

# The Interaction between Hydrogen Peroxide and the DNA–Cu(I) Complex: Effects of pH and Buffers

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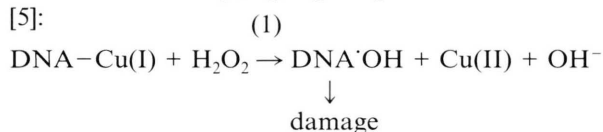
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DNA–Cu(I) Complex, Fenton Reaction, Hydrogen Peroxide Reaction with DNA–Cu(I), Hydroxyl Radical, Piperazine Buffers

The rate of interaction between  $\text{H}_2\text{O}_2$  and the DNA–Cu(I) complex increases with pH and with salt (NaCl) concentration, suggesting that  $\text{HO}_2^-$  is involved. The pH dependence can be fitted, assuming  $k(\text{DNA–Cu(I)} + \text{H}_2\text{O}_2) = 1 \text{ M}^{-1} \text{ s}^{-1}$  and  $k(\text{DNA–Cu(I)} + \text{HO}_2^-) = 10^5 \text{ M}^{-1} \text{ s}^{-1}$  (at low salt concentrations). These interactions cause DNA damage, probably due to the formation of  $\cdot\text{OH}$  radicals near the site of Cu(I) fixation at DNA bases. About 70% of the intermediates DNA $\cdot\text{OH}$ , formed by free  $\cdot\text{OH}$  radicals, were found capable of reducing Cu(II) to regenerate DNA–Cu(I); thus a (limited) reaction chain involving “reductive propagation” by DNA $\cdot\text{OH}$  species appears feasible upon reaction of  $\text{H}_2\text{O}_2$  with DNA–Cu(I).  $\cdot\text{OH}$ -induced intermediates of poly(C) are more efficient (about 80%), those of poly(A) and poly(G) are less efficient (about 38%), in reducing Cu(II). Certain organic buffers, particularly HEPES and PIPES, promote autoxidation in DNA/Cu(II)/ $\text{H}_2\text{O}_2$  systems, and it is shown that  $\cdot\text{OH}$ -induced buffer intermediates as well as secondary stable buffer products can engage in “reductive propagation” of redox cycles.

## Introduction

Copper, an essential element of respiring cells, plays a specific role for instance as active centre of Cu,Zn-superoxide dismutases (SOD) which serve to control reactive oxygen species *via* disproportionation of  $\text{O}_2^{\cdot-}$  radicals into  $\text{O}_2$  and  $\text{H}_2\text{O}_2$  [1, 2]. Copper can on the other hand also enhance free radical damage in biological systems due to Fenton-type reactions of  $\text{H}_2\text{O}_2$  with Cu(I), which appear to generate highly deleterious  $\cdot\text{OH}$  radicals immediately at the site of fixation of Cu(I) in certain biomolecules [3, 4], *e.g.* in proteins or DNA [5]:



DNA may present a prominent target for such damage since copper is considered to be an essen-

tial chromatin component [6–8]. The DNA–Cu(I) complex has been characterized spectroscopically [9, 10] and it was recognized that Cu(I) fixation at DNA bases occurs preferentially in alternating G–C sequences [11]. Due to the exceptional strength of interaction of Cu(I) with DNA, with a stability constant in the order of  $2 \times 10^9 \text{ M}^{-1}$  at  $\text{Cu(I)/base} < 0.2$  [11], the degradation of DNA *via* reaction (1) was hardly affected in aqueous model systems in the presence of proteins. Reaction (1) was found to be slow,  $k_1 = 1.2 \text{ M}^{-1} \text{ s}^{-1}$ , in pH 6.8 phosphate buffer [5, 11].

The corresponding interaction of  $\text{H}_2\text{O}_2$  with SOD–Cu(I), *i.e.* the reduced form of SOD occurring in the catalytic cycle, is known to inactivate the enzyme [12, 3]. A recent investigation of the rate of reaction of SOD–Cu(I) with  $\text{H}_2\text{O}_2$  as a function of pH has led to the conclusion that  $\text{HO}_2^-$ , and not  $\text{H}_2\text{O}_2$  ( $\text{pK}_a = 11.9$ ), is the active species in this system [13].

The main objective of the present study was to determine the rate of reaction (1) as a function of pH and salt concentration in order to establish, as in the case of SOD, the nature of the active species. Another aim was to test the possibility of redox cycling in DNA/copper/ $\text{H}_2\text{O}_2$  systems. The reaction of  $\cdot\text{OH}$  with DNA components is known to generate both oxidizing and reducing intermediates [14, 15],  $\text{DNA}_{\text{ox}}\cdot\text{OH}$  and  $\text{DNA}_{\text{red}}\cdot\text{OH}$ . The

**Abbreviations:** EDTA, ethylenediaminetetraacetic acid–Na-salt; HEPES, N-(2-hydroxyethyl)piperazine–N'-2-ethanesulphonic acid–Na-salt; MES, (2-(N-morpholino)ethanesulphonic acid); PIPES, piperazine–N,N'-bis(2-diethane-sulphonic acid)–Na-salt; TRIS, tris-(hydroxyethyl)aminomethane.

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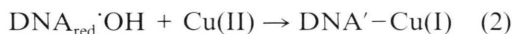
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reaction (1) can hence be expected to induce a limited reaction chain through reaction (2),

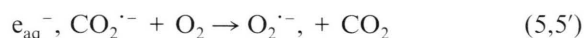
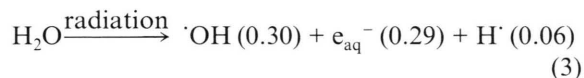


promoting the accumulation of DNA<sub>ox</sub>'OH lesions. Reaction (2) does not necessarily involve a "chemical repair" of the DNA<sub>red</sub>'OH lesion, it may rather produce stable modifications (denoted DNA') such as base hydroxylation.

