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FERRIC AND CUPRIC IONS REQUIREMENT FOR DNA SINGLE-STRAND BREAKAGE BY H_2O_2

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Hydrogen peroxide (H_2O_2), was able to nick the replicative form of the phage fd, without the addition of a reducing agent or of a metal. This DNA single-strand breakage decreased with an increase of the ionic strength, suggesting that H_2O_2 reacted with traces of metal bound to DNA.

When cupric of ferric ions were added, the rate of DNA single-strand breakage by H_2O_2 greatly increased and it was 20–30 times faster with cupric than with ferric ions. The addition of EDTA at an equimolar ratio or in excess of metal prevented partially DNA single-strand cleavage by H_2O_2 in the presence of ferric ions and completely when cupric ions were used. Superoxide dismutase prevented DNA single-strand breakage by H_2O_2 and ferric ions. On the contrary, with cupric ions and H_2O_2, the addition of superoxide dismutase increased the rate of DNA single-strand breakage. That superoxide dismutase was acting catalytically was shown by the loss of its effects after heat inactivation of the enzyme. The results of the present study show that besides its involvement in the Fenton reaction, H_2O_2 is able to reduce the metal bound to DNA, generating the superoxide anion radical or/and its protonated form, the perhydroxyl radical involved in DNA nicking. On the other hand, the ability of cuprous ions unlike ferrous ions to dismutate the superoxide radical may explain some differences observed between iron and copper in the DNA single-strand breakage by H_2O_2.

KEY WORDS: Hydrogen peroxide, Iron, Copper, circular DNA.

INTRODUCTION

Hydrogen peroxide (H_2O_2) a normal metabolite in aerobic cells, is formed by divalent reduction of dioxygen or by dismutation of the superoxide anion radical ($O_2^{\cdot}$). The cellular steady state concentration of H_2O_2 is in the range 10^{-8}–10^{-9} M.[1] Its concentration may increase many times, especially at site of inflammation where the oxidative burst of the phagocyte cells occurs[2] and upon irradiation with near ultraviolet light or visible light.[3]

The role of hydrogen peroxide in the production of genetic damage has been known for twenty years.[4,5,6] However, H_2O_2 is a relatively stable oxidant and does not *per se* cause DNA damage, but reacts with ions such as ferrous (Fe^{2+}) or cuprous (Cu^{1+}) in the Fenton reaction, generating an extremely powerful oxidant; the hydroxyl radical $OH^{\cdot}$.

$$Me^n + H_2O_2 \rightarrow Me^{n+1} + OH^{\cdot} + OH^-$$

The hydroxyl radical is so reactive that it can only diffuse 5–10 molecular diameters before it reacts,[7] so if it is not produced near DNA, it probably will not react with it. However, it has been suggested that the DNA damage occurs at the site where the reduced metal bound to the DNA reacts with H_2O_2:[8]

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$$DNA - Me^{n} + H_2O_2 \rightarrow DNA - Me^{n+1} - OH\cdot + OH^{-}$$
$$\downarrow$$
damage

Hydroxyl radical generated at the metal binding site may attack DNA at either the sugar or the base,[9] ultimately leading to DNA strand breaks. The mechanism of such single-strand breaks involves the addition of $OH^{\cdot}$ to C=C of the base or an abstraction of H-atoms from the various positions of the sugar moiety.[10]

The reduction of the metal is necessary to maintain an ongoing Fenton reaction. Brawn and Fridovich[11] using the xanthine/xanthine oxidase system showed that $O_2^{\cdot}$ is involved in the reduction of ferric iron to ferrous iron. Recent work has shown that Cu^{2+} and H_2O_2 or Fe^{3+} and H_2O_2-catalysed DNA damage was very slow and required to be efficient the presence of a reducing agent such as ascorbate[12] or NADPH.[13]

In this work, the effects of iron (Fe^{3+}) and copper (Cu^{2+}) on the single-strand breaks formation by hydrogen peroxide have been compared using the replicative form of the phage fd (fd RF DNA). The data showed that besides its involvement in the Fenton reaction, H_2O_2 could be also the reducing agent of the metal bound to the DNA, leading to single-strand breakage.

