The spin trapping of pyrimidine nucleotide free radicals in a Fenton system

W. D. FLITTER* and Ronald P. MASON

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709, U.S.A.

The reaction of the hydroxyl radical, generated by a Fenton system, with pyrimidine deoxyribonucleotides was investigated by using the e.s.r. technique of spin trapping. The spin trap t-nitrosobutane was employed to trap secondary radicals formed by the reaction of the hydroxyl radical with these nucleotides. The results presented here show that hydroxyl-radical attack on thymidine, 2-deoxycytidine 5-monophosphate and 2-deoxyuridine 5-monophosphate produced nucleotide-derived free radicals. The results indicate that $\cdot\text{OH}$ radical attack occurs predominantly at the carbon–carbon double bond of the pyrimidine base. The e.s.r. studies showed a good correlation with previous results obtained by authors who used x- or γ -ray irradiation to generate the hydroxyl radical. A thiobarbituric acid assay was also used to monitor the damage produced to the nucleotides by the Fenton system. These results showed qualitative agreement with the spin-trapping studies.

INTRODUCTION

In cells exposed to ionizing radiation it is the DNA that appears to be the main target for the observed lethal and mutagenic effects [1]. It has been proposed that the hydroxyl radical ($\cdot\text{OH}$) plays a major role in the indirect action of radiation on cells.

$\cdot\text{OH}$ is produced when water is exposed to high-energy ionizing radiation. Its properties have been well documented by radiation chemists (for a review, see [2]). $\cdot\text{OH}$ is highly reactive and, as such, will react with any molecule close to its site of formation. The nature of the damage produced by $\cdot\text{OH}$ *in vivo* would, therefore, depend upon its site of formation. For example, if $\cdot\text{OH}$ production occurs close to DNA, it could cause strand breakage or base modification [3].

Most of the $\cdot\text{OH}$ generated *in vivo* would come from the metal ion-dependent breakdown of H_2O_2 according to the general equation:

in which M^{n+} is an unidentified endogenous metal ion. This metal ion could be titanium(III), copper(I), iron (II) or cobalt(II). Realistically only the iron(II) and copper(I) reactions could occur *in vivo*, and these would probably only occur under abnormal physiological conditions. Considerable attention has been focused on the iron-dependent decomposition of H_2O_2 , the so-called 'Fenton reaction'. This reaction is generally written:

This is, in fact, an oversimplification; for a review of the reactions involved, see [4].

Several authors have recently suggested that two separate oxidizing species are produced by the action of

H_2O_2 on iron chelates [5–8]. Koppenol has proposed that iron(II) and H_2O_2 produce a ferryl iron or ferrous H_2O_2 complex [6]. Rush & Koppenol [7] proposed that the reaction between H_2O_2 and ferrous EDTA produces a reactive intermediate which is not $\cdot\text{OH}$, but suggested that it is a ferryl–EDTA complex. This intermediate can undergo further reaction with H_2O_2 to produce an oxidizing species that is claimed to be $\cdot\text{OH}$.

Winterbourn [8] claimed that when ferrous iron is chelated with EDTA, the predominant species formed is $\cdot\text{OH}$, whereas with non-chelated iron a quadrivalent iron complex is formed which is capable of oxidizing ribose. She proposed that iron(II)–EDTA and H_2O_2 produce an iron species and $\cdot\text{OH}$. She further proposed that, with non-chelated iron and H_2O_2 , $\cdot\text{OH}$ production is thermodynamically unfavourable and that the strong oxidant is Fe(IV) .

Youngman & Elstner [9] have proposed that a 'crypto' $\cdot\text{OH}$ could be formed in some systems *in vitro*. Their initial studies used paraquat radicals and H_2O_2 to produce a highly reactive species that was not $\cdot\text{OH}$. Youngman [10] has proposed that the crypto $\cdot\text{OH}$ could be the formation and localized reaction of 'real' $\cdot\text{OH}$, which is also called 'site-specific $\cdot\text{OH}$ formation'.

With this apparent controversy in the literature, exactly what radical products would be formed when deoxyribonucleotides were incubated with a Fenton system was not entirely clear. If the Fenton system acts as a radiomimetic system, then the radical products should be the same as those obtained in radiation studies. If the Fenton system produces other reactive species apart from $\cdot\text{OH}$, then it would be anticipated that the radical products would be different. Here we report the identity of the radical species formed when deoxyribonucleotides were incubated with a Fenton system. The work of radiation chemists has shown that the radical species formed by $\cdot\text{OH}$ attack on DNA or its constituent

Abbreviations used: tNB, t-nitrosobutane; $\cdot\text{OH}$, hydroxyl radical; TBA, 2-thiobarbituric acid.