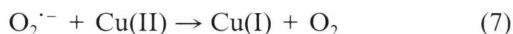
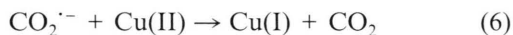
## Materials and Methods

Deoxyribonucleic acid (DNA) from salmon testes, Fe(III)-cytochrome *c*, poly(G) and poly(I) were obtained from Sigma, CuCl<sub>2</sub>·H<sub>2</sub>O and organic buffers from Serva, and poly(A) and poly(C) from Boehringer Mannheim. These and other chemicals were used as received. Solutions were prepared freshly for each experiment with redistilled water using DNA stock solutions prepared at least 24 h in advance. All results refer to room temperature conditions (~ 20 °C).

Cu(I) complexes were generated by radiolytic reduction of Cu<sup>2+</sup> ions, in most cases in air-saturated aqueous solution containing DNA and formate (4 mM), irradiated at about 0.3 Gy/s with a <sup>60</sup>Co-γ-source (Gammacell 220, Atomic Energy of Canada Ltd.). All water radicals are rapidly transformed in this system into O<sub>2</sub>'<sup>-</sup>, at a yield (in brackets) of 0.65 μm Gy<sup>-1</sup> [15],



followed by the reactions (6) to (8) [11]:



Cu(I) complexation with various nucleic acids induces characteristic UV-absorbance changes with distinct maxima, *e.g.* at 295 nm for DNA and at 313 nm for poly(I) [11], and this absorption was used to monitor the slow reaction (1), using a conventional (Perkin Elmer) UV-Vis-spectrophotometer. The stability of Cu(I)-complexes was previously shown to increase in the order poly(U) < poly(dT) < poly(A) < poly(G) ~ poly(C) < DNA

< poly(dG-dC) < poly(I) [11]. The decay of Cu(I) due to disproportionation and oxidation, *via* the reverse of reaction (7), was in the time scale of minutes only in the case of poly(U) and poly(dT), the Cu(I)-complexes of the other nucleic acids were sufficiently stable (hours) to enable rate studies. The stability of DNA–Cu(I) upon addition of various buffers is shown in Table I; Gly, pyrophosphate and the alkaline form of TRIS appear to extract Cu(I) slowly from the DNA.

Cu(I) complexes were prepared by the above means in unbuffered solution (pH ~ 6) at Cu(I)/base ratios well below 0.2, and reaction (1) was then initiated by mixing these systems at a 1:1 (v:v) ratio with solutions of H<sub>2</sub>O<sub>2</sub> in various buffers (5–10 mM). Buffering was necessary since the pH appeared to float during reaction (1) in unbuffered solution. Typical time profiles thus obtained are presented in Fig. 1. The H<sub>2</sub>O<sub>2</sub>/buffer component was added simultaneously both to the irradiated test solution and to the same but unirradiated reference solution (Fig. 1a), since the absorbance decay due to reaction (1) overlapped with absorbing product formation due to autoxidative reactions when reaction (1) was monitored against a H<sub>2</sub>O<sub>2</sub>-free reference solution (Fig. 1b). The autoxidative reactions detected upon addition of H<sub>2</sub>O<sub>2</sub> to unirradiated DNA/Cu(II) solutions (Fig. 1c) depend strongly on the choice of buffer; there was little effect *e.g.* in TRIS buffer but extensive product formation in HEPES and PIPES buffers. It is known that the latter piperazine-derived buffers can stimulate free radical generation by copper and H<sub>2</sub>O<sub>2</sub> [16] and we will come back to this problem in the discussion. Under the conditions applied (Fig. 1a) the time profiles were fairly well exponential, and it was generally checked that the rate of DNA–Cu(I) removal by reaction (1) is linear in H<sub>2</sub>O<sub>2</sub> concentration (Fig. 1d). Several repeats were made for each buffer system, and time profiles were reproducible within about 20%.

## Results and Discussion

### Effect of pH on reaction (1)

Fig. 2. presents a plot of the apparent rate constant of reaction (1) as a function of pH, using a variety of buffers, and conditions as in Fig. 1a. Generally, and for each individual buffer the observed rate constant *k*<sub>1(obs)</sub> increases with pH, as

Table I. Absorbance and stability of DNA–Cu(I) in various buffers.<sup>a</sup>

Buffer	NaCl [mM]	(pH)	OD <sub>295</sub>	Stability <sup>b</sup>
NaCl <sup>c</sup>	2.5	(6.0)	0.140	stable
	102.5	(6.1)	0.145	stable
	2.5	(8.0)	0.100	stable
Phosphate	2.5	(6.2)	0.140	stable
	2.5	(7.6)	0.135	stable
PIPES	2.5	(6.3)	0.135	stable
	2.5	(7.6)	0.105	stable
HEPES	2.5	(6.5)	0.130	stable
	2.5	(8.5)	0.090	stable
MES	2.5	(5.6)	0.140	stable
	2.5	(7.0)	0.135	stable
TRIS	2.5	(7.1)	0.120	stable
	102.5	(7.0)	0.105	less stable
	2.5	(7.6)	0.110	stable
	2.5	(8.7)	0.090 <sup>d</sup>	unstable, 10 min <sup>e</sup>
Borate	2.5	(8.8)	0.100	stable
	2.5	(9.2)	0.075	stable
Gly	2.5	(8.2)	0.130 <sup>d</sup>	unstable, 3 min <sup>e</sup>
Pyrophosphate	2.5	(9.7)	0.075 <sup>d</sup>	unstable, 5 min <sup>e</sup>

<sup>a</sup> Air-saturated solutions of DNA (0.16 g/l), NaCl (5 mM), CuCl<sub>2</sub> (0.1 mM) and HCOONa (4 mM), unbuffered (pH ~ 6), were gamma-irradiated with 100 Gy and diluted immediately (1:1) with 10 mM buffer. Spectra, recorded against the corresponding unirradiated control, showed in each case the characteristic feature of DNA–Cu(I) [11]; only peak absorbances at 295 nm (1 cm cell) are given.