MATERIALS AND METHODS

Materials

The DNA used was the replicative form of the phage fd (fd RF DNA) which was purified as described,[14] ethanol precipitated several times and finally dissolved in 10 mM Tris-HCl pH = 7.9, 10 mM NaCl and stored at -20°C. DNA preparations typically contained 60–80% covalently closed circular (CCC) supercoiled molecules, 20–40% open relaxed circle molecules and virtually no linear molecules.

Superoxide dismutase (SOD, EC 1.15.1.1) 3000 U/mg and D-mannitol were obtained from Sigma Chemical Company, Saint-Louis, MO. Ethylenediamine tetraacetic acid disodium salt (EDTA), hydrogen peroxide, $CuSO_4$, $FeCl_3$ and other salts were of analytical reagent grade.

Detection of DNA single-strand breaks

DNA single-strand breaks were assayed by measuring the conversion of covalently circular double-stranded supercoiled DNA (CCC DNA) to open (relaxed) circular double-stranded DNA and linear double-stranded DNA. The gel electrophoretic mobility of a relaxed circle (form II) in agarose is about half that of supercoiled (form I) with more than 15 turns[15]. The linear double-stranded molecule (form III) migrates intermediately between the form II and the form I.

The fd RF DNA (150 ng) was incubated in 10 mM Tris HCl pH = 7.9, 10 mM NaCl air-saturated with various agents in microfuge tubes (Eppendorf). The final volume was 20 μl. The reactions were initiated by the addition of hydrogen peroxide and immediately kept at 37°C for varying periods of time. The reaction was stopped by the addition of 10 μl of electrophoresis sample buffer (4 M urea, 50% sucrose, 50 mM EDTA and 0.1% bromophenol blue). The samples were loaded on an hor-

izontal 1% agarose slab gel and the electrophoresis was conducted in a mini apparatus (Bethesda Research Laboratories, Bethesda; MD) for 16 hours at 25 volts, at room temperature. The agarose electrophoresis running buffer was 40 mM Tris Acetate pH = 8.4, 10 mM EDTA. Gels were stained for two hours in running buffer containing 0.5 μg/ml ethidium bromide and then destained for several hours. DNA bands were visualized by illuminating the gel with UV light and photographs were taken with a Polaroid Camera equipped with a yellow filter using a black and white Polaroid film type 665. Quantification of bands was achieved by measuring areas of densitometer tracings. Under the conditions of staining used in these experiments, it was found that the decrease in percent of DNA remaining supercoiled was matched with an increase of the DNA being relaxed.

RESULTS

DNA single strand breaks formation by H_2O_2

In this study DNA was precipitated several times and re-suspended in a buffer containing 10 mM Tris-HCl and 10 mM NaCl pH = 7.5 without EDTA. In these conditions, the incubation of supercoiled covalently closed circular DNA (Form I) with H_2O_2 for one hour results in the formation of single-strand and double-strand breaks as shown by neutral agarose gel electrophoresis (Figure 1A). In the absence of H_2O_2 (lane 1) the DNA is essentially all supercoiled. At 10 mM H_2O_2 (lane 6), Form