* Present address and address for correspondence and reprint requests: Department of Biochemistry, Brunel University, Uxbridge UB8 3PH, Middx., U.K.

nucleotides have very short lifetimes. These radicals have been studied by direct e.s.r. spectroscopy using γ -ray radiolysis in the e.s.r. cavity [11], flow techniques using a Ti^{3+} - H_2O_2 $\cdot\text{OH}$ -generating system [12,13] and photolysis of H_2O_2 [14].

More recently the technique of spin trapping has been utilized to try to identify the radicals by $\cdot\text{OH}$ attack on DNA or its constituents. In this technique the short-lived radicals ($\text{R}\cdot$) are generated in a system containing a spin trap. Nitroso and nitron compounds are commonly used as spin traps, and the resultant spin adduct is a relatively stable nitroxide radical which can be conveniently identified by e.s.r. spectroscopy [15]. In the present study t-nitrosobutane (tNB) was used as a spin trap. The general reaction of a radical ($\text{R}\cdot$) with tNB can be written:

The main drawback with the spin-trapping technique is the fact that the relative concentrations of spin-trapped radicals do not reflect the relative concentrations of the initial radicals [16].

Recent work by Kuwabara *et al.* [17] and previous work by Riesz and co-workers [16] has demonstrated that γ -irradiation of DNA and nucleotide solutions produced radical species which could be trapped by tNB.

The 2-thiobarbituric acid (TBA) assay has been widely used to detect oxidative damage in lipids for the last 40 years. More recently it has been applied to more complex biological materials to detect oxidative changes that may take place via non-enzymic free-radical reactions [18]. The TBA assay is now extensively used to detect free-radical-induced damage in systems *in vitro*, with deoxyribose as the detector molecule of choice. $\cdot\text{OH}$ attack on 2-deoxyribose produces a product that, on heating at an acidic pH with TBA, forms a chromogen that absorbs strongly at 532 nm.

In this study we used the TBA assay in conjunction with the e.s.r. technique of spin trapping to study the free-radical damage to deoxyribonucleotides in a Fenton system. Spin trapping permits the identification of at least some of the radicals formed when deoxyribonucleotides are exposed to an iron-dependent $\cdot\text{OH}$ -generating system, whereas the TBA assay is an index of the reaction of $\cdot\text{OH}$ with the 2-deoxyribose component of the nucleotides.

MATERIALS AND METHODS

2-Deoxycytidine 5-monophosphate, 2-deoxythymidine 5-monophosphate (thymidine), 2-deoxyuridine 5-monophosphate (all sodium salts), EDTA disodium salt, FeCl_2 , H_2O_2 (30% solution), tNB and TBA were all purchased from Sigma Chemical Co. and used without further purification. Sodium formate was obtained from Aldrich Chemical Co.

Solutions of tNB were dissolved in a 0.075 M-phosphate buffer, pH 7.4, by stirring overnight in the dark. Solutions were adjusted to a concentration of 2.5 mg/ml (29 mM) and protected from the light.

For the spin-trapping experiments, FeCl_2 (0.1 M) and EDTA (0.2 M) solutions were made with deionized water; both solutions were freshly prepared. Nucleotides (0.2 M)

in 0.075 M-phosphate buffer were prepared immediately before the start of the experiment. The H_2O_2 solution was diluted 1:10 with deionized water.

For TBA assays, FeCl_2 (3 mM) and EDTA (3 mM) solutions were made with deionized water; the H_2O_2 was diluted 1:100 with deionized water.

E.s.r. spectroscopy

ESR spectra were recorded with a Varian E-109 spectrometer using a flat cell for aqueous solutions mounted in a TM_{110} cavity. A modulation frequency of 100 kHz was used, with a scan range of 8 mT, a scan time of 4 min and a time constant of 0.5 s. All experiments were performed at room temperature.

Typical reaction mixtures contained, in the following order, 1.25 ml of the stock solution of the nucleotide under investigation, 17 μl of EDTA, 32 μl of $\text{Fe}(\text{II})$ (as FeCl_2), 750 μl of tNB, 3 μl of H_2O_2 stock solutions and 3.16 ml of 0.075 M-phosphate buffer. Samples were aspirated into the flat cell by using a Gilson rapid sampler [19] immediately after the addition of the H_2O_2 .