<sup>b</sup> The statement “stable” means less than 10% decay within 60 min.

<sup>c</sup> No buffer.

<sup>d</sup> Initial OD.

<sup>e</sup> Approximate half-life;  $t_{1/2}$  strongly decreased with pH and buffer concentration.

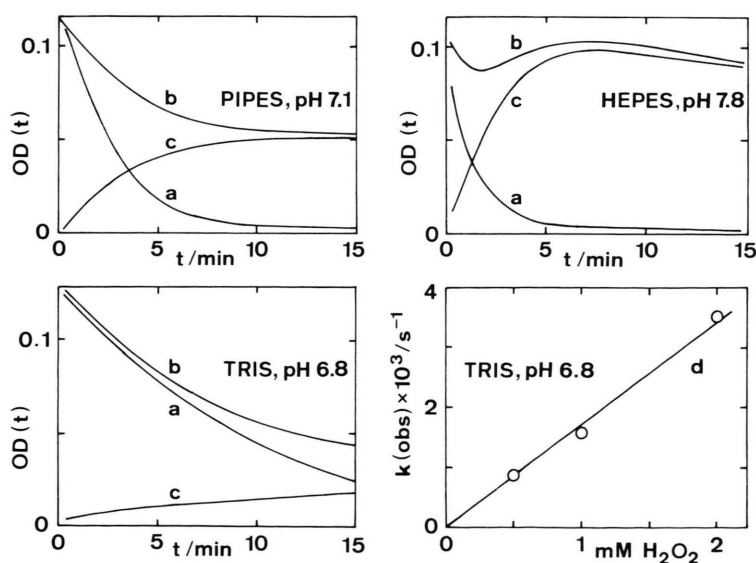


Fig. 1. Kinetics of interaction of H<sub>2</sub>O<sub>2</sub> with DNA–Cu(I), monitored by 295 nm absorbance changes (1 cm cell). The results refer to 100 Gy  $\gamma$ -irradiation of air-saturated solutions containing 0.16 g/l DNA, 5 mM NaCl, 4 mM HCOONa and 100  $\mu$ M CuCl<sub>2</sub> at pH ~ 6 (*i.e.* generation of 65  $\mu$ M (Cu(I))). This solution was subsequently mixed 1:1 (v:v) with solutions of 2 mM H<sub>2</sub>O<sub>2</sub> in 10 mM PIPES, 1 mM H<sub>2</sub>O<sub>2</sub> in 10 mM HEPES, and 2 mM H<sub>2</sub>O<sub>2</sub> in 5 mM TRIS, respectively: (a) H<sub>2</sub>O<sub>2</sub>/buffer component added simultaneously to irradiated test and unirradiated reference solution; (b) same as (a), but without H<sub>2</sub>O<sub>2</sub> in the reference solution; (c) addition of H<sub>2</sub>O<sub>2</sub>/buffer component to the unirradiated system with reference as (b). (d) Test of linearity between  $k_1$ (obs) and [H<sub>2</sub>O<sub>2</sub>] in 2.5 mM TRIS (pH 6.8).

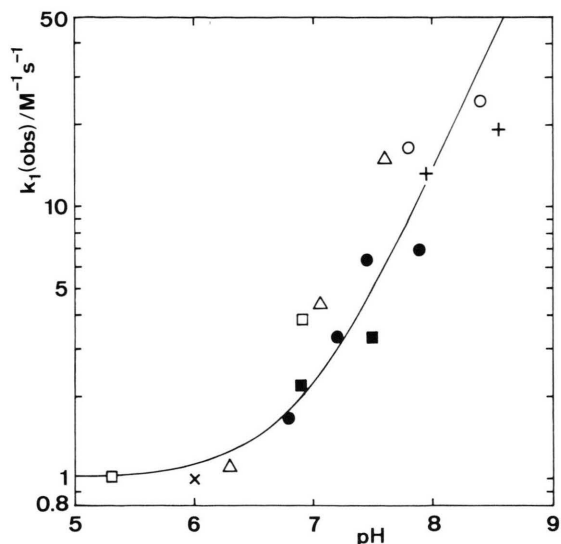
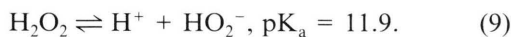
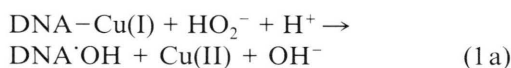


Fig. 2. The rate of reaction between DNA–Cu(I) and H<sub>2</sub>O<sub>2</sub> as a function of pH. Conditions as in Fig. 1a, using 50–100 Gy to generate DNA–Cu(I) and 0.25–2 mM H<sub>2</sub>O<sub>2</sub> added with following buffers: (□) MES, (×) no buffer, (△) PIPES, (●) TRIS, (■) phosphate, (○) HEPES, (+) borate. The theoretical curve was calculated from equation (I), see text.

previously found in the case of reaction between SOD–Cu(I) and H<sub>2</sub>O<sub>2</sub> [13]. In analogy, it can be concluded that the reaction (1) also involves HO<sub>2</sub><sup>−</sup> as active species:



The theoretical curve in Fig. 2 was calculated from the pertinent equation (I),

$$k_1(\text{calc}) = \frac{k_1 + k_{1a}K_9/[\text{H}^+]}{1 + K_9/[\text{H}^+]} \quad (I)$$

assuming  $k_1 = 1.0 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{1a} = 10^5 \text{ M}^{-1} \text{ s}^{-1}$  ( $K_9 = 1.26 \times 10^{-12} \text{ M}$ ). Reaction (1) is apparently not negligible at low pH, but above pH 6 the reaction (1a) becomes increasingly important. The rate constant  $k_{1a}$ , used to fit the experimental data, can be compared with  $k(\text{SOD–Cu(I)} + \text{HO}_2^-) = 2.6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  [13].

