasing periods of time. Three acidic treatments were investigated, namely, 88% formic acid at 140 °C, 60% formic acid at 140 °C, and HF/Pyr at 37 °C. In these series of experiments, the internal

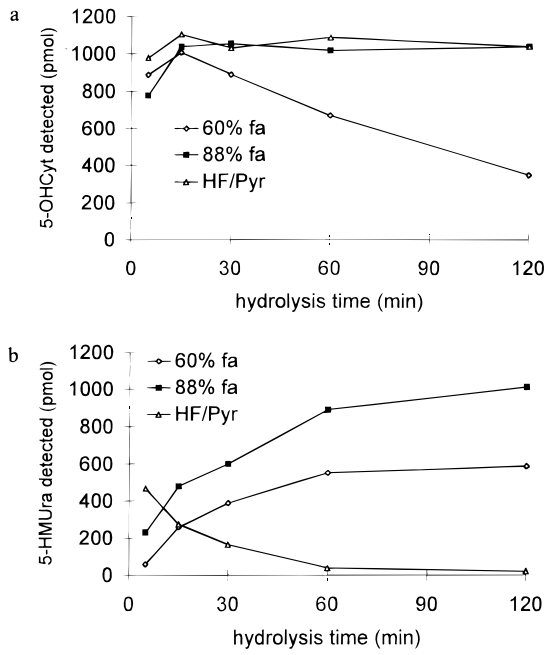

Figure 2. Time course study of acidic hydrolysis of oxidized nucleosides: (a) 5-OHdCyd and (b) 5-HMdUrd. Hydrolysis conditions: 60% fa = 60% formic acid, 140 °C; 88% fa = 88% formic acid, 140 °C; HF/Pyr = 70% hydrogen fluoride in pyridine, 37 °C.

standards required by the GC-MS analysis were added at the end of the hydrolysis in order to estimate the stability of the compounds. For all analyses of DNA, internal standards were added prior to the hydrolysis in order to compensate for any loss of product which could be a major drawback when working at the trace level. It should also be added that hydrolyzed samples were purified by HPLC prior to GC-MS analysis in order to remove remaining nucleosides from the solution. Indeed, the N-glycosidic bond of most of the pyrimidine nucleosides is cleaved during the silylation, leading to the release of the corresponding base moiety. In addition, the salts produced during the neutralization step of the HF/Pyr hydrolysis have to be removed in order to avoid interferences during the GC-MS analysis. Similar observations were made regarding the stability of 5-OHUra, 5-ForUra, and 5-OHCyt (Figure 2a for the latter lesion). The latter products underwent a fast decomposition in 60% formic acid but were stable upon 88% formic acid and HF/Pyr treatments. With the two latter hydrolyzing reagents, the total amount of nucleoside (1 nmol) was recovered as free base, which is indicative of a complete hydrolysis. 5-HMUra (Figure 2b) was stable and quantitatively released upon treatment with 88% formic acid but not with HF/Pyr. The amount of 5-HMUra detected in samples of 5-HMdUrd hydrolyzed in 60% formic was only half of the total amount of nucleoside treated. This indicates that, in spite of the plateau observed for the release of 5-HMUra during the time course study, the compound is also unstable under the latter hydrolysis conditions. It is worth mentioning that the four pyrimidine oxidation products studied are also unstable at the base level in 60% formic acid (data not shown). Similar conclusions were drawn when samples of DNA exposed to 75 Gy of γ radiation in aqueous aerated solution were hydrolyzed for increasing periods of time in the presence of internal standards. In 60% formic acid, the amount of the four oxidized bases detected in the hydrolysate decreased with hydrolysis time. On the other hand, the