TBA assays

TBA solutions were made to a final stock concentration of 1% (w/v) in 0.05 M-NaOH. The HCl stock concentration was 2.8% (v/v). Typical reaction mixtures contained stock solutions as follows: 480 μl of nucleotide, 400 μl of EDTA, 400 μl of $\text{Fe}(\text{II})$, 400 μl of tNB, 10 μl of H_2O_2 and 0.075 M-phosphate buffer to 2 ml. Sodium formate, when used, was at a stock concentration of 666 mM; 300 μl of this solution was added. Reaction mixtures were prepared in new glass screwtop vials. The reaction mixtures were allowed to react for 4 min at room temperature before adding 1 ml of the TBA solution and 0.5 ml of the HCl stock solution. Reaction vials were then placed in a heating block and heated at 100 $^\circ\text{C}$ for 15 min. The samples were then allowed to cool before reading their absorbances at 532 nm against a water blank.

RESULTS AND DISCUSSION

When a radical is spin-trapped, the site of the unpaired electron shifts from the parent radical to the nitroxide group of the radical adduct. This results in some loss of structural information about the parent radical, as most of the free electron spin density is localized on the nitrogen and oxygen of the nitroxide group. The free electron interacts with the nitrogen of the nitroxide and generates a primary triplet splitting. In the present study the nitrogen hyperfine coupling is between 1.4 mT and 1.6 mT as is typical of carbon-centred radicals [20]. Identification of the trapped radical depends on the hyperfine structure within each triplet, which is due to the interaction of the electron localized on the nitroxide with the nuclei of the trapped parent radical.

Fig. 1(a) shows the e.s.r. spectrum obtained when thymidine was exposed to a Fenton system in the presence of the spin trap tNB. The spectrum shown in Fig. 1 is the result of two different radical species, two thymidine-radical adducts. Attack of the $\cdot\text{OH}$ radical on thymidine produced two separate radical species that can be detected by spin trapping with tNB. Fig. 1(b) shows the computer simulation of the spectrum due to the two species. Figs. 1(c)–1(d) show the computer simulations of the two species. Fig. 1(c) shows a spectrum which is a triplet of

Fig. 1. Spectral simulation of the thymidine radical adducts

(a) Experimental e.s.r. spectrum of 48 mM-thymidine in the presence of 600 μ M-EDTA, 600 μ M-Fe(II), 2.8 mM-tNB and 600 μ M-H₂O₂. E.s.r. parameters: modulation amplitude, 0.107 mT; receiver gain, 1.6×10^3 ; time constant, 0.5 s; microwave power, 20 mW. (b) Composite simulation of the thymidine radical spectrum. (c) Thymidine species 1: $a^N = 0.149$ mT, $a^{\beta} = 0.26$ mT, a linewidth of 0.11 mT and a relative concentration of 21.8. (d) Thymidine species 2: $a^N = 0.162$ mT, linewidth of 0.0865 mT, a relative concentration of 78.1, and a g -shift of +0.029 mT.

triplets, with a primary triplet of 1.49 mT and a secondary triplet of 0.26 mT. The smaller secondary splitting can be explained by the interaction of a spin with a nitrogen at the β position. The α , β and γ positions are defined with relation to the unpaired electron on the nitrogen of the nitroxide group [21] as shown diagrammatically below.

A probable assignment for the species responsible for the spectrum shown in Fig. 1(c) would be for the spin

trapping of a carbon-centred radical at the C-6 position, this radical being formed by $\cdot\text{OH}$ attack at the C-5 position.

The assignment of this radical structure for the spectrum in Fig. 1(c) is not entirely straightforward, as various assignments have been made by different authors. Joshi *et al.* [16] assigned this spectrum to a radical centred at N-3 of the base. Kominami *et al.* [24] assigned it to the N-1- or N-3-centred radical, whereas Kuwabara *et al.* [25] assigned it to N-3 or C-6. The C-6 assignment (structure I) was less tenable, as no hyperfine structure for the β -proton at C-6 was evident in the spectrum. However, recent work by Kuwabara *et al.* [26] would seem to indicate that the C-6 assignment is correct, and previous analytical studies by Téoule & Cadet [23] had demonstrated that the end products produced by irradiation of thymidine were formed via a C-6-centred radical.