There is an appreciable scatter in the data (Fig. 2) obtained for various buffers, and we believe that this is mainly due to the buffer-dependent autoxidation, mentioned in the context of

Fig. 1 and discussed below. The effect of autoxidation was accounted for by the simultaneous initiation of these reactions in the test and reference solutions (see Fig. 1a), but it is obvious that they may depend on the initial Cu(I)/Cu(II) ratio which is of course different in the test and reference systems. Minimal interference by autoxidation was obtained using MES (□) at pH 5.3, unbuffered solution (×) at pH 6, and TRIS (●) at pH 7–8, so these data points appear to be the most reliable ones. The previous value of  $k_1(\text{obs}) = 1.2 \text{ M}^{-1} \text{ s}^{-1}$  [11] in pH 6.8 phosphate buffer (detection conditions as in Fig. 1b) is now replaced by  $k_1(\text{obs}) = 2.2 \text{ M}^{-1} \text{ s}^{-1}$  (detection conditions as in Fig. 1a in 5 mM phosphate, pH 6.9). An increase of temperature from 20 to 38 °C speeded up the reaction (1) by a factor of about three. Oxygenation or deoxygenation of the solutions had no effect on  $k_1(\text{obs})$ . It was not possible to extend the present rate studies to the range of  $\text{p}K_a(\text{H}_2\text{O}_2)$  owing to the low absorbance and stability of DNA–Cu(I) (see Table I) in alkaline buffers above pH 9.

It might be suggested that an increase in pH in the range investigated (Fig. 2) destabilizes the DNA–Cu(I) complex, this being the reason for the pH-dependent rate of reaction (1). We have therefore also tested the pH-dependence of the interaction between DNA–Cu(I) and EDTA; the rate of Cu(I) extraction from the DNA was previously found to be independent of EDTA concentration, which led to the suggestion that this process is rate-determined by the reverse reaction (8) [11] (possibly *via* formation of an intermediate ternary complex involving DNA phosphate groups). In contrast to the reaction (1) the rate of reaction of EDTA with DNA–Cu(I) was now found to decrease slightly when going from neutral to pH 9 solutions (unbuffered), which indicates that DNA–Cu(I) is not destabilized in this pH range. It is nevertheless surprising that EDTA interacts with DNA–Cu(I) in a concentration independent manner [11], whereas H<sub>2</sub>O<sub>2</sub> interacts concentration dependent (see Fig. 1d). The reaction (1) does obviously not proceed, as suggested in the case of EDTA, *via* release of Cu(I) from the sites of fixation at bases to the phosphate backbone or to the bulk solution, it rather takes place directly at the Cu(I) fixation sites in the base-pair region. This is indicated also by the observation that 'OH-scavengers are hardly able to prevent DNA



damage *via* reaction (1) [4, 5]. The question whether the interaction between  $\text{H}_2\text{O}_2$  and Cu(I) generates  $\cdot\text{OH}$  radicals or other oxidizing entities such as Cu(III) or  $\text{Cu}^+\text{HO}_2^-$  species appears not to be fully resolved yet [17–20]. The assignment (DNA $\cdot\text{OH}$ ) given in reaction (1) is merely based on the observation that the yield of DNA damage, tested with an unspecific dye intercalation assay, closely resembles the yield of  $\cdot\text{OH}$ -induced damage upon  $\gamma$ -radiolysis of DNA solutions (in absence of copper and  $\text{H}_2\text{O}_2$ ) [11]. Further analytical studies are however required to enable identification of the nature of the oxidizing entity formed in reaction (1).

To sum up, the pH-dependence of the rate of interaction of  $\text{H}_2\text{O}_2$  with DNA–Cu(I) suggests that  $\text{HO}_2^-$  is involved as active species, particularly in alkaline solutions. At low salt concentration (Fig. 2 refers to 5–10 mM salt) the pertinent rate constant is in the order of  $k_{1a} \sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , as compared to  $k_1 \sim 1 \text{ M}^{-1} \text{ s}^{-1}$ .

#### Effect of salt concentration on reaction (1)

Since Cu(I) is buried in the base-pair region, the negatively charged DNA phosphate backbone can be expected to impede interactions of the  $\text{HO}_2^-$  anion. Such interactions should be facilitated, however, at high salt concentration owing to counter ion condensation at the phosphate groups. The plot of  $k_1(\text{obs})$  vs.  $[\text{NaCl}]$ , presented in Fig. 3,

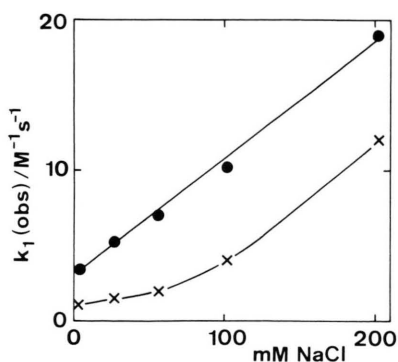


Fig. 3. The rate of reaction between DNA–Cu(I) and  $\text{H}_2\text{O}_2$  as a function of salt concentration. Reactions were initiated as in Fig. 1a, (●) in TRIS buffer at pH 7.1, and (×) in unbuffered solution at pH 6.1. The salt was added with the  $\text{H}_2\text{O}_2$ /buffer component after irradiation.

shows indeed a remarkable increase of the reaction rate with salt concentration, which gives further support to the importance of reaction (1a). The reaction (1), on the other hand, should not be salt dependent and it appears reasonable therefore that only a modest initial salt effect is seen at pH 6.1 (Fig. 3 (×)) where reaction (1) predominates (see above). The rate of interaction between DNA–Cu(I) and EDTA in TRIS buffer (pH 7) was not speeded up at high salt concentration, as would have been expected if the salt effect was due to destabilization of the complex. It can be concluded that under physiological conditions of pH and ionic strength (190 mM) the overall rate constant of reaction (1)/(1a) is of the order of  $k_1(\text{obs}) \sim 15$  to  $20 \text{ M}^{-1} \text{ s}^{-1}$ , with (1a) as the main reaction path.

