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ASCORBIC ACID OXIDATION AND DNA SCISSION CATALYZED BY IRON AND COPPER CHELATES

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The asorbic acid (AH^-) auto-oxidation rates catalyzed by copper chelates of 1,10-phenanthroline (OP) or by iron chelates of bleomycin (BLM) are only slightly higher than the oxidation rates catalyzed by the metal ions. AH^- oxidation in the presence of DNA is accompanied by degradation of the DNA. The rates of DNA scission by the metal chelates are markedly higher than the rates induced by the free metal ions. AH^- oxidation is slowed down in the presence of DNA which forms ternary complexes with the chelates. The ternary complexes react slowly with AH^- but induce DNA double strand breaks more efficiently than the free metal chelates. With OP, DNA is degraded by the reaction of the ternary complex, DNA-$(OP)_2Cu(I)$, with H_2O_2.

AH^- oxidation in the presence of DNA was biphasic, showing a marked rate increase after DNA was cleaved. We suggest that this sigmoidal pattern of the oxidation curves reflects the low initial oxidative activity of the ternary complexes, accelerating as DNA is degraded.

Using O_2^- produced by pulse radiolysis as a reductant, we found that AH^- oxidation with $(OP)_2Cu(II)$ induced more DNA double strand breaks per single strand break than bipyridine-copper.

The site specific DNA damaging reactions indicated by these results are relevant to the mechanism of cytotoxic activities of bleomycin and similar antibiotics or cytotoxic agents.

KEY WORDS: Ascorbic acid oxidation, DNA degradation, bleomycin, phenanthroline, copper, iron, O_2^-.

INTRODUCTION

In biological systems, active oxygen radicals such as O_2^-, $OH^{\cdot}$, and oxidants of comparable reactivity, are implicated in many harmful reactions.[1–3] Metal ions, particularly iron and copper, are involved in generating oxygen radicals and in their reactions.[4–6] The activity of the metal ions with oxygen and oxy-radicals depends on chelators present in the system.[7,8] In biological systems the concentrations of free transition metal ions appear to be very low and the metal ions are mostly bound to ligands such as proteins or DNA.[9] The reactivity of such complexes with di-oxygen and oxy-radicals is therefore of considerable interest.

In biological O_2^- generating systems, the formation of reactive species such as hydroxyl radical is catalyzed mostly by Fe(III) salts and chelates which are reduced by the superoxide anion and then react with H_2O_2 to produce more reactive species.[5,6] Fe(III) salts and chelates may also be reduced by ascorbic acid to give rise to oxy-radicals, in the presence of oxygen or H_2O_2, by an apparently O_2^- independent mechanism, although O_2^- seems to occur as a transient during auto-oxidation of ascorbate.[10]

Copper ions and copper chelates such as 1,10-phenathroline, or 2,2′-bipyrydine can

also promote the formation of $OH^{\cdot}$ radicals, or species of equivalent reactivity, in reactions requiring H_2O_2 and a reducing agent such as O_2^- or ascorbate.[11–15]

DNA-iron and -copper complexes are involved in DNA damaging reactions. In order to study the reactivity of such DNA-metal complexes with oxygen and oxy-radicals, we chose copper and iron complexes with ligands which form ternary complexes with DNA and cause DNA damage by well studied mechanisms.[13,15–17] The copper complex of 1,10-phenanthroline is known to induce DNA damage *in vivo*[18] and to cleave DNA *in vitro* in presence of reducing agents and H_2O_2 or O_2.[13] The iron chelate chosen was bleomycin. Bleomycin is a widely used cytotoxic antibiotic which causes DNA damage *in vivo*[19,20] and degrades DNA *in vitro*[17] in reactions that require oxygen and a reducing agent. Ascorbic acid or O_2^- generated by pulse radiolysis were used as reductants. Recent kinetic studies[16,21] have shown that the ternary complexes, with DNA-phenanthroline-copper, DNA-bleomycin-iron and DNA-bipyridine-copper are reduced very slowly in comparison with the free metal complexes, and that the reduction of the ternary complexes proceeds, through the reduction of the DNA-free metal chelates. It was suggested[16,21] that DNA damaging reactions, induced by these chelates, also proceed by means of the ternary complexes in site-specific reactions which may produce DNA double strand breaks more efficiently than those induced by the DNA-free chelates. We therefore studied the catalytic auto-oxidation of ascorbate in the presence of DNA and the accompanying cleavage of DNA. We tried to correlate ascorbate oxidation and DNA double strand scission, in order to evaluate the relative efficiencies of the metal ions, metal chelates and metal chelate ternary complexes in relation to both processes. Our results show that DNA degradation proceeds through the reduced ternary complexes, which, although poor catalysts of ascorbate oxidation, cleave DNA more efficiently than the DNA-free metal complexes.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade. They were dissolved in triple distilled water and used without further purification: potassium phosphate, sodium chloride, ferric ammonium sulfate, cupric sulfate and H_2O_2 (Merck); tris (Serva); EDTA (BDH), superoxide dismutase (SOD), catalase, diethylenetriaminopentaacetic acid (DTPA) and agarose type II (Sigma); 1,10-phenanthroline, (OP), 2,2′-bipyridine (BPY) and ascorbic acid (Fluka).