The spectrum shown in Fig. 1(d) is a triplet of 1.62 mT without any resolved β -hyperfine splittings from hydrogen or nitrogen. The proposed structure is shown in structure (II). Any other structure should give rise to hyperfine splitting from an adjacent atom [22]. This species would be formed by $\cdot\text{OH}$ attack on the C-6 of the carbon-carbon double bond, forming a carbon-centred free radical at C-5. Confirmation of these assignments can be taken from the fact that Téoule & Cadet (23) have shown that the preferred site for $\cdot\text{OH}$ attack on the pyrimidines is at the C-5=C-6 double bond.

Fig. 2. E.s.r. spectrum of thymidine radical adducts with the experimental controls

- (a) Experimental spectrum of the thymidine radical, as in Fig. 1(a). (b) Same as (a), but no thymidine (phosphate buffer replacing nucleotide). (c) Same as (a), but no EDTA. (d) Same as (a), but no iron. (e) Same as (a), but no tNB. (f) Same as (a), but no H_2O_2 .

$\cdot\text{OH}$ attack at either C-5 or C-6 can lead to the formation *in vivo* of thymine glycol [27]. Fig. 2(b) shows that the signal is entirely dependent upon the presence of the nucleotide. A signal of reduced intensity can be obtained in the absence of the iron chelator EDTA (Fig. 2c). EDTA is added to '*in vitro*' Fenton systems for several reasons. EDTA binds iron very tightly, pulling iron away from low-molecular-mass biological complexes [28], but the iron-EDTA chelates are still very effective catalysts of free $\cdot\text{OH}$ production [29].

Iron can bind to a wide range of ligands, including nucleotides [30], DNA and membrane lipids [31]. All these ligands are capable of supporting $\cdot\text{OH}$ production. It would seem likely that the $\cdot\text{OH}$ formed in this way would attack the ligand binding the metal ion. Indeed this has brought forward the concept of site-specific damage. Although there is some evidence to support this concept with copper ions [32,33], there is little direct

Fig. 3. Spectral simulation of the dCMP radical adducts

- (a) Experimental e.s.r. spectrum of 48 mM-dCMP in the presence of 600 μM -EDTA, 600 μM -Fe(II), 2.8 mM-tNB and 600 μM - H_2O_2 . E.s.r. parameters: modulation amplitude, 0.107 mT, receiver gain, 4×10^4 ; time constant, 0.5 s; microwave power, 20 mW. (b) Composite simulation of the dCMP radical. (c) 2-Deoxycytidine species 2: $a^{\text{N}} = 0.154$ mT, $a_{\beta}^{\text{N}} = 0.09$ mT, $a_{\beta}^{\text{H}} = 0.555$ mT, a linewidth of 0.098 mT, and a relative concentration of 77. (d) 2-Deoxycytidine species 1: $a^{\text{N}} = 0.154$ mT, $a_{\beta}^{\text{H}} = 0.44$ mT, linewidth of 0.098 mT, a relative concentration of 23, and a g -shift of +0.027 mT.

evidence to support the same idea with iron ions. The idea that an iron-EDTA system would serve as a radiomimetic system has been postulated by Gutteridge [34]. In the system used here, the iron-EDTA should serve as a radiomimetic system, producing free $\cdot\text{OH}$. This explains why our results are so consistent with the radiation results. In the absence of EDTA one might expect the $\cdot\text{OH}$ generation to be site-specific; that is, the radical would be formed at the site of the iron-binding ligand. This, in turn, would suggest that it would be difficult to detect this radical with the spin-trapping technique and that the radical species produced could be different from that produced by the 'free' $\cdot\text{OH}$ generating systems. However, this does not appear to be the case. Indeed, the only difference between the signals in Figs.

Fig. 4. E.s.r. spectrum of dCMP radical adducts with the experimental controls

(a) Experimental spectrum of dCMP as in Fig. 3(a). (b) Same as (a), but no cytidine. (c) Same as (a), but no EDTA. (d) Same as (a), but no iron. (e) Same as (a), but no spin trap (tNB). (f) Same as (a), but no H_2O_2 .

2(a) and 2(c) is the signal intensity. This implies that either the radical produced by the site-specific generation of $\cdot\text{OH}$ is the same as that produced by free $\cdot\text{OH}$, or that free $\cdot\text{OH}$ is still being produced in this system. It has been proposed that nitrosoalkanes can act as ligands with Fe(II). Mansuy *et al.* [35] have shown that primary and secondary aliphatic nitroso compounds can form complexes with haemoglobin and myoglobin as well as iron(II)-porphyrins, and suggest that the bond is formed between the iron centre and the nitroso group of the nitrosoalkanes. This could suggest that the spin trap (a tertiary nitrosoalkane) is binding the iron in such a way that it can still catalyse the production of the $\cdot\text{OH}$ from H_2O_2 .