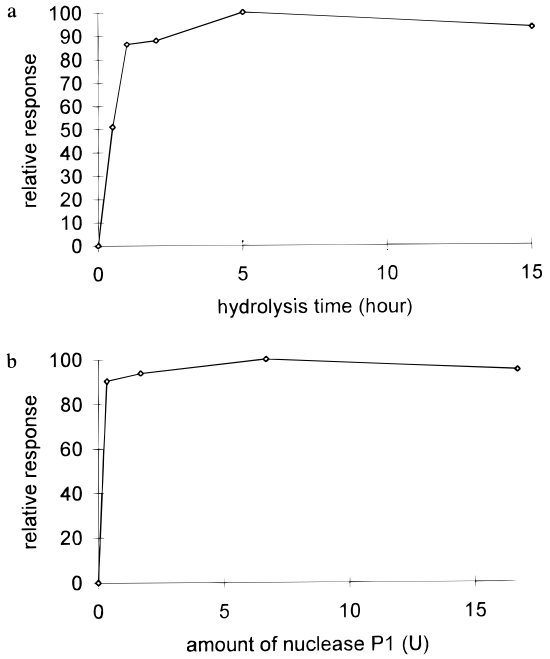

Figure 3. Effect of the conditions of enzymatic digestion on the modification rate of 5-OHdCyd measured in DNA by HPLC-EC. (a) 10 units of nuclease P1 for increasing period of time; (b) increasing amount of nuclease P1 for 2 h.

release of the four oxidized bases reached a plateau after 20 min in 88% formic acid. A similar observation was made for the release of oxidized bases induced by HF/Pyr, with the exception of 5-HMUra which was not stable under these conditions. The quantitative aspect of the DNA enzymatic hydrolysis by nuclease P1 was also checked (Figure 3). A time course study (from 0.5 to 18 h) with 2 U of the enzyme was performed. Optimization of the enzymatic hydrolysis conditions was also investigated by incubating DNA samples with increasing amounts of enzyme (from 0.3 to 17 U) for 2 h. Samples were then analyzed for their content in 5-OHdCyd by HPLC-EC. It was found that both the amount of hydrolyzed DNA and the value of the yield of lesion reached a plateau either after 1 h when 10 U of nuclease P1 was used or when DNA sample was incubated with 2 U of the latter enzyme for 2 h. Therefore, it was concluded that treatment of the samples by 10 U of nuclease P1 for 2 h is most likely to allow a complete digestion. Study of the stability of dUrd diols (Figure 4A) showed that they are completely stable under the enzymatic digestion conditions used. Indeed, the HPLC elution profiles were identical after and before incubation in the buffer used for nuclease P1 and alkaline phosphatase. In contrast, 5-OHUra was the only detectable product in dUrd diol samples hydrolyzed by 88% formic acid. 5-HMura and the faster eluted dUrd diol exhibit identical HPLC retention time but are easily distinguishable on the basis on their UV spectrum. In addition to 5-OHUra, a second major compound, which UV spectra exhibited only one maximum centered around 210 nm like dUrd diols, was detected in dUrd diol samples after treatment by HF/Pyr. The yield of the latter hydrolysis product was approximately half of that of 5-OHUra. Additional structural information was inferred from a GC-MS analysis showing the presence of a compound whose mass spectrum corresponds to that of the tetrasilylated Ura diol (Figure 4B). It is worth mentioning that dUrd diols are partly converted into 5-OHUra during the silylation step of the GC-MS analysis. Therefore, it