Calf thymus DNA type I (Sigma), lambda DNA and its Hind III digest (BRL) was dissolved in 1 mM phosphate buffer pH 7.4. The concentration was determined optically using $\varepsilon_{258} = 6700\,M^{-1}cm^{-1}$.

Bleomycin sulfate (BLM) was the gift of Bristol Laboratories and contained approximately 60% bleomycin A_2, 30% bleomycin B_2 and 10% other bleomycins. The average molecular weight was assumed to be 1550. Solutions were prepared in 1 mM phosphate buffer at pH 7 and standardized optically using $\varepsilon_{292} = 1.45 \times 10^4\,M^{-1}cm^{-1}$. Solutions of BLM-Fe(II), in buffer, were prepared by mixing oxygen-free solutions of BLM, in phosphate buffer at pH 7, with oxygen-free solutions of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in water. BLM-Fe(III) was prepared by mixing 10^{-3} M $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ in 10^{-2} N H_2SO_4, with a small excess of BLM, followed by neutralization with NaOH.

Solutions of $(BPY)_2Cu(II)$, were prepared by mixing cupric sulfate with 2.2 equivalents of the ligand.

Solutions of $(OP)_2Cu(II)$ were prepared by mixing cupric sulfate with 2.5 or with 5 equivalents of OP.

Methods

Ascorbic acid oxidation was measured by following the decrease in absorption at 266 nm using a Uvikon 860 spectrophotometer. Unless otherwise stated, the reaction mixtures were incubated in air at 37°C and contained 0.1 mM ascorbic acid, 1 mM H_2O_2 and various concentrations of metal salts or metal-chelates in a total volume of 3 ml. Reactions were started by the addition of ascorbic acid.

DNA degradation reactions in the presence of Cu(II) or $(OP)_2Cu(II)$ were stopped by the addition of EDTA (1 mM) and catalase to a final concentration of 50 mg/l. DNA cleavage in the presence of BLM-Fe(III) was stopped by the addition of 1 mM DTPA and 20 mM ascorbic acid or potassium iodide.

Double strand DNA was denatured by incubation in NaOH (0.2 N) for 15 minutes at 37°C.

Electrophoretic studies of DNA scission were carried out on horizontal 0.8% agarose slab gels prepared in tris-acetate buffer pH 8 containing 2 mM EDTA and 0.5 mg/l ethidium bromide at 40 mA for 3 to 4 hours.

Pulse radiolysis experiments were carried out with a Varian 7715 linear accelerator using 0.1–1.5 μsec pulse durations with a 200 mA current of 5 MeV electrons.