Fig. 2(d) shows the spectrum produced in the absence of added iron. The weak background signal is due to the

Fig. 5. Spectral simulation of the dUMP radical adducts

(a) Experimental e.s.r. spectrum of 48 mM-dUMP in the presence of 600 μM -EDTA, 600 μM -Fe(II), 2.8 mM-tNB and 600 μM - H_2O_2 . E.s.r. parameters: modulation amplitude 0.107 mT; receiver gain, 5×10^3 ; time constant, 0.5 s, microwave power, 20 mW. (b) Composite spectral simulation of dUMP. (c) dUMP species 1: $a^{\text{N}} = 0.154$ mT, $a_{\beta}^{\text{N}} = 0.106$ mT, $a_{\beta}^{\text{H}} = 0.4$ mT, linewidth of 0.1 mT, and a relative concentration of 71.4. (d) dUMP species 2: $a^{\text{N}} = 1.54$ mT, $a_{\beta}^{\text{H}} = 0.340$ mT, linewidth of 0.088 mT, a relative concentration of 28.5 and a g -shift of +0.025 mT.

adventitious transition-metal ions present in the reaction mixture. The spectrum in Fig. 2(e) shows that a signal cannot be obtained in the absence of the spin trap. Fig. 2(f) shows a signal of decreased intensity in the absence of added H_2O_2 . In this case some H_2O_2 is presumably produced via autooxidation of the ferrous iron. EDTA is known to accelerate the oxidation of iron(II) to iron (III) [36,37].

Fig. 3(a) shows the e.s.r. spectrum generated when 2-deoxycytidine 5-monophosphate is incubated with a Fenton system in the presence of the tNB. The spectrum is a triplet of doublets, with a primary nitrogen triplet and a secondary hydrogen splitting. Close inspection of Fig. 3(a) will reveal that the cytidine spectrum is,

in fact, a composite of two species of 2-deoxycytidine 5-monophosphate. The two radical adducts produce spectra with nearly identical nitroxide nitrogen hyperfine couplings. Fig. 3(b) shows the composite simulation of the 2-deoxycytidine 5-monophosphate spectrum. Fig. 3(c) shows the simulated spectrum of a radical species with a large triplet of 1.54 mT, a small unresolved triplet of 0.09 mT and a doublet of 0.55 mT. This spectrum would be consistent with radical formation at the C-6 position of the base, with the primary triplet formed by trapping a carbon-centred radical, the smaller triplet coming from the β -nitrogen at N-1 of the base, and the secondary doublet coming from the β -hydrogen. Such a radical would have the structure shown in (III)

Fig. 3(d) shows the simulation of a species with a primary splitting of 1.56 mT and a hydrogen splitting of 0.44 mT. This spectrum would be consistent with that produced by a radical spin trapped at the C-5 position of the base moiety; this radical is shown in structure (IV). Such an assignment has also been reported by Kuwabara *et al.* [25]. Joshi *et al.* [16] also proposed that $\cdot\text{OH}$ addition occurred at the C-6 position of the double bond of the base.

As Fig. 4 shows, the signal is entirely dependent on the presence of the nucleotide. In the absence of EDTA, a decreased signal is produced once again, suggesting that

Fig. 6. E.s.r. spectrum of dUMP radical adducts with the experimental controls

(a) Experimental spectrum of dUMP; conditions were as in Fig. 5(a). (b) Same as (a), but no uridine. (c) Same as (a), but no EDTA. (d) Same as (a), but no iron. (e) Same as (a), but no tNB. (f) Same as (a), but no H_2O_2 .

either the spin trap tNB is binding the iron in such a way that it can undergo Fenton chemistry or that the radical formed by the site-specific production of $\cdot\text{OH}$ is the same as that produced by free $\cdot\text{OH}$ attack on the nucleotide. No signal is seen in the absence of added iron, nor is there a signal in the absence of the spin trap. A weaker spectrum is found in the absence of added H_2O_2 . This is the same triplet of doublets seen with the complete system, although at a much reduced intensity (Fig. 4f). Once again, the H_2O_2 is presumably produced via the autoxidation of the Fe(II), a process that is accelerated in the presence of EDTA.