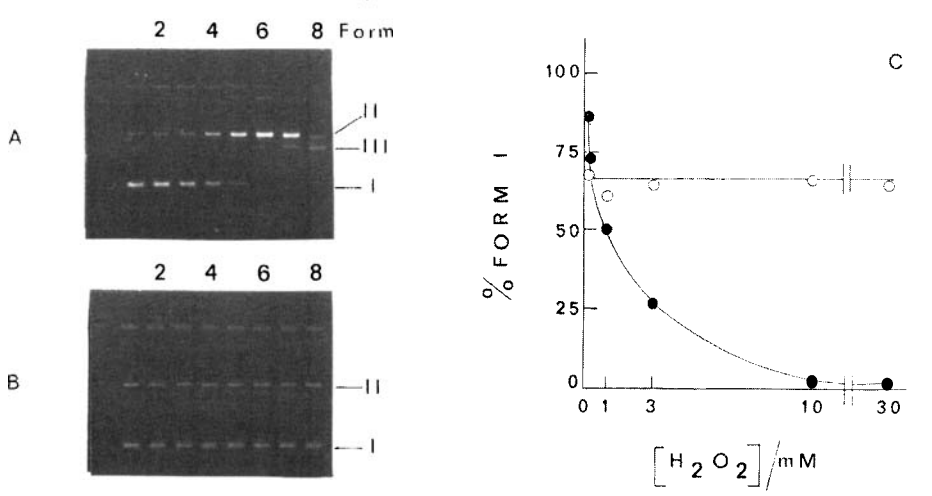

FIGURE 1 Neutral agarose gel showing the production of single-strand and double-strand breaks in fd RF DNA by H_2O_2. All reaction mixtures contained 150 ng fd RF DNA in 10 mM Tris-HCl, 10 mM NaCl, pH = 7.5. Incubation proceeded at 37°C for 1 hour. *Panel A*. Lane 1, no addition; lanes 2–8 increasing concentrations of H_2O_2 (0.1, 0.3, 1, 3, 10, 30 and 100 mM, respectively). *Panel B*. Lane 1, 1 μM EDTA; lanes 2–8 increasing concentrations of H_2O_2 (0.1, 0.3, 1, 3, 10, 30 and 100 mM, respectively) with 1 μM EDTA. I, II and III are covalently closed circular, open circular and linear forms of fd RF DNA, respectively.. *Panel C*. Densitometric analysis of the lanes of the gel in panel A (without EDTA) ● and in panel B (with 1 μM EDTA) ○.

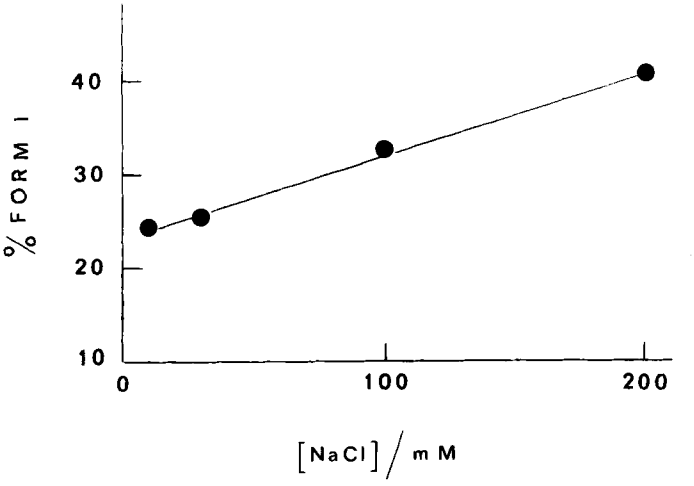

FIGURE 2 Effect of increase of the ionic strength on the nicking of supercoiled DNA by H_2O_2. 150 ng of fd RF DNA in 10 mM Tris-HCl pH = 7.9 and various concentrations of NaCl were incubated with 3 mM H_2O_2 for 1 hour at 37°C.

I was entirely converted into the relaxed form (Form II) and one can see that an increase of H_2O_2 concentration above 10 mM (lanes 7–8) results in the appearance of the linear form (Form III). The addition of 1 μM EDTA to the incubation mixture completely blocks DNA degradation by H_2O_2 (Figure 1B), indicating an involvement of traces of metal bound to DNA, since neither copper nor iron have been detected by atomic absorption spectroscopy measurements in the buffer 10 mM Tris HCl/ 10 mM NaCl (detection limit : 0.05 μM). Figure 1C shows the densitometric scanning of the negatives corresponding to the Figures 1A and 1B, and it appears that the decrease of the DNA supercoiled form was exponential with H_2O_2 concentration.