RESULTS AND DISCUSSION

Effect of copper and copper phenanthroline chelates on the oxidation of ascorbate

The catalytic effect of copper ions and copper phenanthroline chelates on the auto-oxidation of AH^- was followed spectrophotometrically at 266 nm. The oxidation rate was roughly first order with respect to [ascorbate] and [Cu(II)] and was dependent on the concentration of $[H_2O_2]$. These observations are in accord with the proposed mechanisms of ascorbate oxidation.[11,12]

OP enhanced the catalytic activity of copper ions. The highest oxidation rate of AH^- was observed with [OP]/[Cu(II)] = 5. This rate was about two fold higher than the rate obtained in the absence of OP (Table I). The rate decreased to about 30% at [OP]/[Cu(II)] = 1 and approximately to 60% when [OP]/[Cu(II)] = 20 (data not shown). Using the respective stability constants we evaluated the concentrations of the various copper phenanthroline chelates. Under our experimental conditions the predominant complex was always $(OP)_2Cu(II)$. The observation that the catalytic activity increased with the relative concentration of $(OP)_2Cu(II)$ suggests that this species is more active than unchelated copper, $(OP)Cu(II)$ or $(OP)_3Cu(II)$.

The rates of AH^- oxidation, catalyzed by Cu(II) or $(OP)_2Cu(II)$, were dependent on the ionic strength (I) of the reaction mixtures. Increasing the ionic strength from 0.002 to 0.1 with NaCl or $NaClO_4$ raised the rate of oxidation in the presence of $(OP)_2Cu(II)$ by 50% and lowered the rate with copper ions (Table I).

In order to examine the role of H_2O_2 in the reaction with $(OP)_2Cu(II)$ we added H_2O_2 under aerobic and anaerobic conditions. In the presence of air (where H_2O_2 is

TABLE I
Ascorbate oxidation rates in presence of metal chelates; effects of H_2O_2 and ionic strength

[NaCl] mM	Catalyst: [metal]-μM:	Oxidation rate – μM/min							
		$CuSO_4$ 1	OP-Cu 1	BPY-Cu 1	BLM-Cu 10	BLM-Mn 10	BLM-Fe(III) 10	EDTA-Fe(III) 10	OP-Fe(II) 10
–		21.0	27.6	17.0	0.5	0.7	57.4	5.2	8.3
10	$-H_2O_2$	15.6	30.8	17.4	0.6	0.5	47.3	5.2	8.8
100		7.3	37.1	17.3	0.5	0.7	40.2	5.9	13.4
–		35.9	40.1	19.4	1.9	1.0	79.9	26.8	20.9
10	$+H_2O_2$	32.6	43.4	23.8	1.6	0.8	83.0	31.1	27.8
100		10.5	66.2	23.8	1.9	1.3	60.9	45.6	25.7

Ascorbic acid 0.1 mM; H_2O_2 1 mM; OP/Cu(II) ratio was 5/1. Other chelates were in 1/1 ratio. Phosphate buffer pH 7.4 1 mM: Initial rates (3–30 sec) at 37°C.

known to be formed from the oxidation of AH^- and from the oxidation of $(OP)_2Cu(I)$ by O_2) addition of H_2O_2 almost doubled the oxidation rate, whereas catalase (50 mg/l) almost fully blocked it. No reaction took place in the absence of both air and H_2O_2, while the addition of H_2O_2 to an anoxic reaction system resulted in a reaction rate similar to that observed in the presence of air (Table II).

Neither 100 mg/l superoxide dismutase, nor 0.01 M OH-radical scavengers such as mannitol or t-BuOH had any significant effect on the oxidation rate of AH^-, indicating neither involvement of superoxide, except as a precursor of H_2O_2, nor the involvement of homogeneously distributed hydroxyl radicals in the process. These results also indicate that with $(OP)_2Cu(II)$ it is H_2O_2 rather than molecular oxygen which is responsible for the oxidation process, whereas O_2 serves merely as a precursor of H_2O_2 (Table II).

Effect of DNA on the rate of copper-catalyzed ascorbate oxidation

In the presence of DNA the catalytic activity of Cu(II) was drastically decreased. This effect strongly depended on [DNA] and increased with the increase in [DNA]/[Cu(II)]. For example, with 0.06 mM DNA and 5 μM copper the initial rate of oxidation decreased almost 200 fold as compared to the activity in the absence of DNA. As shown in Figure 1, the value of the reaction rate did not level off even under high excess of DNA ([DNA]/[Cu(II)] > 20). The reaction rate was linear with 1/[DNA], with a zero intercept (inset Figure 1). This indicates that DNA-bound copper ions were practically ineffective in catalyzing AH^- oxidation. We have found that DNA-Cu(I) is stable in the presence of oxygen and reacts very slowly with H_2O_2. The effect of DNA was the same whether or not H_2O_2 was included in the reaction mixture.