Fig. 5(a) shows the e.s.r. spectrum produced when 2-deoxyuridine 5-monophosphate was incubated with a Fenton system in the presence of tNB. The spectrum shown in Fig. 5(a) can be attributed to two 2-deoxyuridine 5-monophosphate radicals. Both species have a nitroxide splitting of 1.54 mT, but different β -hyperfine splittings, and one species has an additional nitrogen splitting (Fig. 5c). This was proved by using a

computer-simulation technique. Fig. 5(b) shows the computer simulation of the composite 2-deoxyuridine 5-monophosphate-derived radical adducts. Figs. 5(c) and (d) show the computer simulations of the separated uridine-derived radicals.

Fig. 5(c) shows the spectrum of a spin-trapped radical with a primary triplet of 1.54 mT, a secondary nitrogen splitting of 0.106 mT, and a secondary hydrogen splitting of 0.4 mT. This simulation would be consistent with $\cdot\text{OH}$ addition at C-5, creating a radical centred at the C-6 of the base moiety (structure V). Fig. 5(d) shows the spectrum generated when the radical is centred at the C-5 position (structure VI).

Once again it can be seen that the generation of the radical adduct is nucleotide-, iron-, and tNB-dependent (Fig. 6). For the reasons outlined previously, spectra with reduced intensity are obtained in the system with no EDTA and no H_2O_2 .

The results presented indicate that $\cdot\text{OH}$ attack occurs predominantly at the pyrimidine base of the nucleotide. However, it should be noted that the $\cdot\text{OH}$ can attack the sugar moiety of the nucleotide. Scholes *et al.* [38], using stationary radiolysis, have noted that 25% of the oxidizing species react with the pentose moiety, but, for whatever reason, sugar-derived radical adducts are not detected in our work with nucleotides and we cannot explain this.

The results presented indicate that $\cdot\text{OH}$ formed via a Fenton system can attack deoxyribonucleotides to produce secondary radicals. These radicals have been identified by means of spin trapping and suggest that $\cdot\text{OH}$ attack occurs predominantly at the carbon-carbon double bond of the heterocyclic base.

The spectra presented here are in good agreement with those produced by Riesz and his colleagues [16] and Kuwabara and co-workers [25,39] (Table 1). Both these authors used irradiation as their source of $\cdot\text{OH}$, suggesting that the primary reactive species generated by the Fenton system is indeed the $\cdot\text{OH}$. We could find no evidence to suggest an involvement of a ferryl-iron species.

The TBA reaction is widely used in the oxygen-radical field to detect oxidative deterioration in bulk lipids and complex biological molecules. The TBA reaction depends upon carbonyl compounds (formed by the breakdown of lipids, amino acids or carbohydrates) reacting with two molecules of TBA to form a chromogenic adduct. Malonaldehyde is one such carbonyl compound that can react to form a chromogenic adduct with TBA. Malonaldehyde is formed as a minor component of lipid peroxidation and can be formed from carbohydrates exposed to high-energy radiation. Gutteridge [18] has demonstrated that TBA-reactive material can be produced from deoxyribose on exposure to Fe(II). Since this work by Gutteridge [18], many investigators have used the deoxyribose/TBA assay as a measure of $\cdot\text{OH}$ formation. Recently Cheeseman *et al.* [40] have demonstrated conclusively that this TBA-reactive material is malonaldehyde.

The results on the formation of TBA-reactive material from the three pyrimidine nucleotides are shown in Table 2. Since only thymidine contains the three adjacent carbon atoms necessary for malonaldehyde formation outside of the 2-deoxyribose moiety, the TBA-reactive material detected in our studies is presumably also malonaldehyde. As can be seen, the formation of TBA reactive material is almost entirely dependent on the presence of the nucleotide and Fe(II). Interestingly, there is still a considerable amount of TBA-reactive material formed when EDTA is removed from the reaction system, in agreement with the spin-trapping studies. This would suggest that either tNB is binding iron, or that TBA-reactive material is produced by the site-specific generation of $\cdot\text{OH}$. Removal of the spin trap from the reaction system has little overall effect. At first, the absence of any effect of tNB is surprising, but the concentration of the spin trap (5.8 mM) is much less than that of the nucleotides (48 mM) and, since the $\cdot\text{OH}$ generally reacts with all organic chemicals at near diffusion-limited rates, no significant scavenging occurs. The removal of H_2O_2 produces only a small decrease in the amount of TBA-reactive material. Apparently sufficient H_2O_2 can be produced by the autoxidation of Fe(II). The spin-trapping experiments generally show a greater dependence on added H_2O_2 , but even with spin trapping, significant radical formation can be detected without added H_2O_2 (Fig. 4e).