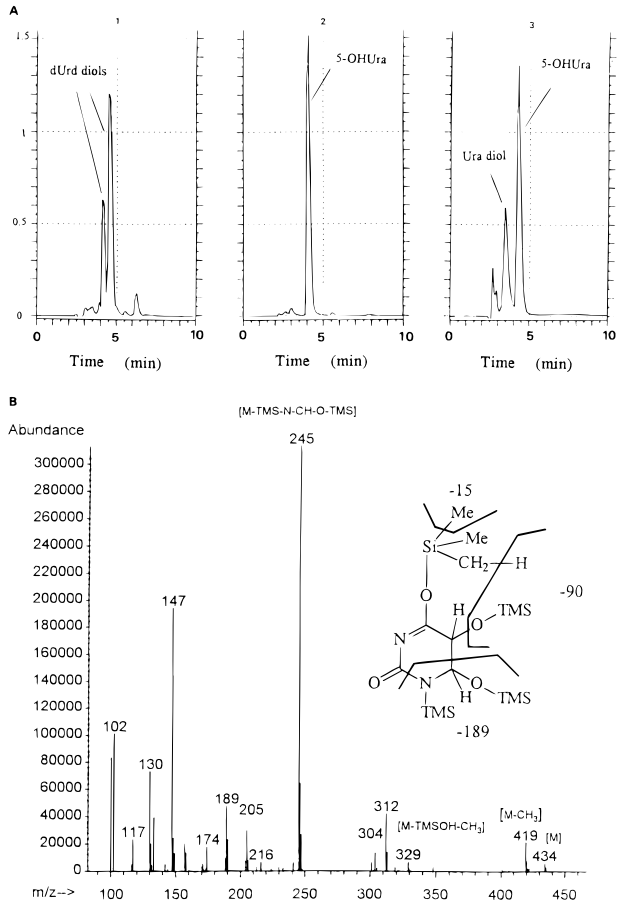

Figure 4. Stability of dUrd diols under different hydrolysis conditions. (A) HPLC analysis of samples treated under conditions of (1) enzymatic digestion, (2) 88% formic acid, (3) HF/Pyr. (B) Electron impact mass spectrum of the Ura diol obtained by GC-MS after trimethylsilylation of dUrd diol samples hydrolyzed by HF/Pyr.

is not possible to directly estimate the yield of each of the hydrolysis products on the basis of the area of the peaks of the GC-MS elution profile. Altogether, it was concluded that Ura diol is a major product of the HF/Pyr mediated hydrolysis of dUrd diols.

Measurements of Oxidized Pyrimidine Bases in γ-Irradiated DNA Samples. A first series of results was obtained for DNA samples exposed to γ rays in aerated aqueous solution and then hydrolyzed by either 88% formic acid or HF/Pyr. 5-HMUra, 5-ForUra, 5-OHUra, and 5-OHCyt were measured by GC-MS. The modification rate was found to be proportional to the dose for the four radiation-induced lesions over the dose range (0−225 Gy) investigated. In addition, the results dealing with the formation of 5-ForUra upon either HF/Pyr or 88% formic acid mediated release were similar (Figure 5). Such a comparison could not be made for 5-HMUra since the latter lesion is not stable in HF/Pyr. The homogeneity of the results obtained for 5-ForUra following either formic acid or HF/Pyr was not observed for 5-OHUra and 5-OHCyt (Figure 6a). When 88% formic acid was used, 5-OHUra was found to be predominant over 5-OHCyt (ratio 5-OHUra/5-OHCyt = 2.39). In contrast, when DNA was hydrolyzed by HF/Pyr, 5-OHCyt was detected in a higher yield than 5-OHUra (5-OHUra/5-OHCyt = 0.78). It should be added that the yield of 5-OHCyt and 5-OHUra was higher upon formic acid hydrolysis than following HF/Pyr treatment. The effect was stronger for 5-OHUra since the yield increases by a

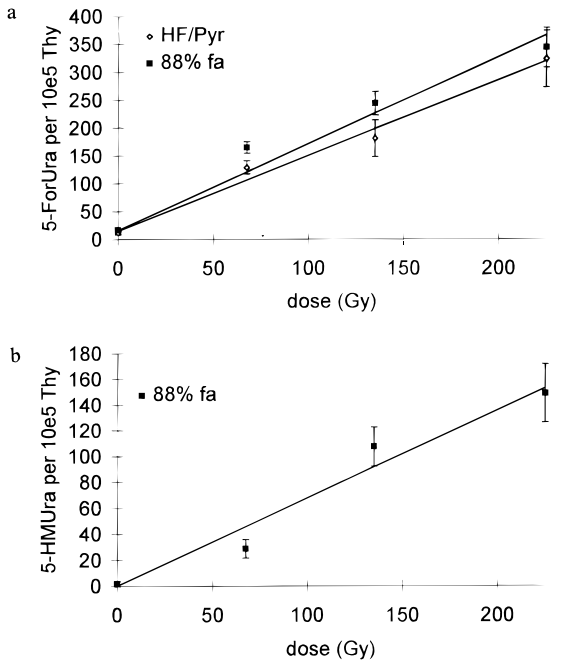

Figure 5. Formation of (a) 5-ForUra and (b) 5-HMUra in DNA exposed to γ rays in aqueous aerated solution determined by GC-MS. Hydrolysis conditions: 88% fa = 88% formic acid, 140 °C, 45 min; HF/pyr = 70% hydrogen fluoride in pyridine, 37 °C, 45 min. The error bars correspond to the average of 3 independent determinations.