Effect of DNA on the rate of $(OP)_2Cu(II)$-catalyzed ascorbate oxidation

When DNA was included in the reaction mixture, the rate of ascorbate oxidation catalyzed by $(OP)_2Cu(II)$ was reduced. However, unlike the case of unchelated cop-

TABLE II

Effects of anoxia, catalase, SOD and $OH^\cdot$ scavengers on the rate of ascorbate oxidation catalyzed by $(OP)_2Cu(II)$

Additives	Oxidation rate (μM min)	
	$-H_2O_2$	$+H_2O_2$
	29.0	43.4
Anoxic condition	0.0	37.0
Catalase	2.3	9.2
Heated catalase	12.3	–
Na azide + catalase	5.8	18.5
Na azide	28.4	41.2
SOD	27.6	39.7
Mannitol	26.0	39.0
t-BuOH	29.5	40.6

Ascorbic acid 0.1 mM; (OP 5 μM, $CuSO_4$ 1 μM); H_2O_2 1 mM; phosphate buffer pH 7.4 1 mM at 37°C. Anoxic conditions – under N_2. Catalase 50 mg/l; Na azide 1 mM; SOD 100 mg/l; mannitol and t-BuOH 10 mM.

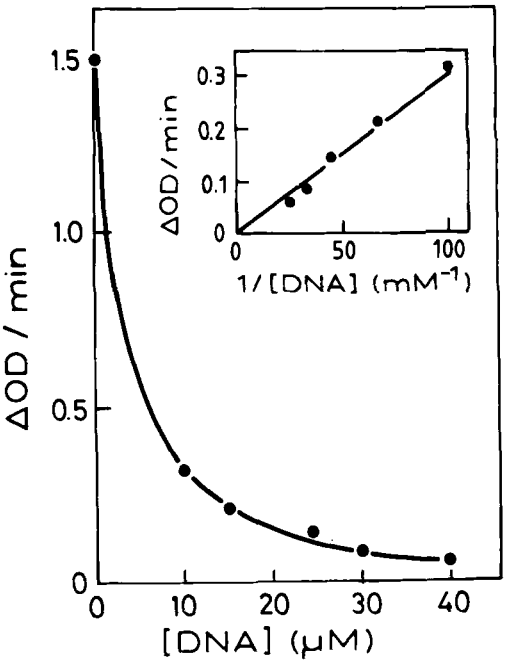

FIGURE 1 Effect of DNA on the rate of ascorbate oxidation catalyzed by copper ions. Ascorbic acid 0.1 mM; $CuSO_4$ 5 μM; H_2O_2 1 mM; phosphate buffer pH 7.4 1 mM, 37°C. Initial reaction rates were measured in the presence of varying [DNA]. Inset: The same data plotted versus 1/[DNA].

per, higher [DNA] were required in order to reduce the oxidation rate in the same proportion (Figures 2 and 5). The final absorption values after the reaction was completed in the presence of DNA leveled off at a higher OD than in the absence of DNA (Figure 2). This hyperchromic effect is due to the higher absorption of degraded DNA at 266 nm as compared to native DNA.

Increasing the ionic strength in the absence of DNA increased the oxidation rate by 37% without H_2O_2 and by 65% in the presence of H_2O_2 (Table I). The effect of ionic strength was much larger in the presence of DNA. Increasing I from 0.002 to 0.1, in the presence of "saturating" concentrations of DNA (> 0.15 mM), increased the initial rate of AH^- oxidation ca. 25 fold, both with and without addition of H_2O_2. AH^- oxidation and DNA cleavage rates continue to decrease with the increase in [DNA] above concentrations at which most of the $(OP)_2Cu(II)$ seems to be bound to DNA (0.2–0.6 mM) (Figure 8). These observations suggest that the ternary complex DNA-$(OP)_2Cu(II)$ may not be reduced directly by AH^-. It is plausible that the reduced ternary complex DNA-$(OP)_2Cu(I)$, is formed through reactions (1) or (2) depending on the [DNA], and DNA double strand breaks are produced by reaction (3).

