Reaction of reducing hydroxyl radical adducts of pyrimidine nucleotides with riboflavin and flavin adenine dinucleotide (FAD) via electron transfer: a pulse radiolysis study

Changyuan Lu, Side Yao*, Zhenhui Han, Weizhen Lin, Wenfeng Wang, Wenlong Zhang, Nianyun Lin

Laboratory of Radiation Chemistry, Shanghai Institute of Nuclear Research, Chinese Academy of Sciences, P.O. Box 800-204, Shanghai, 201800, PR China

Abstract

Using the techniques of pulse radiolysis with time-resolved spectrophotometric detection, it has been demonstrated that the interaction of reducing OH radical adducts of dCMP, TMP and UMP with riboflavin (RF) and flavin adenine dinucleotide (FAD) does proceed via an electron transfer reaction. From buildup kinetics of radical species, the rate constants of electron transfer from reducing OH adducts of pyrimidines to RF and FAD have been determined, respectively. It could be deduced that RF and FAD would reduce the probability of repair of the damaged DNA in the presence of enzymes and antioxidants, accordingly RF and FAD might have a radiosensitization effect on DNA damage. © 2000 Elsevier Science B.V. All rights reserved.

---

* Corresponding author. Tel.: +86-21-5955-3998; fax: +86-21-5955-3021.
E-mail address: sideyao@sinr.ac.cn (S. Yao)

1. Introduction

The carcinogenic, mutagenic and lethal effect on living matter exposed to ionizing radiation is thought to be almost exclusively due to the DNA damage of the cell nucleus. The most important types of the multiple radiation-induced lesions that occur in DNA are: (1) double-strand break; (2) single-strand break; (3) chemical modification of DNA bases and sugars; and (4) inter- and intra-strand cross-linkings [1–4]. A significant contribution to DNA damage results from attack by radicals formed from ionization of the water molecules in the close vicinity of DNA. Of these radicals, the hydroxyl radical is thought to be the most important. In mammalian cells, $^\bullet$OH is reported to be responsible for approximately two-thirds of lethal events [5].

Among the radiation-induced DNA damage, hydroxyl adducts to DNA base constitute an interesting subsequent species, since they can result from both direct and indirect effects [3–10]. The direct effect, where the energy is deposited directly in the bases of DNA, leads to the formation of primary radical cations that can undergo rapid irreversible hydroxylation to the corresponding OH adduct radicals [9]. On the other hand, the indirect effect, where the energy is deposited in the water phase surrounding the DNA, results in the formation of $^\bullet$OH, $^\bullet$H and e_{aq}^- [3,4].

The interaction of the $^\bullet$OH radical with purines and pyrimidines has been widely studied under various experimental conditions. The reaction is less clearcut for purine bases than for pyrimidine bases, although OH radicals add to purines with a pronounced preference for addition to C4, C5 and C8 positions [2–11]. As indicated by ESR and the spin-trapping method, in preference to abstraction hydrogen from the sugar moiety, the $^\bullet$OH adds predominantly to the C5−C6 double bond of pyrimidine nucleotides with a pronounced preference for the C5 position (approx. 60−90%) because of the highest electron density, giving rise to the formation of C6-yl and C5-yl radicals. To thymine, a small fraction of $^\bullet$OH can abstract H from C5-methyl to give the UCH$_2^\bullet$ radical. And the site of radical addition has been shown to strongly influence the redox properties of the

resulting radicals determined by reaction with *N*, *N*, *N*′, *N*′ - tetramethyl - *p* - phenylenediamine (TMPD) or tetranitromethane (TNM), respectively. In general, C6-yl radical is reducing, and C5-yl radical is oxidizing, whereas the UCH$_2^\bullet$ radical is neither strongly reducing nor strongly oxidizing [4–8]. The reducing adduct can be rapidly converted into an oxidizing one undergoing a base-catalyzed dehydration reaction, while C5−OH and C6−OH can isomerize each other undergoing an acid-catalyzed reaction [6,10]. It has been suggested that the oxidizing adduct can be fast repaired via an electron transfer process by enzymes and antioxidants, such as thiols, hydroxycinnamic acid derivatives, to form the anion of OH adducts which can regenerate the parent molecule of bases by dehydroxycation [11,12]. Whereas the reducing adduct can be oxidized to form the cation of OH adducts by oxidants which can react with water to form final products (such as glycol) and cause DNA damage [13–16].