#### Buffer interactions in DNA/Cu(II)/ $\text{H}_2\text{O}_2$ -systems

The autooxidative processes observed in unirradiated DNA/Cu(II)/ $\text{H}_2\text{O}_2$  systems (Fig. 1c) deserve some further consideration since certain additives (here buffers) seem to engage in redox chains promoting the reaction (1). Such autooxidation was also seen in phosphate, TRIS and unbuffered solution, but it was most pronounced in HEPES and PIPES buffer (see Fig. 1c). Furthermore, the effect was pH dependent for the individual buffers; in MES buffer, for instance, there was no autooxidation (within 15 min) at pH 5.5 but extensive autooxidation at pH 6.9, similar to HEPES. Deoxygenation of the systems had no effect on the time profiles shown in Fig. 1c. Absorption spectra taken during autooxidation of the unirradiated DNA/Cu(II)/ $\text{H}_2\text{O}_2$  system in HEPES buffer are shown in Fig. 4. After 5 min incubation the absorbance difference spectrum corresponded mainly to DNA–Cu(I) with a maximum at 295 nm and a (negative) minimum at 250 nm (*cf.* ref. [11]), but at longer times a further positive absorbance grew in at around 270 nm probably due to DNA and buffer decomposition products.

$\text{H}_2\text{O}_2$  was stable in all buffers tested. However, when Cu(II) was added to  $\text{H}_2\text{O}_2$ /buffer solutions (in absence of DNA) absorbing products were formed, in the range 200–350 nm and in the time scale of 30 min, specifically in HEPES and PIPES buffer, but not in phosphate, TRIS or unbuffered solution. Our experience indicates that HEPES, PIPES and the deprotonated form of MES are ac-

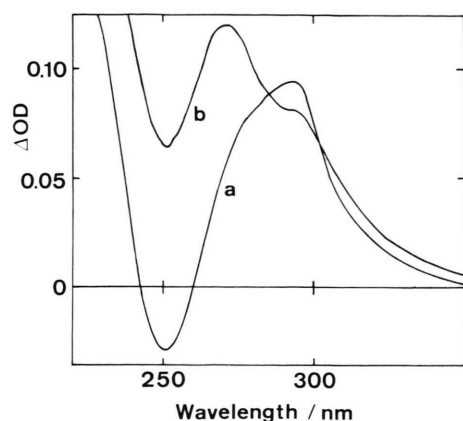


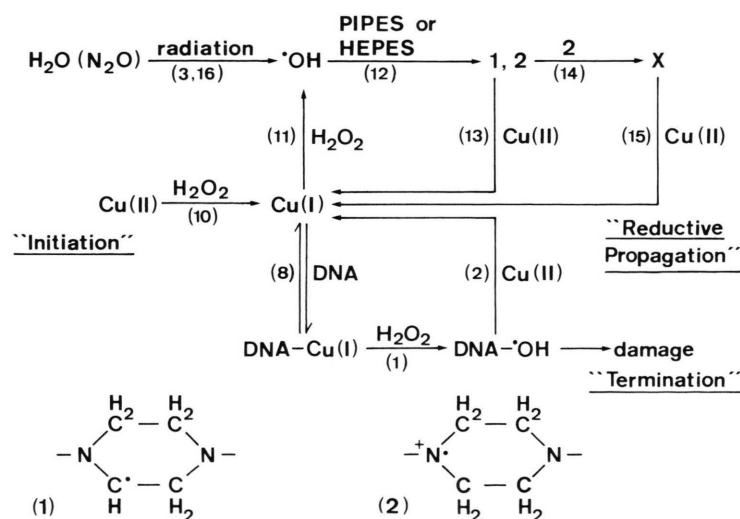
Fig. 4. Absorption changes due to autoxidative redox reactions in (unirradiated) solutions containing 0.08 g/l DNA, 2.5 mM NaCl, 2 mM  $\text{HCOONa}$ , 50  $\mu\text{M}$   $\text{CuCl}_2$ , 5 mM HEPES (pH 7.8) and 0.5 mM  $\text{H}_2\text{O}_2$ . The spectra were recorded against a corresponding reference solution without  $\text{H}_2\text{O}_2$ , (a) 5 min and (b) 30 min after  $\text{H}_2\text{O}_2$  addition. A time profile for this system at 295 nm is given in Fig. 1c (HEPES).

tive in promoting copper-dependent redox reactions of  $\text{H}_2\text{O}_2$ .

The ability of HEPES and PIPES to stimulate free radical generation by copper ions and  $\text{H}_2\text{O}_2$  was previously attributed to solubilization of the metal ion and formation of a redox active buffer–Cu(II) complex that is effective in generating  $\cdot\text{OH}$  from  $\text{H}_2\text{O}_2$  [16]. We have now observed that the deprotonated forms of HEPES and PIPES form

complexes with Cu(II) which absorb around 250 nm (*cf.* ref. [16]), but no such absorptions were detectable in a pH range where these buffers are protonated; this may explain some discrepancies in literature with regard to Cu(II) complexation by HEPES and PIPES [16, 21]. In presence of DNA the weak buffer–Cu(II) absorption was replaced by the stronger DNA–Cu(II) absorption around 280 nm (in these experiments two-compartment tandem cells were used which enable absorbance measurements of mixed against unmixed components). Since DNA forms the stronger complex with Cu(II) we do not think that buffer–Cu(II) complexation is the principal reason for the efficient autoxidation in DNA/Cu(II)/ $\text{H}_2\text{O}_2$  systems in presence of HEPES or PIPES (Fig. 1c) – it may nevertheless be important in other systems [16].