$$(OP)_2Cu(I) + \text{DNA-}(OP)_2Cu(II) \longrightarrow (OP)_2Cu(II) + \text{DNA-}(OP)_2Cu(I) \quad (1)$$

$$(OP)_2Cu(I) \overset{\text{DNA}}{\rightleftharpoons} \text{DNA-}(OP)_2Cu(I) \quad (2)$$

$$\text{DNA-}(OP)_2Cu(I) + H_2O_2 \longrightarrow \text{DNA-}(OP)_2Cu(II) + OH^- + OH^{\cdot} \quad (3)$$

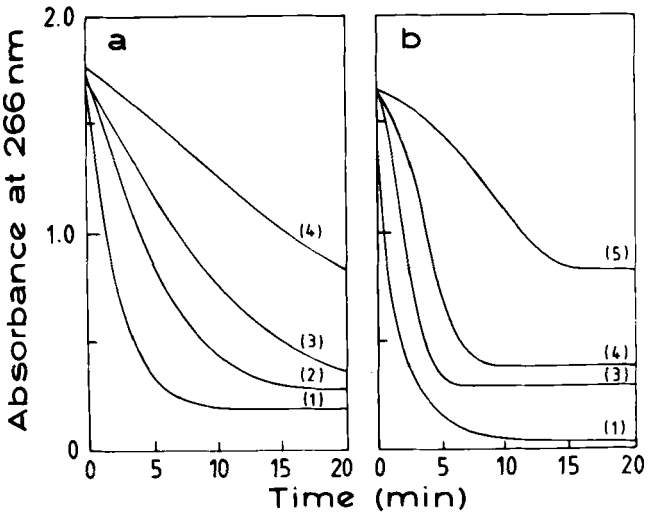

FIGURE 2 Ascorbate oxidation catalysed by $(OP)_2Cu(II)$, effect of DNA concentration and H_2O_2 on the rate and shape of the oxidation curves. Ascorbic acid 0.1 mM; (OP 10 μM, $CuSO_4$ 2 μM); phosphate buffer pH 7.4 1 mM, at 37°C with varying concentrations of DNA: 1 – 0; 2 – 0.05 mM; 3 – 0.10 mM; 4 – 0.15 mM; 5 – 0.25 M. Figure 2(a) – no H_2O_2; Figure 2(b) – with H_2O_2 1 mM.

This assumption is in accord with direct determinations of the rate constants for the reduction of $(OP)_2Cu(II)$ and DNA-$(OP)_2Cu(II)$ by O_2^-.[16,21] The effect of ionic strength on the rate of AH^- oxidation in the presence of DNA (Figure 5) may be due to the decreased stability of the ternary complex, at high I, which increases the concentration of free $(OP)_2Cu(II)$ in the system.

Under low ionic strength and in the presence of both DNA and H_2O_2, the oxidation rate of AH^- by $(OP)_2Cu(II)$ was biphasic and the reaction curve was sigmoidal (Figures 2 and 3). Whereas, when neither H_2O_2 nor DNA was added, or under conditions where the reaction rate was very fast or very slow, no sigmoidal reaction curve was seen. The pattern of the sigmoidal reaction curve depended on [DNA]. Increasing [DNA] increased the duration of the slow phase (Figure 2). We considered the possibility that the sigmoidal oxidation curves in the presence of DNA might be an artifact, resulting from the cleavage of DNA before most of the ascorbate was oxidized. However, the contribution of the DNA hyperchromicity at 266 nm did not exceed 20% (Figure 2). Moreover, the sigmoidal oxidation curve was also observed when oxidation of ascorbic acid was followed indirectly by the ferriphenanthroline assay,[22] confirming its authenticity.