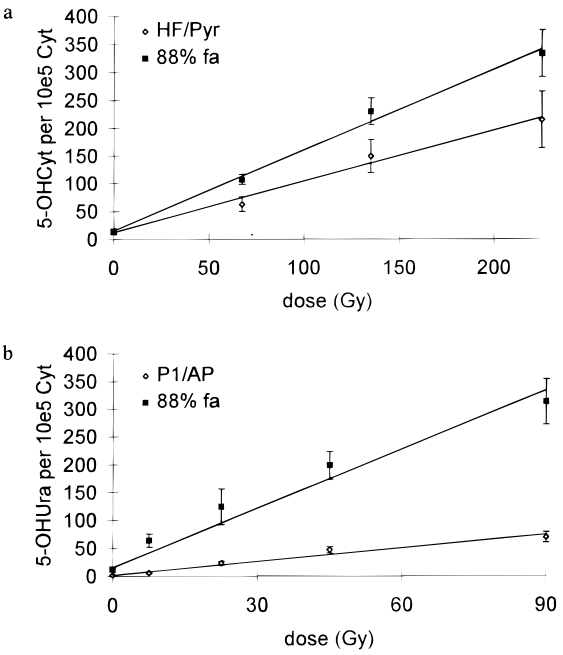

Figure 6. Formation of (a) 5-OHCyt and (b) 5-OHUra in DNA exposed to γ radiation in aqueous aerated solution. Hydrolysis conditions: 88% fa = 88% formic acid, 140 °C, 45 min; HF/Pyr = 70% hydrogen fluoride in pyridine, 37 °C, 45 min; P1/AP = enzymatic digestion by nuclease P1 and alkaline phosphatase. The two former series of samples were analyzed by GC-MS. The enzymatically digested samples were analyzed by HPLC-EC. The error bars correspond to the average of 3 independent determinations.

factor of 4.4 whereas it was only 1.6-fold higher for 5-OHCyt. To further assess the role of the hydrolysis technique, another series of γ-irradiated DNA samples were analyzed by both GC-MS following 88% formic acid hydrolysis and HPLC-EC after enzymatic digestion. The latter technique has been successfully applied to the detection of both 5-OHdCyd and 5-OHdUrd in isolated and cellular DNA *(19)*. It was observed that the level of both 5-OHUra (Figure 6b) and 5-OHCyt was higher upon formic acid hydrolysis than after enzymatic digestion (ratio: 4.0 and 1.6, respectively). In addition, 5-OHCyt was the predominant lesion upon enzymatic digestion (ratio 5-OHUra/5-OHCyt = 0.75) whereas 5-OHUra was detected in the highest yield after formic acid hydrolysis (5-OHUra/5-OHCyt = 1.87).

Discussion

GC-MS Methodology. GC-MS analysis is a now widely used technique for the detection of oxidative base lesions within DNA after acidic hydrolysis. However, until recently, yields of 8-oxoGua measured by using this approach were much higher than those obtained by HPLC-EC *(20)*. Observation of an artifactual oxidation of guanine moieties during the silylation step of the GC-MS analysis is likely to explain these discrepancies *(12, 13)*. This observation has then been extended to four other oxidized nucleobases, including 5-HMUra, 5-OHCyt, 5-ForUra, and 8-oxo-7,8-dihydroadenine *(14)*. In order to overcome this problem, GC-MS measurements of the radiation-induced oxidized DNA bases released upon acidic treatment of DNA were carried out after HPLC prepurification of the samples. This step was aimed at removing the normal bases from their oxidized derivatives prior to silylation. Under these conditions, the amount of oxidized bases detected by GC-MS is only accounted for by their release from DNA. Hydrolysis at high temperature could be another source of artifactual oxidation of the DNA samples. However, this would lead to a continuous increase of the amount of oxidized bases measured in the same sample with increasing periods of acidic treatment. In contrast, the time course study of the release of the four lesions studied in 88% formic acid reached a plateau. This is strongly indicative that no oxidation occurs during the hot acidic hydrolysis. Similar observations were made for HF/Pyr and enzymatic treatments. Another aspect of the present GC-MS assay is the use of isotopically labeled internal standard. This is necessary to avoid any bias due to the possible loss of sample in the experimental workup and to compensate for variation in the silylation rate or injection efficiency. The accuracy of the improved GC-MS assay allowed the precise study of the effect of the hydrolysis conditions. It was observed that 60% formic acid, a reagent used by some authors *(21, 22)*, leads to an extensive degradation of the four lesions studied. In contrast, when the concentration of formic acid was raised to 88% *(23, 24)*, the four molecules were stable over a period of time allowing their quantitative release from DNA. These results can be explained by the molecular ratio between water and formic acid which is 1.4 in 60% formic acid and 0.5 in 88% formic acid. This means that dissociation of the carboxylic acid group will be higher in the less concentrated solution. Similar conclusions on the degradative effects of diluted formic acid on oxidized DNA bases were reported in a recent work *(25)*. In addition to the obvious loss of sensitivity, the use of a hydrolyzing reagent able to decompose the molecules of interest induces a drastic decrease in the reliability of the measurement. It can be stated that since both the lesion and its internal standard are degraded at a similar rate, the ratio between the amount of these two compounds will remain constant over the hydrolysis period. However, the normal oxidized base is slowly released from