Riboflavin (RF) and flavin adenine dinucleotide (FAD), the most common active part of several dehydrogenases and oxidases, are important endogenous cellular photosensitizers in vivo and in vitro [17,18]. Photoexcitation of RF and FAD may potentially occur in the organs and tissues permeable to light, such as the skin or eye, make DNA and other cell-matrix components damage causing inflammation and accelerating aging. The photosensitization mechanism was not elucidated completely by our group and others until recently with time-resolved spectra and kinetics results [18–21]. Although RF and FAD are widely distributed in human tissues and fluids in free and conjugated forms, very little is known about the function of RF and FAD on DNA damage induced by ionizing radiation. However, it is important to understand further the effect of flavin in vivo and in vitro.

In the last paper, we have shown that RF and FAD, as weaker oxidants, can sensitize DNA damage induced by ionizing radiation via either directly scavenging solvated electron at close to diffusion-control rate formed from radiation-induced ionization or fast electron transfer from electron adducts of nucleotides [22]. In the present paper, with a view to further understanding

the radiosensitizing effect of RF and FAD, an attempt has been made to study the effect of RF and FAD on DNA damage caused by hydroxyl radicals. As the radiosensitivity of pyrimidines is stronger than that of purines, pyrimidine nucleotides (UMP was considered, too) were chosen as the nucleic acid model [13,16]. The results show that electrons are easily transferred from reducing hydroxyl adducts of pyrimidine nucleotides to RF and FAD, which demonstrates that RF and FAD are radiosensitive to DNA and can enhance DNA radiation damage. The radiosensitivity mechanism of RF and FAD were summarized according to direct and indirect effects of ionizing radiation.

2. Materials and methods

2.1. Materials

Riboflavin (RF) was obtained from Huamei Biochemical Co. (Shanghai, China) and used as received. Flavin adenine dinucleotide (FAD), 2′-deoxycytidine-5′-monophosphate (dCMP), thymidine-5′-monophosphate (TMP) and uridine-5′-monophosphate (UMP) as potassium salt or free acid were purchased from Sigma Chemical Co. and used without further purification. NaOH, $HClO_4$ and phosphate (analytic grade reagent) were commercially available and used as received.

Unless otherwise indicated, all solutions were freshly made with triply distilled water and protected from light at all times. The pH value of the solution was adjusted by adding NaOH, $HClO_4$ or phosphate solution. Just before the pulse radiolysis experiments, the solutions were deaerated with high purity nitrous oxide by administering for at least 20 min. All experiments were performed at room temperature ($\sim 20°C$).
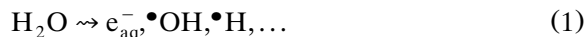
2.2. Pulse radiolysis experiments

Pulse radiolysis experiments were performed utilizing a 10-MeV linear accelerator which delivers an electron pulse with a duration of 8 ns. The analysing light beam passed perpendicularly through a 2-cm quartz cuvette. The transmitted light entered a monochromator equipped with a R955 photomultiplier. The signals were collected using an HP54510B 300-MHz transient recorder and then processed with a PC-586 personal computer. The dosimetry of the electron pulse was determined by the thiocyanate dosimeter using $G[(CNS)_2^{\bullet-}] = 6.0$ in a 10 mM KCNS solution saturated with nitrous oxide by taking $\varepsilon_{480} = 7600$ dm^3 mol^{-1} cm^{-1} for $(CNS)_2^{\bullet-}$ [23]. The details of the setup were described in a previous paper [24]. In this work, the dose per electron beam pulse was 8 Gy.
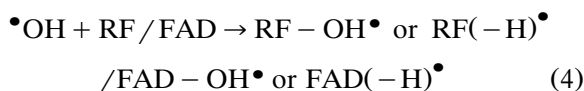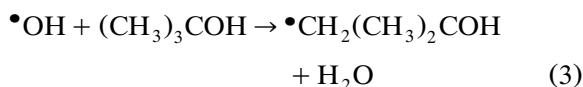
3. Results and discussion