If H_2O_2 reacts with traces of metal bound to DNA, as suggested above, it is expected that the rate of degradation of DNA by H_2O_2 decreases with an increase of the ionic strength. The results in Figure 2 show that this is the case. At a low ionic strength (10 mM NaCl), the percentage of DNA remaining supercoiled was 25% whereas at a high ionic strength (200 mM NaCl) it was 40%.

Effects of copper (Cu^{2+}) and iron (Fe^{3+}) on DNA degradation induced by H_2O_2

In order to check the role played by transition metals involved in the so-called Haber-Weiss reaction, the kinetics of the degradation of supercoiled DNA by H_2O_2 in the absence of added metal and in the presence of 50 μM Fe^{3+} or 50 μM Cu^{2+} were followed. Without H_2O_2, neither copper nor iron alone are able to promote some nicks whereas in the presence of 3 mM H_2O_2, Fe^{3+} and particularly Cu^{2+} promote a striking increase of the rate of DNA degradation (Figure 3). The time taken to introduce one nick per molecule was about 50 minutes when no metal was added and 30 seconds and 15 minutes, respectively, in presence of 50 μM Cu^{2+} and 50 μM Fe^{3+}.

Because the kinetics of DNA degradation by H_2O_2 with Fe^{3+} was about 20 to 30 times slower than with Cu^{2+}, an incubation time of 15 minutes and 45 seconds was

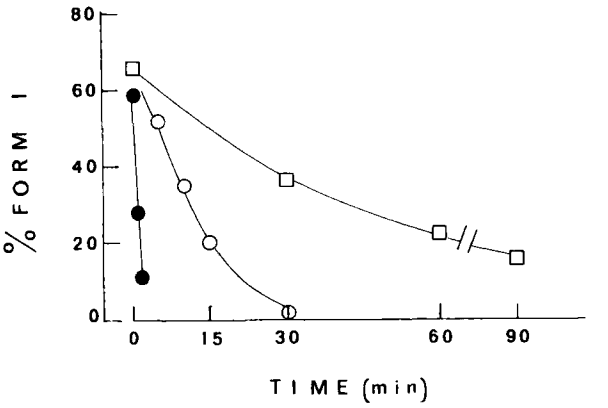

FIGURE 3 Effects of copper and iron on the kinetics of nicking of supercoiled DNA by H_2O_2. 150 ng of fd RF DNA in 10 mM Tris-HCl, 10 mM NaCl, pH = 7.5, was incubated with 3 mM H_2O_2 at 37°C; □, no metal added; ○, 50 μM Fe^{3+}; ●, 50 μM Cu^{2+}.

chosen respectively to study the effect of an increasing concentration of Fe^{3+} and Cu^{2+} on the DNA single-strand breaks induced by H_2O_2. As shown in Figure 4A, in the presence of 3 mM H_2O_2, the decrease of Form I was exponential with the metal concentration and the slope of the dose response curve was slightly larger with Fe^{3+} than with Cu^{2+}. Without H_2O_2, no DNA degradation was observed, even at the higher concentration of metal used.

The effect of an increasing H_2O_2 concentration shows a pattern different with Fe^{3+} and with Cu^{2+} (Figure 4B). In the presence of 50 μM Fe^{3+}, the decrease of Form I seems to be proportional with the logarithm of H_2O_2 concentration. With 50 μM Cu^{2+}, the hydrogen peroxide dose response could be divided in two parts : At low H_2O_2 concentration (0.3 mM), no DNA degradation was seen and at a H_2O_2 concentration above 1 mM, the rate of DNA degradation greatly increased with the logarithm of H_2O_2 concentration. At 30 mM H_2O_2 and 50 μM Cu^{2+} DNA was completely degraded (data not shown).