Table 1. Radiation-Induced Formation of 5-HMUra, 5-ForUra, 5-OHUra, and 5-OHCyt following Different Hydrolysis Procedures[a]

compd	HF/Pyr	88% formic	P1/AP digestion
5-HMUra	unstable	0.0042	0.0035
5-ForUra	0.0077	0.0083	nd[b]
5-OHCyt	0.0043	0.0069	0.0048
5-OHUra	0.0035	0.0154	0.0035

[a] Yields expressed in μmol·J^{-1}. [b] nd, not determined.

DNA by the hydrolysis reagent. Therefore, the overall stability of the normal oxidized pyrimidine in the hydrolysis mixture partly depends on its stability when still linked to DNA. This will not be compensated by the addition of internal standard which is added as a free base from the very beginning of the hydrolysis. A third hydrolysis technique, based on the use of hydrogen fluoride in solution in pyridine, was investigated. This reagent has been applied in our laboratory to various modified DNA bases, including (6−4) photoproducts *(26)* and adducts of α,β-unsaturated aldehydes to 2′-deoxyguanosine *(27)* and 8-oxodGuo *(2)*. HF/Pyr was also found to lead to the quantitative hydrolysis of the N-glycosidic bond of modified nucleosides such as thymidine diols *(28)* and 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine *(29)*. HF/Pyr was found to be a mild reagent allowing the hydrolysis of lesions which were unstable under other conditions. In the present study, HF/Pyr was successfully applied to the quantitative release of 5-OHCyt, 5-OHUra, and 5-ForUra. Altogether, this work shows that optimization of the whole analytical procedure has to be achieved when a new lesion is studied. GC-MS, like any other detection technique, cannot be used as a universal method of measurement.

Relative Yield of Formation of 5-ForUra and 5-HMUra. In the following discussion, the formation of oxidized bases is discussed in terms of radiochemical yield of formation (G) (Table 1). The formation rate of the two oxidized thymine derivatives studied, namely, 5-ForUra and 5-HMUra, was inferred from GC-MS analysis. 5-ForUra was measured by GC-MS using two different hydrolysis techniques involving 88% formic acid and HF/Pyr, respectively. The data obtained by using these two approaches are similar (G: 0.0083 and 0.0077 μmol·J^{-1}, respectively). This is strongly indicative that we obtained reliable results with respect to the hydrolysis method used. For 5-HMUra, only 88% formic acid hydrolysis was performed. The G value obtained (G = 0.0042) is in good agreement with the value obtained previously by GC-MS analysis following enzymatic digestion (G = 0.0035) *(14)*. Altogether, these results show that 5-ForUra is generated in a 2.1-fold higher yield than 5-HMUra. This is in agreement with menadione mediated photosensitization experiments at the nucleoside level. 5-FordUrd was isolated in a 3.7-fold higher yield than 5-HMUra *(15)*. This is expected since both oxidizing conditions lead to the formation of the same methyl centered thymine radical which further gives rise to 5-HMUra and 5-ForUra under aerobic conditions.