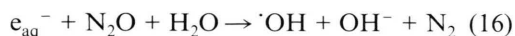
Some further reactions which could explain the buffer activity in promoting autoxidation under conditions as in Fig. 1c and 4 are presented in Scheme 1. We certainly believe that the initiating reaction (10), which probably involves  $\text{O}_2^{\cdot-}$  ( $+2\text{H}^+$ ) formation [22] and additionally the reaction (7), depends on the Cu(II) ligands (such as buffer,  $\text{Cl}^-$ , DNA etc.), which may either promote or inhibit this reaction. However, once Cu(I) is formed, a redox cycle may occur in presence of additives such as piperazine-buffers through the reactions (11) to (15).  $\cdot\text{OH}$  radicals generated in the bulk solution (reaction (11)) can be expected to interact mainly with the buffer, since reaction (12) is much faster [23] than reaction of  $\cdot\text{OH}$  with DNA



Scheme 1. Possible reaction pathways and intermediates (1, 2) involved in auto-oxidation of DNA/Cu(II)/ $\text{H}_2\text{O}_2$  systems, with piperazine-derived buffers as example (only the active products of each reaction are indicated).

[24]. Furthermore, free radical oxidation of piperazine is known to produce 40% carbon-centred  $\alpha$ -amino radicals (**1**) with reducing properties and 60% nitrogen-centred radicals (**2**) with oxidizing properties [25], thus the reaction (13) involving species **1** appears feasible.

The results shown in Fig. 5 serve to demonstrate the reaction paths (12) to (15). In this experiment we have generated  $\cdot\text{OH}$  by  $\gamma$ -radiolysis of a N<sub>2</sub>O-saturated solution, *via* reactions (3) and (16) (see e.g. [15]),



the solution containing DNA, Cu(II) and PIPES; H<sub>2</sub>O<sub>2</sub> was omitted so as to eliminate the reactions (10), (11), (1) and (2) (see Scheme 1). Conditions were chosen that  $\cdot\text{OH}$  primarily interacted with the buffer (reaction (12)), and secondary Cu(I) generation was monitored by formation of DNA–Cu(I), drawn curves, in comparison with the routine method of DNA–Cu(I) generation in formate solution *via* reactions (3) to (8), broken curves. Surprisingly the final yield of DNA–Cu(I) is almost

three times higher in the PIPES than in the formate system, and it is particularly noteworthy that the build-up of DNA–Cu(I) in PIPES buffer mainly occurs after the (50 s) period of irradiation (Fig. 5, insert). The higher yield of DNA–Cu(I) in the PIPES system is not only seen in the characteristic 295 nm absorbance peak (and the 250 nm minimum), but also by the long-wavelength tail which only appears when DNA is densely populated with Cu(I) [11].

The post-irradiation build-up of DNA–Cu(I) in the irradiated PIPES system (Fig. 5) reveals that Cu(II) can be reduced not only by the intermediate **1** (Scheme 1) but also by stable secondary buffer products **X**, which probably are formed by self-termination of the intermediates **2**; the reaction (14) might for instance lead to N–N coupled dimers. The high yield of Cu(I) in the case of PIPES (as compared to formate) furthermore suggests that each species **X** is capable of reducing at least two equivalents of Cu(II). When PIPES was replaced by HEPES, results similar to those in Fig. 5 were obtained; no post-irradiation Cu(II) reduction was detected, however, with the other buffers applied in this study.

The (unknown)  $\cdot\text{OH}$ -induced products **X** of HEPES and PIPES appear to be stable for hours. This was demonstrated by irradiating the buffers alone in N<sub>2</sub>O-saturated solution and delayed addition to DNA–Cu(II) or other reducible systems. Fig. 6 shows as an example the reduction of Fe(III)-cytochrome *c* (reduction potential:  $E^\circ = 0.27$  V at pH 7) by stable  $\cdot\text{OH}$ -induced buffer products.

It appears evident from the above results that in presence of H<sub>2</sub>O<sub>2</sub> a redox cycle is feasible through the reactions (11) to (15), involving “reductive propagation” by  $\cdot\text{OH}$ -products of piperazine-buffers (Scheme 1). It might be expected that with DNA as target the reactions (8) and (1) efficiently terminate this redox cycle, since Cu(I) fixation *via* reaction (8) is very much faster ( $k_8 \sim 4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  [11]) than the bulk reaction (11) ( $k_{11} = 4.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  [18]). The results shown in Fig. 1 c and 4 reveal that piperazine-buffers (particularly HEPES) nevertheless promote the formation of DNA–Cu(I) in unirradiated DNA/Cu(II)/H<sub>2</sub>O<sub>2</sub> systems; it is likely therefore that DNA becomes highly populated with Cu(I) and releases under this condition some of the Cu(I) back into the bulk