Effects of free radical scavengers and other agents

To gain insight into the mechanisms of DNA single-strand break formation by H_2O_2 in presence of Fe^{3+} or Cu^{2+}, the effects of a chelating agent and free radical scavengers were examined. Table 1 shows that the addition of EDTA at a molar ratio 0.5:1, 1:1 and 2:1 to the incubation mixture containing 50 μM Fe^{3+} partially blocked the DNA degradation by H_2O_2, whereas in the presence of 50 μM Cu^{2+} EDTA completely blocked the DNA degradation at a molar ratio 1:1 and 2:1. Furthermore, the decrease of the rate of DNA degradation by H_2O_2 in the presence of Fe^{3+} or Cu^{2+} was dependent on the increase of the ionic strength (Table 2), suggesting that H_2O_2 reacts with metals in the vicinity of DNA.

Superoxide dismutase, unlike heat-inactivated superoxide dismutase, dose-dependently inhibits DNA single-strand breaks induced by 50 μM Fe^{3+} and 3 mM H_2O_2. On the contrary, when the incubation mixture contained 50 μM Cu^{2+} and 3 mM

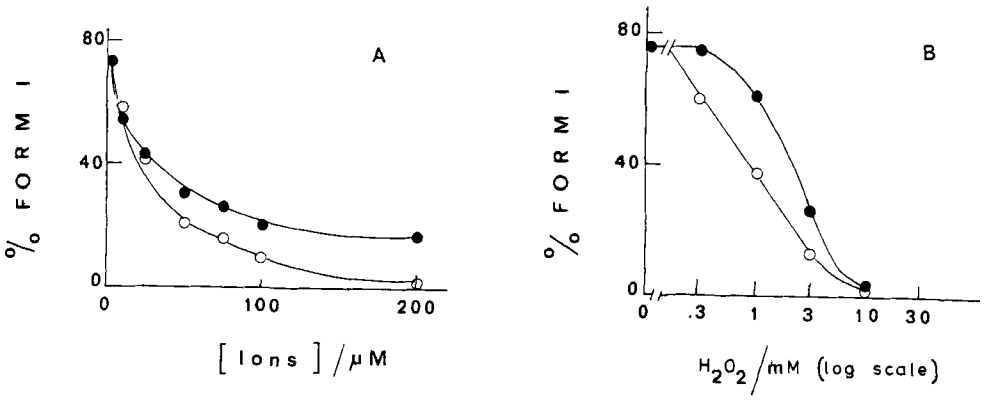

FIGURE 4 *Panel A* Dose effect of Cu^{2+} and of Fe^{3+} on the nicking of supercoiled DNA by H_2O_2. 150 ng of the fd RF DNA was incubated in 10 mM Tris-HCl, 10 mM NaCl, pH = 7.5 either with Cu^{2+} and 3 mM H_2O_2 (●) for 45 seconds or with Fe^{3+} and 3 mM H_2O_2 (○) for 15 minutes at 37°C. *Panel B.* Dose effect of H_2O_2 on the nicking of supercoiled DNA in presence of copper or iron. 150 ng of the fd RF DNA was incubated in 10 mM Tris-HCl, 10 mM NaCl, pH = 7.5 either with 50 μM Cu^{2+} and H_2O_2 (●) for 45 seconds or with 50 μM Fe^{3+} and H_2O_2 (○) for 15 minutes at 37°C.

H_2O_2, the addition of SOD increased the rate of DNA degradation (Table 2). The addition of mannitol, a scavenger of the hydroxyl radical produced some protection only when the transition metal used is Cu^{2+}.

DISCUSSION

This study shows that in the absence and in the presence of added metal such as Fe^{3+} or Cu^{2+}, H_2O_2 is able to promote DNA single-strand breakage without the addition

TABLE I

Effect of EDTA on the DNA single-strand breakage by H_2O_2 and iron or by H_2O_2 and copper. 150 ng of fd RF DNA was incubated in 10 mM Tris-HCl, 10 mM NaCl, pH = 7.5. Fe^{3+} and Cu^{2+} were present at 50 μM and H_2O_2 at 3 mM. The reaction proceeded at 37°C for 30 minutes prior to electrophoresis and quantitation as described under "Materials and Methods".