If the TBA assay is repeated in the presence of an $\cdot\text{OH}$ scavenger, and assays are performed in the presence and absence of both EDTA and tNB, some interesting observations can be made (Table 3). If we consider the complete system, addition of sodium formate at a final concentration of 100 mM produces a 71% inhibition in

Table 1. Hyperfine splitting constants for pyrimidine nucleotide-derived carbon-centred radical adducts

Nucleotide free radical	Splitting constant (G)			Reference
	Nitroxide nitrogen	Secondary splittings a_{β}^H	a_{β}^N	
dTMP				
C-5	16.2			Present work
C-6	14.9		2.6	Present work
C-5	15.8			[39]
C-6	14.3		3.0	[39]
C-5	15.95			[16]
C-6	14.5		2.7	[16]
dCMP				
C-5	15.4	4.4		Present work
C-6	15.4	5.55	0.9	Present work
C-5	15.0	4.4		[16]
C-6	15.1	5.8		[16]
C-5	14.5	4.0		[39]
dUMP				
C-5	15.4	3.4		Present work
C-6	15.4	4.0	1.06	Present work
C-5	15.6	3.5		[39]
C-5	15.6	3.3	1.5	[39]

Table 2. Development of TBA-reactive material from deoxyribonucleotides on exposure to a Fenton system

Treatment	Nucleotide...	TBA-reactive material formed (%)		
		dTMP	dCMP	dUMP
Complete system		100	100	100
– Nucleotide		6.1	16.6	6.1
– EDTA		44	75	79
– Fe(II)		2.1	7.7	9
– tNB		103	83	73
– H ₂ O ₂		78.9	76	95

Table 3. Effect of 100 mM-formate on the development of TBA-reactive material from dTMP in the presence on absence of EDTA and tNB

Absorbance values are means for two separate experiments (performed in duplicate) that differed by less than 10%. The final concentration of the reactants were: dTMP, 48 mM; Fe(II), 600 μ M; EDTA, 600 μ M; tNB, 5.8 mM; sodium formate, 100 mM; and H₂O₂, 600 μ M.

Treatment	A_{532}	Inhibition by formate (%)
Complete system	0.346	
+ Formate	0.097	71
Complete system – EDTA	0.291	
+ Formate	0.152	47
Complete system – tNB	0.379	
+ Formate	0.112	70
Complete system – tNB – EDTA	0.126	
+ Formate	0.097	23

the development of TBA-reactive material. This result suggests that formate is acting as an 'OH scavenger. In the system with no EDTA, there is a decrease in the amount of TBA-reactive material produced, but even in this system, formate can still produce a degree of inhibition (47%). This result suggests that not all of the damage inflicted on the nucleotide is produced by a site-specific generation of 'OH, and that some 'free' 'OH must still be produced. This would infer that a species capable of binding iron is still present. The only candidate for this role is the spin trap tNB.

In the absence of the spin trap (but in the presence of EDTA), formate still acts as an 'OH scavenger, producing an inhibition of 70%. However, when both EDTA and tNB are removed from the reaction system, the protective effect of formate is greatly reduced. This clearly suggests that, in this system, 'OH is produced by a site-specific mechanism. That is, the iron necessary for the Fenton reaction is bound to the nucleotide. The results of the TBA assays suggest that the spin trap, tNB, can serve to bind iron in such a way that it can still undergo the necessary redox chemistry to produce 'OH free in solution.

The work reported in the present paper demonstrates that a Fenton system can produce the same secondary radicals from pyrimidine nucleotides as high-energy ionizing radiation, thus implicating 'OH as the damaging species and showing that the Fenton–EDTA system can act as a radiomimetic system. This study also shows that 'OH attack on the nucleotide produces TBA-reactive material and that there is a good qualitative agreement between the formation of TBA-reactive material and nucleotide-derived free radicals.