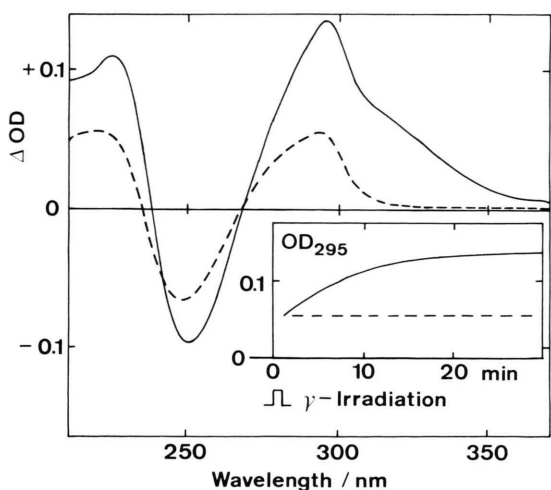


Fig. 5. Absorption changes due to DNA–Cu(I) formation upon 20 Gy  $\gamma$ -irradiation of N<sub>2</sub>O-saturated solutions containing 0.08 g/l DNA and 50  $\mu\text{M}$  CuCl<sub>2</sub>: (—) in presence of 5 mM PIPES (pH 6.3), and (---) in presence of 5 mM HCOONa (in pH 6.8 phosphate buffer). The difference spectra were recorded against the corresponding unirradiated system. The 295 nm time profile for the PIPES system shows that DNA–Cu(I) formation mainly occurs after the (50 s) irradiation period; the corresponding spectrum was taken 30 min after irradiation.

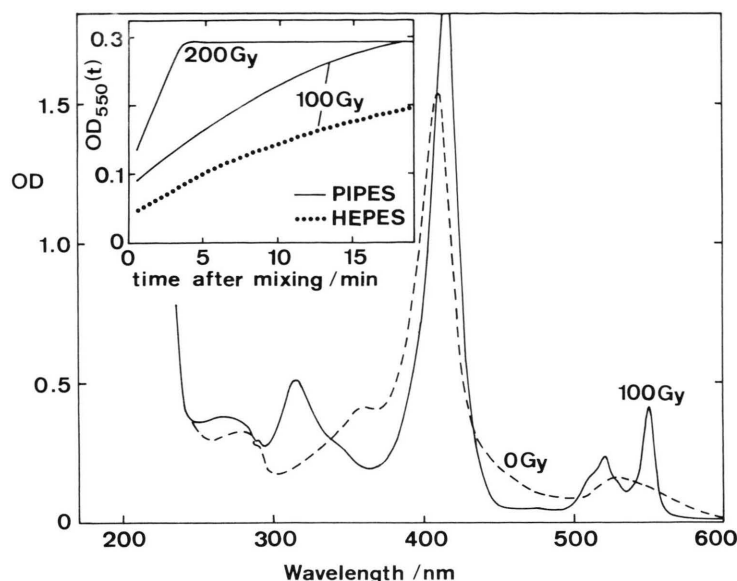


Fig. 6. Fe(III)-cytochrome *c* reduction by stable  $\cdot\text{OH}$ -induced products (**X**) of piperazine buffers. 10 mM HEPES (pH 7.2) or PIPES (pH 6.9),  $\gamma$ -irradiated in  $\text{N}_2\text{O}$ -saturated solution with the doses indicated, was subsequently diluted 1:1 (v:v) with a  $40\text{ }\mu\text{M}$  Fe(III)-cytochrome *c* solution, and time profiles at 550 nm (8.75 mm cell) were run against the corresponding unirradiated system. The Fe(II)-cytochrome *c* spectrum (—) was recorded (against  $\text{H}_2\text{O}$ ) 25 min after mixing with irradiated PIPES, in comparison with the spectrum of Fe(III)-cytochrome *c* (---) in unirradiated buffer.

solution. Certain additives (here HEPES and PIPES) may actually compete with DNA in complexation of Cu(I), and may thereby promote or inhibit reaction (11). It may be possible also that oxidizing DNA intermediates ( $\text{DNA}_{\text{ox}}\cdot\text{OH}$ ) formed in reaction (1) interact with the buffer to form the products **1** and **2**. The additional redox cycle involving reducing intermediates ( $\text{DNA}_{\text{red}}\cdot\text{OH}$ ), *i.e.* reaction (2) (Scheme 1), is considered in the next paragraph.

In view of the present and previous results [16] it appears likely that piperazine buffers can engage in deleterious free radical redox cycles in combination with copper ions and  $\text{H}_2\text{O}_2$  according to Scheme 1, not only in the above systems, but also when applied *in vivo*. We have also recognized that stable  $\cdot\text{OH}$ -induced products **X** of HEPES and PIPES interact slowly with  $\text{H}_2\text{O}_2$  (in the absence of Cu(II) and DNA), and it appears possible that this reaction again generates  $\cdot\text{OH}$  radicals.

#### Cu(II) Reduction by $\cdot\text{OH}$ products of DNA

A redox cycle involving the reactions (1), (2) and (8) (Scheme 1) appears feasible since  $\cdot\text{OH}$  produces oxidizing as well as reducing DNA intermediates, the latter of which may engage in reaction (2) (see Introduction). The results presented in Fig. 7 serve to estimate the efficiency of reaction (2). In these

experiments we have generated  $\cdot\text{OH}$  by  $\gamma$ -radiolysis (*via* reactions (3) and (16)) of  $\text{N}_2\text{O}$ -saturated solutions containing DNA, Cu(II) and phosphate (without  $\text{H}_2\text{O}_2$ ), under conditions where  $\cdot\text{OH}$  reacts in  $>94\%$  with DNA (see ref. [24] for rate constants). The difference spectrum (Fig. 7a) shows again the characteristic peak (295 nm) and minimum (250 nm) of the DNA–Cu(I) complex, indicating that DNA  $\cdot\text{OH}$  intermediates indeed cause Cu(II) reduction; there was however no evidence for a post-irradiation DNA–Cu(I) build-up, as in the case of HEPES and PIPES buffered systems (Fig. 5). In the absence of Cu(II) the reaction of  $\cdot\text{OH}$  with DNA produced an absorbance increase around 270 nm (not shown), which apparently contributes also to the absorbance change in the DNA/Cu(II) system (270 nm shoulder). Due to the overlap of DNA–Cu(I) with other DNA product absorptions, it was not possible to estimate the Cu(I) yield from the 295 nm absorbance peak in Fig. 7a. Solutions, prepared as in Fig. 7a, were therefore mixed with solutions of poly(I), which serves as Cu(I) detector molecule: Cu(I) is transferred quantitatively (within about 10 min) from DNA to poly(I), and the poly(I)–Cu(I) complex exhibits an absorption peak above 310 nm [11], *i.e.* beyond the spectra of DNA and DNA products. The dose dependence of poly(I)–Cu(I) absorption thus obtained (insert, b) is compared