The sigmoidal kinetics might stem from several sources: (1) As proposed by Sigman for the NADH-$(OP)_2Cu(II)$ system,[13] a certain equilibrium distribution among copper chelates such as DNA-$(OP)_2Cu(II)$ and DNA-$(OP)_2Cu(I)$ or some other equilibrium involving copper ions and AH^- needs to be achieved before an optimal reaction rate can be reached. The time required to reach this equilibrium would represent the slow phase of the reaction. (2) Since, $(OP)_2Cu(II)$ is a better catalyst of AH^- oxidation than DNA-$(OP)_2Cu(II)$, it is possible that the fast rate in the sigmoidal curve is achieved after $(OP)_2Cu(II)$ is liberated from the ternary complex upon degradation of DNA. The plausibility of these explanations for the sigmoidal oxidation curve were tested by adding fresh samples of both AH^- and DNA after most of the initial ascorbate had been oxidized. The reaction curve shown in Figure 4(a) was started without

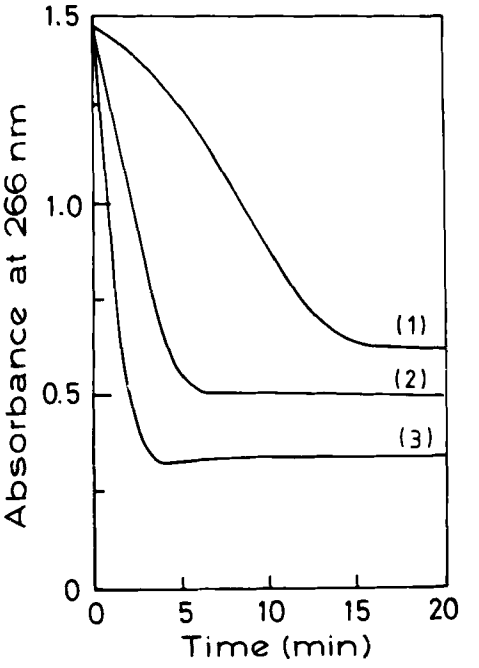

FIGURE 3 Effect of ionic strength on ascorbate oxidation catalyzed by DNA-$(OP)_2Cu(II)$. Ascorbic acid 0.1 mM; DNA 0.25 mM. (OP 10 μM, $CuSO_4$ 2 μM); H_2O_2 1 mM; phosphate buffer pH 7.4 1 mM at 37°C, with varying concentrations of NaCl: 1 — 0; 2 — 10 mM; 3 — 100 mM.

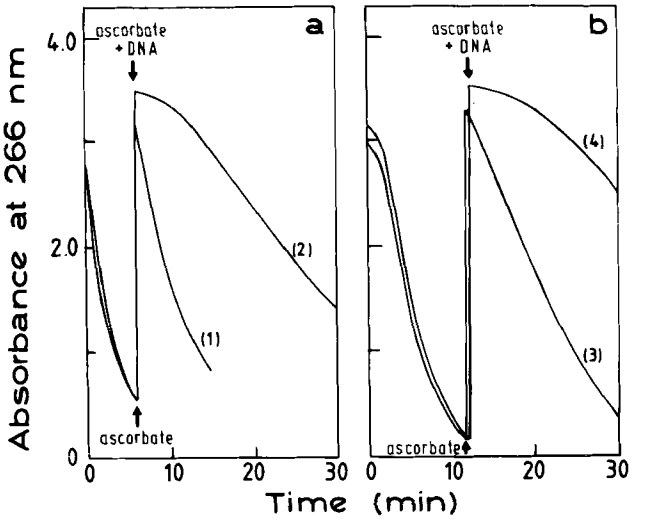

FIGURE 4 Effect of DNA addition on the second round of AH^- oxidation catalyzed by $(OP)_2Cu(II)$. Ascorbic acid 0.2 mM; H_2O_2 0.5 mM; (OP 5 μM, $CuSO_4$ 2 μM) DNA 0.125 mM; phosphate buffer pH 7.4 1 mM. a — Reactions started without DNA. At the time indicated by arrows ascorbate was added to reaction (1) and ascorbate plus DNA was added to reaction (2). b — Reactions started in the presence of DNA. At the time indicated by arrows ascorbate was added to reaction (3) and ascorbate plus DNA was added to reaction (4).