The radiolysis of dilute aqueous solution saturated with N_2 leads to the production of three primary highly reactive intermediates (e_{aq}^-, $^{\bullet}OH$, $^{\bullet}H$) besides other less reactive molecular compounds (H_{aq}^+, H_2, H_2O_2) [23]. Under nitrous oxide saturation, e_{aq}^- can be quantitatively converted into $^{\bullet}OH$ radicals.

$$H_2O \rightsquigarrow e_{aq}^-, {}^{\bullet}OH, {}^{\bullet}H, \ldots \qquad (1)$$

$$e_{aq}^- + N_2O + H_2O \rightarrow {}^{\bullet}OH + N_2 + OH^- \qquad (2)$$

3.1. Reaction of $^{\bullet}OH$ with RF and FAD

The $^{\bullet}OH$ reaction was carried out in N_2O-saturated 0.1 mM RF (or FAD) aqueous solution buffered with 4.0 mM phosphate at pH 9. The transient absorption spectrum is displayed in Fig. 1 and would disappear while t-BuOH existed in solution, which was suggested to be from the contribution of the reaction of the OH radical with RF (or FAD). The bimolecular rate constants 1.1×10^{10} and 5.8×10^9 dm^3 mol^{-1} s^{-1} for the reaction of $^{\bullet}OH$ with RF and FAD, respectively, were derived from the first-order build-up of the transient absorbance observed at 570 nm or bleaching at 440 nm, which were in good agreement with the values reported earlier based on steady state radiolytic competition kinetic method within the limits of experimental error

[25–27]. Based on the conductivity and time-resolved spectra, the mechanism of •OH addition or abstraction hydrogen from the flavin moiety by OH radical has been suggested [25–27].

$$•OH + (CH_3)_3COH \rightarrow •CH_2(CH_3)_2COH$$
$$+ H_2O \qquad (3)$$

$$•OH + RF/FAD \rightarrow RF-OH^• \; or \; RF(-H)^•$$
$$/FAD-OH^• \; or \; FAD(-H)^• \qquad (4)$$

3.2. Reaction of reducing OH adducts of pyrimidine with RF and FAD

Fig. 2a,b shows the transient absorption spectra after pulse radiolysis of an N_2O-saturated aqueous solution containing 4.0 mM dCMP, 4.0 mM phosphate and 0.1 mM RF and FAD at pH 9, respectively. The reaction rate constants of the OH radical with dCMP, TMP and UMP have been determined to be 5.0×10^9, 5.2×10^9 and $4.5 \times 10^9 \; dm^3 \; mol^{-1} \; s^{-1}$, respectively [23,28]. According to the competition reaction, the fraction of OH radicals which react with dCMP, TMP and UMP was expected to be $> 98\%$ for 4.0 mM pyrimidine aqueous solutions containing 0.1 mM
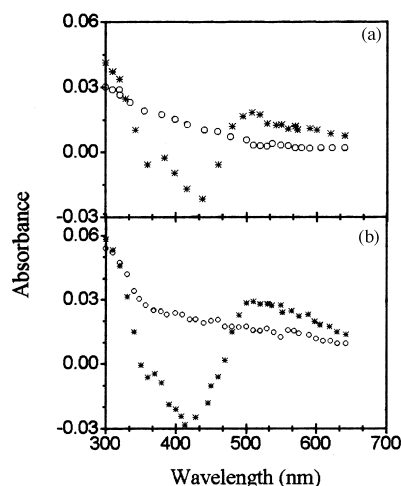

Fig. 2. Transient absorption spectra obtained in the pulse radiolysis of 4.0 mM dCMP and 0.1 mM (a) RF, (b) FAD aqueous solution saturated with N_2O at pH 9 recorded at (○) 1.0 μs, (*) 30.0 μs, respectively.