Conditions	Form I %	Inhibition(%)
Experiment I		
Fe^{3+}	60.5	
Fe^{3+} + H_2O_2	0	0
Fe^{3+} + EDTA (25 μM) + H_2O_2	30	49
Fe^{3+} + EDTA (50 μM) + H_2O_2	36	59
Fe^{3+} + EDTA (100 μM) + H_2O_2	36.5	60
Experiment II		
Cu^{2+}	58	
Cu^{2+} + H_2O_2	0	0
Cu^{2+} + EDTA (25 μM) + H_2O_2	0	0
Cu^{2+} + EDTA (50 μM) + H_2O_2	56	96
Cu^{2+} + EDTA (100 μM) + H_2O_2	62.5	107

TABLE II

Effect of various agents on the DNA single-strand breakage by H_2O_2 and iron or by H_2O_2 and copper. 150 ng of the fd RF DNA was incubated in 10 mM Tris-HCl, 10 mM NaCl (except when indicated otherwise), pH = 7.5 either with Fe^{3+} (50 μM) and H_2O_2 (3 mM) for 15 minutes or with Cu^{2+} (50 μM) and H_2O_2 (3 mM) for 30 seconds. The reaction proceeded at 37°C prior to electrophoresis.

Compounds	Fe^{3+}		Cu^{2+}	
	Form I %	Inhibition %	Form I %	Inhibition %
None	73.5		82	
H_2O_2	5	0	44	0
H_2O_2 (NaCl 30 mM)	11.5	9.5	55	29
H_2O_2 (NaCl 100 mM)	32	39.5	70	68.5
H_2O_2 + SOD (0.1 μg/ml)	5.5	0	49	13
H_2O_2 + SOD (1 μg/ml)	33.5	41.6	41.5	−6.5
H_2O_2 + SOD (10 μg/ml)	57	76	32	−31
H_2O_2 + SOD boiled (10 μg/ml)	7	3	42	−5
H_2O_2 + Mannitol (1 mM)	< 5	0	54.5	27.5
H_2O_2 + Mannitol (10 mM)	< 5	0	52	21
H_2O_2 + Mannitol (20 mM)	< 5	0	60	42

of a reducing agent. These data suggest the following sequence of reactions where H_2O_2 is both the reducing and the oxidizing agent:

$$Fe^{3+} \text{ or } Cu^{2+} + H_2O_2 \rightarrow Fe^{2+} \text{ or } Cu^{1+} + HO_2\cdot + H^+ \quad (2)$$

$$Fe^{2+} \text{ or } Cu^{1+} + H_2O_2 \rightarrow Fe^{3+} \text{ or } Cu^{2+} + OH\cdot + OH^- \quad (3)$$

The Fe^{3+} and Cu^{2+} catalysed decomposition of hydrogen peroxide involving the reactions (2) and (3) have been described already.[16,17,18]

At neutral pH, these metals precipitate out as unreactive polynuclear structure.[19] They only remain in solution by being complexed to low-or high-molecular weight cellular components, and consequently may serve as catalytic centers for free radical production.[20,21] As DNA preparations contain substantial amount of copper[22] and iron[23] which could be chelated by the phosphate of the DNA backbone[24,25] or sequestered by nucleotides,[26] it was expected that H_2O_2 could react with metals bound to DNA. In such a situation, one should consider reactions (2a, 2b) and (3a, 3b) instead of reactions (2) and (3).

$$DNA - Fe^{3+} + H_2O_2 \rightarrow DNA - Fe^{2+} + HO_2\cdot + H^+ \quad (2a)$$

or

$$DNA - Cu^{2+} + H_2O_2 \rightarrow DNA - Cu^{1+} + HO_2\cdot + H^+ \quad (2b)$$

$$DNA - Fe^{2+} + H_2O_2 \rightarrow DNA - Fe^{3+} \ldots OH\cdot + OH^- \quad (3a)$$

or

$$DNA - Cu^{1+} + H_2O_2 \rightarrow DNA - Cu^{2+} \ldots OH\cdot + OH^- \quad (3b)$$