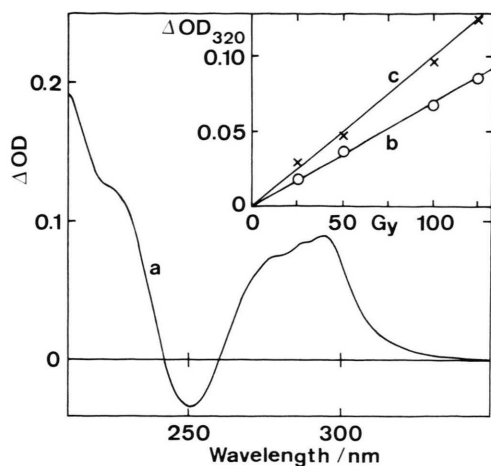


Fig. 7. Absorption changes due to interaction of  $\cdot\text{OH}$ -induced DNA intermediates with Cu(II) (reaction (2)). The spectrum (a) was obtained by 100 Gy  $\gamma$ -irradiation of a  $\text{N}_2\text{O}$ -saturated solution containing 0.16 g/l DNA and 40  $\mu\text{M}$   $\text{CuCl}_2$  in 5 mM phosphate (pH 6.8), and subsequent 1:1 (v:v) dilution with  $\text{H}_2\text{O}$ , with the corresponding unirradiated system as reference. The dose dependence (b) was obtained by  $\gamma$ -irradiation of  $\text{N}_2\text{O}$ -saturated solutions containing 0.25 g/l DNA, 100  $\mu\text{M}$   $\text{CuCl}_2$  and 5 mM phosphate (pH 6.8), and subsequent 1:1 dilution with 0.25 g/l poly(I): the poly(I)–Cu(I) absorption at 320 nm was used to monitor Cu(I) formation (see text). The curve (c) is the poly(I)–Cu(I) reference, obtained by directly irradiating 0.25 g/l poly(I) in presence of 100  $\mu\text{M}$   $\text{CuCl}_2$ , 10 mM  $\text{HCOONa}$  and 5 mM phosphate (pH 6.8), and subsequent 1:1 dilution with unirradiated 0.25 g/l DNA.

with that for direct generation of poly(I)–Cu(I) *via* reactions (3), (4), (16) and (6) (insert, c). The comparison reveals that about 70% of the DNA  $\cdot\text{OH}$  intermediates produce Cu(I) *via* reaction (2) – the Cu(I) yield in the DNA system may even be underestimated since  $\text{H}^\cdot$  contributes to poly(I)–Cu(I) formation in the reference solution (c), due to reaction (4'), whereas  $\text{H}^\cdot$  reacts mainly with DNA in the test solution (b).

The DNA experiment shown in Fig. 7 was repeated also with several homo-polynucleic acids, so as to find an answer to the question which of the DNA constituents engage in reaction (2). With poly(C) about 80% of the  $\cdot\text{OH}$ -products were able to reduce Cu(II), poly(A) and poly(G) both gave an efficiency of about 38%; poly(dT) could not be test-

ed owing to the low stability of the Cu(I) complex. This result gives the impression that  $\text{DNA}_{\text{red}}\cdot\text{OH}$  intermediates (see Introduction) are mainly formed by interaction of  $\cdot\text{OH}$  with pyrimidine bases, and in lower yield by  $\cdot\text{OH}$  interactions with purine bases and the sugar moiety. Generation of reducing intermediates by free radical oxidation is in fact known to be more efficient with pyrimidine than with purine bases [15], and reduction of tris(2,2'-bipyridyl)ruthenium(III), a much stronger oxidant than Cu(II), has been demonstrated upon one-electron oxidation of various nucleic acids [26].

Since the reaction (2) was found to operate quite efficiently, it can be expected that a limited reaction chain, (2)–(8)–(1) (see Scheme 1), takes place in presence of  $\text{H}_2\text{O}_2$ , provided the reaction (1) generates similar intermediates as free  $\cdot\text{OH}$  radicals. According to the efficiency of reaction (2) of about 70% the chain length of this cycle can be estimated to be of the order of 3. The reaction chain involving “reductive propagation” by DNA  $\cdot\text{OH}$  intermediates may explain previous results [11] which revealed that the efficiency of damage *via* reaction (1) was higher, particularly in the case of poly(dG-dC), than that of damage by free  $\cdot\text{OH}$  radicals in the absence of copper ions and  $\text{H}_2\text{O}_2$ .

Copper is believed to play a major role in DNA quaternary structure by linking threads of DNA to structural proteins, and it has been proposed that ionizing radiation and certain cytotoxic agents induce structural alterations and cellular misfunction by disturbing the Cu(II)/Cu(I) equilibrium within the chromatin [7]. The high yield of Cu(II) reduction by  $\cdot\text{OH}$ -induced DNA intermediates appears to be relevant in this context, even if numerous other reducing species, formed by ionizing radiation, by drugs and by metabolic processes, can affect the Cu(II)/Cu(I) equilibrium, *e.g.*  $\text{O}_2^{\cdot-}$  *via* reaction (7).

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