RF or FAD, respectively. So the transient absorption spectra recorded at 1.0 μs after the electron pulse stem predominately from OH adducts of dCMP (dCMP—OH•). Following rapid decay of dCMP—OH• absorbance, a concomitant increase of absorbance at 520 nm with a bleaching at 440 nm has been observed. The transient absorption spectra are very similar to that of the
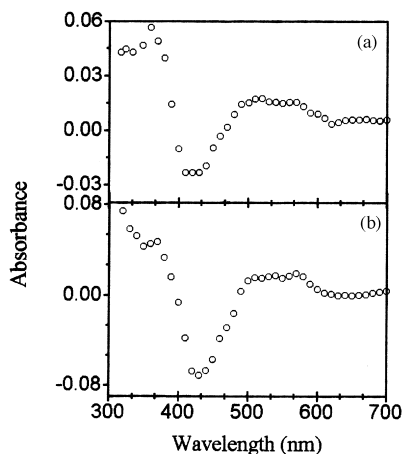

Fig. 1. Transient absorption spectra obtained in the pulse radiolysis of N_2O-saturated aqueous solution containing 0.1 mM (a) RF, (b) FAD buffered with 4.0 mM phosphate at pH 9 recorded 5.0 μs after the electron beam pulse.
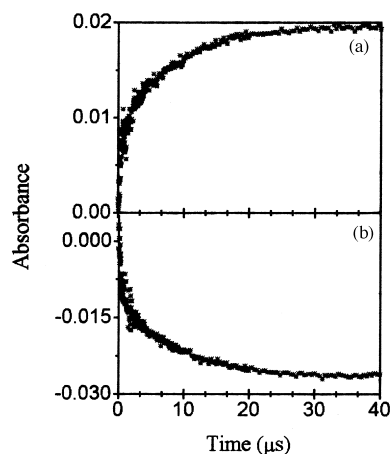

Fig. 3.. (a) Growth trace of the transient absorbance observed at 520 nm and (b) bleaching curve recorded at 440 nm obtained from pulse radiolysis of N_2O saturated containing 4.0 mM dCMP and 0.1 mM RF aqueous solution at pH 9.

Table 1
The rate constants (dm^3 mol^{-1} s^{-1}) for interaction of RF or FAD with reducing OH radical adducts of pyrimidines

Substrate	[dCMP—OH]$^{\bullet}_{red}$	[TMP—OH]$^{\bullet}_{red}$	[UMP—OH]$^{\bullet}_{red}$
RF	1.3×10^9	1.9×10^9	1.7×10^9
FAD	6.8×10^8	8.2×10^8	5.3×10^8

radical anion of RF or FAD (pK_a = 8.3), respectively [22,29], which arose from an electron transfer reaction from reducing OH adducts to RF or FAD.

Fig. 3 shows the trace curves of absorbance observed at 520 and 440 nm, which are mainly attributed to the radical anion of RF (formation and bleaching). Based on synchronization between two curves, it is obvious that electron transfer from reducing OH adducts of dCMP to RF does occur. By varying RF or FAD concentrations (concentrations in the range 0.02–0.15 mM), the rate constant for the electron transfer has been determined from the pseudo-first-order formation kinetics observed at 520 nm or bleaching observed at 440 nm in N$_2$O-saturated solution (see Table 1).

The electron transfer reaction from reducing OH adducts of TMP or UMP to RF and FAD has also been studied in the same way and is shown in
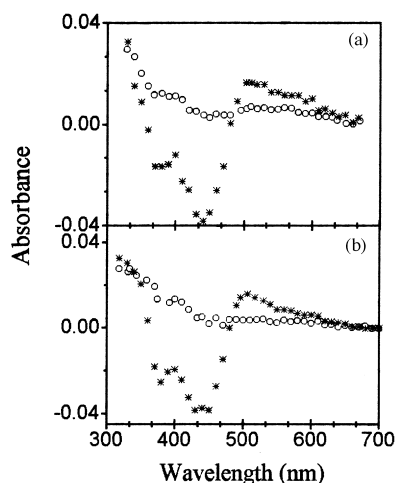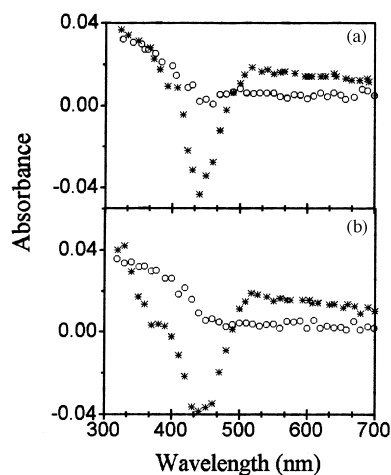

Fig. 5. Transient absorption spectra obtained in the pulse radiolysis of 4.0 mM UMP and 0.1 mM (a) RF, (b) FAD aqueous solution saturated with N$_2$O at pH 9 recorded at (○) 1.0 μs, (*) 30.0 μs, respectively.