REFERENCES

1. Roots, R. & Okada, S. (1972) *Int. J. Radiat. Biol.* **21**, 329–342

2. Willson, R. L. (1978) in *Biochemical Mechanism of Liver Injury* (Slater, T. F., ed.), pp. 123–224, Academic Press, London
3. Mello Filho, A. C. & Meneghini, R. (1984) *Biochim. Biophys. Acta* **781**, 56–63
4. Walling, C. (1982) *Proc. Int. Symp. Oxidases Relat. Redox Systems 3rd* 85–97
5. Koppenol, W. H. & Liebman, J. F. (1984) *J. Phys. Chem.* **88**, 99–101
6. Koppenol, W. H. (1985) *J. Free Radicals Biol. Med.* **1**, 281–285
7. Rush, J. D. & Koppenol, W. H. (1986) *J. Biol. Chem.* **261**, 6730–6733
8. Winterbourn, C. C. (1987) *Free Radical Biol. Med.* **3**, 33–39
9. Youngman, R. J. & Elstner, E. F. (1981) *FEBS Lett.* **129**, 265–268
10. Youngman, R. J. (1984) *Trends Biochem. Sci.* **9**, 280–283
11. Eiben, K. & Fessenden, R. W. (1971) *J. Phys. Chem.* **75**, 1186–1201
12. Fitchett, M., Gilbert, B. C. & Jeff, M. (1985) *Philos. Trans. R. Soc. London Ser. B* **311**, 517–529
13. Schmidt, J. & Borg, D. C. (1976) *Radiat. Res.* **65**, 220–237
14. Livingston, R. & Zeldes, H. (1966) *J. Chem. Phys.* **44**, 1245
15. Mason, R. P. (1984) in *Spin Labeling in Pharmacology* (Holtzman, J. L., ed.), pp. 87–129, Academic Press, New York
16. Joshi, A., Rustgi, S. & Riesz, P. (1976) *Int. J. Radiat. Biol.* **30**, 151–170
17. Kuwabara, M., Inanami, O. & Sato, F. (1986) *Int. J. Radiat. Biol.* **49**, 829–844
18. Gutteridge, J. M. C. (1981) *FEBS Lett.* **128**, 343–346
19. Mason, R. P. (1984) *Methods Enzymol.* **105**, 416–422
20. Buettner, G. R. (1987) *Free Rad. Biol. Med.* **3**, 259–303
21. Riesz, P. & Rustgi, S. (1979) *Radiat. Phys. Chem.* **13**, 21–40
22. Lion, Y., Kuwabara, M. & Riesz, P. (1982) *Photochem. Photobiol.* **35**, 53–62
23. Téoule, R. & Cadet, J. (1978) in *Effects of Ionizing Radiation on DNA* (Hüttermann, J., Köhnlein, W. & Téoule, R., ed.), pp. 171–203, Springer-Verlag, New York
24. Kominami, S., Rokushika, S. & Hatano, H. (1977) *Radiat. Res.* **72**, 89–99
25. Kuwabara, M., Yoshii, G. & Itoh, T. (1983) *Int. J. Radiat. Biol.* **44**, 219–224
26. Kuwabara, M., Inanami, O., Endoh, D. & Sato, F. (1987) *Biochemistry* **26**, 2458–2465
27. Cathcart, R., Schwieters, E., Saul, R. L. & Ames, B. N. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5633–5637
28. Gutteridge, J. M. C., Rowley, D. A. & Halliwell, B. (1981) *Biochem. J.* **199**, 263–265
29. McCord, J. M. & Day, E. D., Jr. (1978) *FEBS Lett.* **86**, 139–142
30. Floyd, R. A. (1981) *Biochem. Biophys. Res. Commun.* **99**, 1209–1215
31. Halliwell, B. & Gutteridge, J. M. C. (1984) *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford
32. Samuni, A., Chevion, M. & Czapski, G. (1981) *J. Biol. Chem.* **256**, 12632–12635
33. Gutteridge, J. M. C. & Wilkins, S. (1983) *Biochim. Biophys. Acta* **759**, 38–41
34. Gutteridge, J. M. C. (1987) *Biochem. J.* **243**, 709–714
35. Mansuy, D., Chottard, J. C. & Chottard, G. (1977) *Eur. J. Biochem.* **76**, 617–623
36. Cohen, G. & Sinet, P. M. (1980) in *Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase* (Bannister, J. V. & Hill, M. A. O., eds.), pp. 27–37, Elsevier/North-Holland, Amsterdam
37. Halliwell, B. & Gutteridge, J. M. C. (1981) *FEBS Lett.* **128**, 347–352
38. Scholes, G., Ward, J. F. & Weiso, J. (1960) *J. Mol. Biol.* **2**, 379
39. Kuwabara, M., Minegishi, A., Ito, A. & Ito, I. (1986) *Photochem. Photobiol.* **44**, 265–272
40. Cheeseman, K. H., Beavis, A., Esterbauer, H. (1988) *Biochem. J.* **252**, 649–653

Received 10 October 1988/15 February 1989; accepted 21 February 1989