However, it was shown that few or no DNA degradation was observed when purified DNA was exposed to H_2O_2 alone.[12,13] However, traces amount of EDTA which could be copurified with DNA despite the extensive dialysis,[27] the use of phosphate buffer or of a high ionic strength, could inhibit or compete for metal DNA binding. In the conditions of their experiments, traces of metal were removed from DNA and H_2O_2 could not react in a site-specific manner. But, in our experimental conditions, in the absence of additional metal, the addition of 1 μM EDTA was sufficient to inhibit DNA degradation by H_2O_2 (Figure 1B). Furthermore, the increase of the ionic strength partially blocks DNA single-strand break formation by H_2O_2. However, the possibility cannot be dismissed that high ionic strength could hinder the relaxation of a fraction of nicked DNA.

In order to investigate the sequence of reactions 2a, 2b and 3a, 3b, the effect of Cu^{2+} and Fe^{3+} on the DNA degradation by H_2O_2 was studied. It appears that DNA degradation by H_2O_2 was about 30 times faster with Cu^{2+} than with Fe^{3+}. Similar results were described previously.[4,12]

The less extensive DNA damage with iron could be due to its lower solubility so that the level of available iron in the system might be only a small proportion of the total added ions. Copper, on the other hand, is much more soluble so that the concentration of redox-active copper is most probably equal to the total added amount. Moreover, the differences between iron and copper may be also due to different rates of reduction of the metal complex by hydrogen peroxide and/or to different rate constants for the reduced metal-DNA complex in the Fenton reaction, yielding highly reactive hydroxyl radicals.[28] At higher ionic strength the decrease of the rate of DNA degradation induced by Cu^{2+} plus H_2O_2 or by Fe^{3+} plus H_2O_2 stresses that metal ions are tightly bound to DNA and are available to react with H_2O_2.

The addition of EDTA at an equimolar concentration or in excess of metal ions partially prevents DNA single-strand clevage by H_2O_2 with Fe^{3+} and completely by H_2O_2 with Cu^{2+}. Two combined explanations can be provided. Firstly, EDTA could remove Cu^{2+} more easily than Fe^{3+} from DNA, and consequently the number of cupric ions reacting with H_2O_2 at the DNA metal binding sites may be lower. Furthermore, the effect of mannitol which exhibits a protection only with copper stresses this hypothesis, since the protection afforded by mannitol could be related to its ability to chelate metal but not to its hydroxyl radical scavenger property.[29,30] Secondly, H_2O_2 is able to react with the Fe^{3+} - EDTA chelate, generating the hydroxyl radical.[17] As a matter of fact, Fe^{3+} – EDTA in solution includes seventh coordination site which is freed or occupied by an easily dissociable ligand such as water[31] and so is readily reduced by hydrogen peroxide. The free hydroxyl radicals, because reacting at near diffusion-controlled rates, were probably not involved in the DNA cleavage induced by the Fe^{3+} – EDTA/H_2O_2 system. However Koppenol *et al.*[32] showed a possible reaction mechanism leading to the ferryl ion:

$$Fe^{3+} - EDTA + H_2O_2 \rightarrow Fe^{2+} - EDTA + O^{\cdot}_2 + 2H^+ \quad (4)$$

$$Fe^{2+} - EDTA + H_2O_2 \rightarrow FeO^{2+} - EDTA + H_2O \quad (5)$$

Such a ferryl ion could be involved in DNA single-strand breakage.[13]

The difference between Fe^{3+} – EDTA and Cu^{2+} – EDTA, with regard to their reactivity with H_2O_2, could be due to the absence of available coordination sites able to react with H_2O_2 for Cu^{2+} – EDTA. This was supported by the fact that Cu^{2+} – EDTA unlike other copper complexes is unable to catalyse the dismutation of the superoxide anion radical,[33] suggesting that all coordination sites are hindered in such a complex.