Fig. 4. Transient absorption spectra obtained in the pulse radiolysis of 4.0 mM TMP and 0.1 mM (a) RF, (b) FAD aqueous solution saturated with N$_2$O at pH 9 recorded at (○) 1.0 μs, (*) 30.0 μs, respectively.

Figs. 4 and 5, respectively. As in Fig. 2, following the disappearance of the spectrum of OH adducts of TMP or UMP the characteristic spectrum of the radical anions of RF or FAD formed in a few microseconds after beam pulse. From the pseudo-first-order formation kinetics observed at 520 nm or bleaching at 440 nm depending linearly on RF and FAD concentrations, the rate constants determined have been listed in Table 1. Furthermore, with the system addition of t-BuOH, no detectable transient absorbance has been observed, which means that electron transfer from reducing OH adducts of pyrimidine to RF and FAD must take place.

3.3. Molecular mechanism of electron transfer and radiosensitivity

The OH radicals produced via the radiolysis of water react with pyrimidines at the C5–C6 double
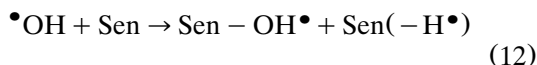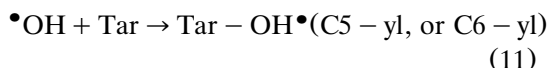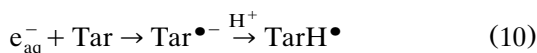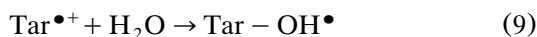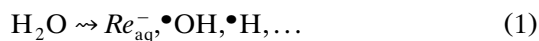
$$(5)$$
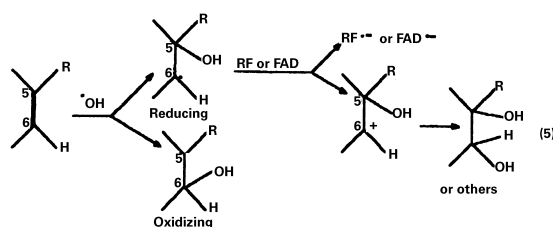
bond to form reducing or oxidizing hydroxyl adduct radicals. The reducing hydroxyl adducts could be one-electron oxidized by electron-affinic RF or FAD to form the cation of hydroxyl adducts, which is too short-lived to be monitored and can be converted into pyrimidine glycol by hydrolytic OH^- incorporation [14,30]. Recently, the result of HPLC in our laboratory confirmed that there is an increase in the formation of thymine glycol, an indicator of oxidative damage, upon RF (or FAD)-mediated thymine aqueous solution under γ irradiation.

If the experiments are carried out in a nearly acid environment, the reaction yield of the electron transfer is higher. Under these conditions, C5—OH and C6—OH can undergo acid-catalyzed isomerization [10]. Then the OH adducts can be oxidized or reduced depending on the reaction condition. Hence, in the presence of oxidants, the oxidizing C6—OH will be isomerized to reducing C5—OH and thereby increase the reaction yield [4,10].


$$(6)$$
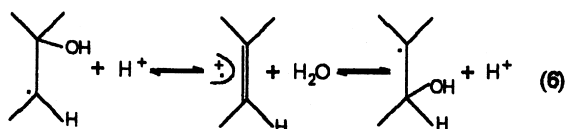
In combination with previous papers [20−22], the radiosensitization mechanism of RF and FAD (Sen) to DNA (Tar) can be summarized from direct and indirect effects at the molecular level:

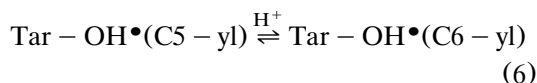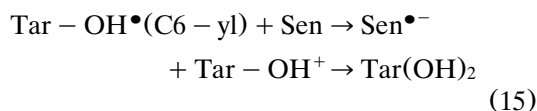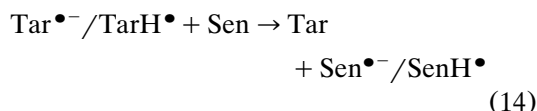1. the primary radiation process [16];

$$H_2O \rightsquigarrow Re^-_{aq}, {}^\bullet OH, {}^\bullet H, \ldots \tag{1}$$

$$Tar \rightsquigarrow e^-_{aq} + Tar^{\bullet +} \tag{7}$$

$$Sen \rightsquigarrow e^-_{aq} + Sen^{\bullet +} \rightarrow Sen(-H)^\bullet \tag{8}$$

$$Tar^{\bullet +} + H_2O \rightarrow Tar - OH^\bullet \tag{9}$$

$$e^-_{aq} + Tar \rightarrow Tar^{\bullet -} \xrightarrow{H^+} TarH^\bullet \tag{10}$$

$${}^\bullet OH + Tar \rightarrow Tar - OH^\bullet (C5 - yl, \text{ or } C6 - yl) \tag{11}$$

$${}^\bullet OH + Sen \rightarrow Sen - OH^\bullet + Sen(-H^\bullet) \tag{12}$$

2. The sensitivity process

a. direct scavenging of the solvated electron [22]

$$e^-_{sol} + Sen \rightarrow Sen^{\bullet -} \xrightarrow{H^+} SenH^\bullet \tag{13}$$
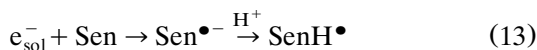
b. oxidation of target radicals (radical anion, reducing OH adducts of target) via electron transfer [22]

$$Tar^{\bullet -}/TarH^\bullet + Sen \rightarrow Tar + Sen^{\bullet -}/SenH^\bullet \tag{14}$$

$$Tar - OH^\bullet (C6 - yl) + Sen \rightarrow Sen^{\bullet -} + Tar - OH^+ \rightarrow Tar(OH)_2 \tag{15}$$

$$Tar - OH^\bullet (C5 - yl) \xrightleftharpoons{H^+} Tar - OH^\bullet (C6 - yl) \tag{6}$$

The direct effect of ionizing radiation (the net results of Eqs. (7) and (10)) is to produce positive (hole) and negative centers. The electron-affinic RF and FAD serve as electron sinks to prevent recombination reactions between centers, thus enhancing DNA damage [22].

c. The oxidation of target or antioxidant (AO) molecules by sensitizer's radicals produced

from the primary process of ionizing radiation (see Eqs. (8) and (12)) [20,21]. The redox potential of flavin is approximately 2.3 V [25], which is higher than those of biomolecules and antioxidants. The repair of target damage can be inhibited by reaction of antioxidants with the sensitizer's radicals.

$$Sen(-H)^{\bullet} + Tar/AO \rightarrow Sen + Tar^{\bullet+}/AO^{\bullet+} \tag{16}$$
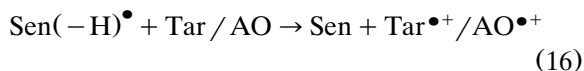
$$\rightarrow DNA\ damage \tag{17}$$

It is noted that these ways complement each other to sensitize DNA damage.

4. Conclusion

Our experimental results have demonstrated that the reducing OH radical adducts of pyrimidine: dCMP, TMP and UMP can be oxidized to form cations of OH adducts (carbocation) by riboflavin (RF) and flavin adenine dinucleotide (FAD) via electron transfer reaction. Then the carbocation can be converted into final decomposition products. Riboflavin and FAD which exert an oxygen-mimic effect, have been expected for the first time to have significant radiosensitivity on DNA of hypoxic cells. A kinetic study of the electron transfer reaction between riboflavin, FAD and DNA base radicals induced by ionizing radiation has provided novel confirmations as well as development of charge transfer sensitization mechanisms [16].

Riboflavin, FAD and other flavin compounds have been suggested to photosensitize efficiently an oxygen-deficient cell [18–21], the application potency of tumor therapy would be extended if the photosensitizing and radiosensitizing abilities of riboflavin and FAD were combined. To further elucidate the sensitive effect of flavin on DNA

radiation damage, especially in tumor cells, steady and biomedical experiments are needed.