Formation of 5-OHUra, 5-OHCyt, and Cytosine and Uracil Diols. The yields of 5-OHCyt and 5-OHUra in γ-irradiated DNA are highly dependent on the hydrolysis technique used. When DNA was hydrolyzed by 88% formic acid, the yield of both lesions was much higher than when milder conditions (HF/Pyr or enzymatic digestion) were used. Following formic acid hydrolysis, the G values for 5-OHCyt and 5-OHUra were 0.0069 and 0.0154, respectively. In the case of the enzymatic treatment, HPLC-EC detection was applied instead of GC-MS because internal isotopically labeled standards for the nucleosides were not available. However, the difference observed in the results cannot be explained by the detection technique used since we previously showed that, for the same hydrolysis technique, the use of either HPLC-EC or improved GC-MS led to the same level of 5-OHCyt in calf thymus DNA *(14)*. Moreover, similar G values for 5-OHCyt were obtained by GC-MS following HF/Pyr hydrolysis and HPLC-EC following enzymatic digestion (G = 0.0043 and 0.0048, respectively). This observation also applied to 5-OHUra (G = 0.0035 and 0.0035, respectively). This further supports the fact that the apparent discrepancies in the results cannot be explained by the detection methods used. These differences which are dependent on the hydrolysis conditions can be rationalized in terms of formation of 5-hydroxylated derivatives from unstable 5,6-saturated products of cytosine during the strong acidic treatment. Likely candidates for these precursors are the cytosine diols. As far as enzymatic digestion and formic acid hydrolysis are concerned, this is strongly supported by studies involving dUrd diols. The use of formic acid leads to the quantitative release of 5-OHUra whereas no decomposition of dUrd diols was observed under the conditions of enzymatic hydrolysis. Results are more ambiguous for HF/Pyr hydrolysis. When used for the hydrolysis of dUrd diols, HF/Pyr leads to the partial conversion of dUrd diols as 5-OHUra. However, the latter hydrolysis conditions also allow the release of uracil diol, as shown by HPLC and GC-MS. Therefore, it can be concluded that HF/Pyr is a milder hydrolyzing agent than hot 88% formic acid. The difference in the results between nucleoside and DNA may be accounted for by the role of the phosphate groups. The presence of the latter electron withdrawing group has been shown to decrease the hydrolysis rate in modified dinucleoside monophosphates *(26)*. The involvement of cytosine and uracil diols is also supported by other experimental data. In acidic media, cytosine diols can undergo two main transformations. One reaction is a fast deamination which leads to the corresponding uracil derivative. In addition, both cytosine and uracil diols can dehydrate, producing the related 5-hydroxylated derivatives. This is in agreement with the observation of a much higher increase in the amount of 5-OHUra by comparison to 5-OHCyt in strong acidic conditions. This is likely due to the fact that deamination is faster than dehydration. Altogether, it can be proposed that 5-OHCyt and 5-OHUra detected using mild hydrolysis conditions are produced by a direct mechanism, as proposed for cytosine by von Sonntag *(30)*, whereas the higher amount detected after 88% formic acid hydrolysis accounts for both directly produced 5-hydroxylated derivatives as well as cytosine and uracil diols. Based on the values obtained following either enzymatic digestion or HF/Pyr hydrolysis, the G values of 5-OHCyt and 5-OHUra were determined as 0.0046 and 0.0035 μmol·J^{-1}, respectively. The differences between the yields of 5-OHUra and 5-OHCyt determined using enzymatic and strong hydrolysis conditions provide an estimation of the overall yield of cytosine and uracil diols produced by γ irradiation (G = 0.0131). These observations are in agreement with semiquantitative results based on the analysis of the supernatant fraction of γ-irradiated DNA incubated with endonuclease III

where uracil diols were detected in higher amount than 5-OHCyt and 5-OHUra *(3)*.

Conclusion

The yields of formation of 5-ForUra, 5-HMUra, 5-OHUra, and 5-OHCyt obtained in this study range between those of 8-oxoGua and 8-oxoAde, which were determined by HPLC-EC to be 0.0178 and 0.0009 μmol·J^{-1}, respectively *(2)*. This suggests that the oxidized pyrimidine derivatives studied in the present work are among the main lesions induced by ionizing radiation in DNA. Work is in progress to extend the assays used in the present study to the measurement of other radiation-induced DNA base damage in order to obtain reliable values for their yields of formation.

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