If the reactions 2a and 2b occur at the metal binding site, as suggested above, it is expected that the superoxide radical $O^{\cdot}_2$ or/and its conjugate acid, the perhydroxyl radical $HO_2\cdot$ were generated near the DNA molecule. The pKa for the $O^{\cdot}_2/HO_2\cdot$ couple is about 4.8, so at a physiological pH almost 1% of any superoxide formed is present in the protonated form $HO_2\cdot$. $HO_2\cdot$ unlike $O^{\cdot}_2$, is *per se* a reactive oxygen species and can, for instance, initiate lipid peroxidation[34] because the pH decreases in close proximity to membranes. In the same manner according to Mannings's theory, the local concentration of protons condensed around the DNA treated as a polyanion was not greatly influenced by the pH in the bulk solution.[35,36] Thus, this local concentration may lower the pH in the vicinity of DNA allowing $HO_2\cdot$ to abstract an hydrogen atom from the sugar moiety or the base leading to single-strand break. If that is the case, it would be expected that the addition of superoxide dismutase greatly inhibits the rate of DNA degradation by H_2O_2. That is true only when the metal used is Fe^{3+}. Since heat-inactivated superoxide dismutase did not exhibit an effect, one can correlate the effect of superoxide dismutase to its enzymatic activity. The ability of the copper–DNA complex to dismutate the superoxide radical to H_2O_2 [37] in reactions 6 and 7 through a ping pong mechanism led us to discard the involvement of $HO_2\cdot$ in DNA single-strand break induced by H_2O_2 in presence of copper, since the rate of reactions 6 and 7 may be much faster than the slow rate of reaction 2b.

$$Cu^{2+}\text{-DNA} + HO_2\cdot \rightarrow Cu^{1+}\text{-DNA} + O_2 + H^+ \quad (6)$$

$$Cu^{1+}\text{-DNA} + HO_2\cdot + H^+ \rightarrow Cu^{2+}\text{-DNA} + H_2O_2 \quad (7)$$

On the other hand, the addition of superoxide dismutase to the mixture containing Cu^{2+} and H_2O_2 increases the rate of DNA degradation. This result could be explained by the competition between superoxide dismutase and Cu^{1+}–DNA in reaction 7 allowing an increase of the rate of the reaction 3b by limiting the reoxidation of Cu^{1+}–DNA to Cu^{2+}–DNA. This interpretation was stressed by the pattern of the H_2O_2 dose-response curve with copper. At a low hydrogen peroxide concentration, the value of $k_7[O^{\cdot}_2]$ would be high as compared to the value of $k_{3b}[H_2O_2]$ and so, the rate of DNA degradation is slow. On the contrary, at the higher hydrogen peroxide concentration, $k_{3b}[H_2O_2]$ becomes much higher than $k_7[O^{\cdot}_2]$ and so the rate of DNA degradation greatly increases. Afterwards, repeated cycles of reduction and reoxidation of copper occur at the same site or step by step leading to formation of multi-hits.[38]

In summary, reactions (2a, 2b and 3b) may be envisioned, depending on the metal and on the H_2O_2 concentration used:

$$\text{DNA} - Fe^{3+} + H_2O_2 \rightarrow \text{DNA} - Fe^{2+} + HO_2\cdot + H^+ \quad (2a)$$

$$\downarrow$$

damage

$$\left\{\begin{array}{ll} \mathrm{DNA - Cu^{2+} + H_2O_2 \rightarrow DNA - Cu^{1+} + HO_2\cdot + H^+} & \text{(2b)} \\ \mathrm{DNA - Cu^{1+} + H_2O_2 \rightarrow DNA - Cu^{2+} \ldots OH\cdot + OH^-} & \text{(3b)} \end{array}\right.$$

$$\downarrow$$

damage

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