References

[1] A.O. Colson, M.D. Sevilla, Int. J. Radiat. Biol. 67 (1995) 627–645.
[2] J. Cadet, M. Berger, Int. J. Radiat. Biol. 47 (1985) 127–143.
[3] S. Steenken, Chem. Rev. 89 (1989) 503–520.
[4] C. von Sonntag, H.P. Schuchmann, Int. J. Radiat. Biol. 49 (1986) 1–34, and references therein.
[5] P. Wardman, Radiat. Phys. Chem. 30 (1987) 423–432.
[6] C. von Sonntag, Radiat. Phys. Chem. 30 (1987) 313–330.
[7] S. Fujita, S. Steenken, J. Am. Chem. Soc. 103 (1981) 2540–2545.
[8] D.K. Hazra, S. Steenken, J. Am. Chem. Soc. 105 (1983) 4380–4386.
[9] M.S. Sevilla, M.L. Engelhardt, Chem. Soc. Faraday Discuss. 63 (1978) 255–263.
[10] M. Al-Sheikhly, C. von Sonntag, Z. Naturforsch. 38b (1983) 1622–1629.
[11] W.F. Wang, L. Jian, Z.H. Zuo et al., Radiat. Phys. Chem. 46 (1995) 41–45.
[12] W.Y. Li, Z.H. Zuo, R.L. Zheng et al., Radiat. Phys. Chem. 49 (1997) 429–432.
[13] V.T. Kagiya, S.I. Nishimoto, Free Rad. Res. Commun. 6 (1989) 191–194.
[14] M.M.M. Bamatraf, P. O'Neill, B.S.M. Rao, J. Am. Chem. Soc. 120 (1998) 11852–11857.
[15] V.T. Kagiya, K. Sakano, S.I. Nishimoto, Radiat. Phys. Chem. 29 (1987) 451–454.
[16] N.Y. Lin, Radiat. Phys. Chem. 28 (1986) 211–217.
[17] P.F. Heelis, Chem. Soc. Rev. 11 (1982) 15–39.
[18] H. Kasai, Z. Yamaizumi, M. Berger, J. Cadet, J. Am. Chem. Soc. 114 (1992) 9692–9694.
[19] K. Ito, S. Inoue, K. Yamamoto, S. Kawanish, J. Biol. Chem. 268 (1993) 13221–13227.
[20] C.Y. Lu, W.F. Wang, W.Z. Lin, Z.H. Han, S.D. Yao, N.Y. Lin, J. Photochem. Photobiol. B: Biol. 52 (1999) 111–116.
[21] C.Y. Lu, W.Z. Lin, W.F. Wang, Z.H. Han, S.D. Yao, N.Y. Lin, Phys. Chem. Chem. Phys. 2 (2000) 329–335.
[22] C.Y. Lu, S.D. Yao, W.Z. Lin, W.F. Wang, Z.H. Han, Z.D. Zheng, Radiat. Phys. Chem. (2000) in press.
[23] G.V. Buxton, C.L. Greenstock, W.P. Helman, A.B. Ross, J. Phys. Chem. Ref. Data. 17 (1988) 513–890, and references therein.
[24] S.D. Yao, S.G. Sheng, J.H. Cai, J.S. Zhang, N.Y. Lin, Radiat. Phys. Chem. 46 (1995) 105–110.

[25] K. Kishore, P.N. Moorthy, S.N. Guha, Radiat. Phys. Chem. 38 (1991) 119−125.

[26] K. Kishore, P.N. Moorthy, K.N. Rao, Radiat. Phys. Chem. 4 (1982) 241−245.

[27] A. Saha, P.C. Mandal, S.N. Bhattacharyya, Bull. Chem. Soc. Jpn. 64 (1991) 2532−2538.

[28] H.B. Michaels, J.W. Hunt, Radiat. Res. 58 (1973) 57−70.

[29] E.J. Land, A.J. Swallow, Biochemistry 8 (1969) 2117−2125.

[30] K.D. Asmus, D.J. Deeble, A. Garner, K.M. Idriss Ali, G. Scholes, Br. J. Cancer Suppl. 37 (1978) 46−49.