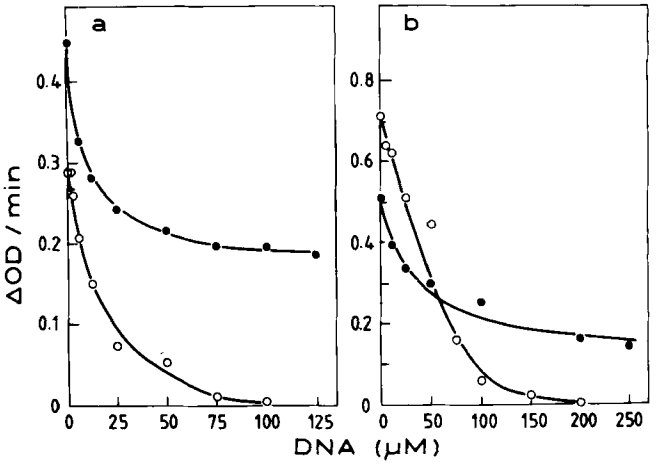

FIGURE 5 Effect of DNA concentration and ionic strength on the rate of ascorbate oxidation catalyzed by $(OP)_2Cu(II)$ or BLM-Fe(III). Ascorbic acid 0.1 mM; phosphate buffer pH 7.4 1 mM and various concentrations of calf thymus DNA. a – (OP 5 μM, $CuSO_4$ 1 μM), b – BLM-Fe(III) 10 μM, (○) – no NaCl added, (●) – 100 mM Nacl added.

DNA. After about 80% of the ascorbate was oxidized, DNA was added, together with a second dose of AH^-. The sigmoidal curve produced was similar to the curve obtained in reactions started in the presence of DNA. The reaction curve shown in Figure 4(b) was started with DNA and AH^-. We allowed the oxidation of ca. 90% of the initial ascorbate to proceed and then added a second dose of ascorbate. The fast phase was achieved immediately, no sigmoidal curve was produced, and the oxidation rate resembled that observed in the absence of DNA. However, when a second sample of both DNA and AH^- was added, the sigmoidal behavior was manifested again.

These observations rule out the possibility that the sigmoidal curve and the lag period represent the time needed for the achievement of a required equilibrium or the increased ionic strength consequent on DNA degradation. This behavior agrees, instead, with the assumption that the rate of AH^- oxidation is slow as long as highly polymerized DNA is present and that the reaction accelerates as DNA is degraded.

AH^- oxidation by iron-bleomycin

Unlike $(OP)_2Cu(II)$, the bleomycin chelate of copper did not catalyze the oxidation of AH^-. This is not surprising in view of the high redox potential of BLM-Cu(II).[24] In fact, the addition of BLM to the AH^- Cu(II) system blocked the catalytic activity of copper ions. Similarly, BLM-Mn(II) had almost no effect on the auto-oxidation of AH^-. On the other hand, BLM greatly enhanced the rate of AH^- oxidation by Fe(II) and Fe(III). The reaction was approximately first order with respect to [BLM-iron]. The oxidation rate at low $[AH^-]$ increased linearly with $[AH^-]$. However, upon increasing $[AH^-]$ beyond 0.1 mM the rate of the reaction leveled off and the reaction order was between 1 and zero, indicating a complex reaction mechanism. AH^- oxidation did not take place in the absence of oxygen and, in contrast to the reaction catalyzed by $(OP)_2Cu(II)$, addition of H_2O_2 to the anoxic system did not replace

oxygen. In the presence of air, the addition of H_2O_2 enhanced the effect of BLM-Fe(II) and BLM-Fe(III), increasing the initial rate by approximately 30%. Copper ions blocked the catalysis by BLM-iron yet BLM-copper had no effect at all. Upon increasing the ionic strength (from 0.002 to 0.1) the activity of BLM-iron was moderately decreased (−30%), in contrast to the effect of increased ionic strength on the catalytic activity of $(OP)_2Cu(II)$ (Table I and Figure 5). The BLM-iron catalysis of AH^- oxidation was not affected by SOD, catalase or OH radical scavengers.

Effect of DNA on AH^- oxidation catalyzed by BLM-iron

The addition of DNA decreased the catalytic activity of both BLM-Fe(II) and BLM-Fe(III), indicating that the ternary complex DNA-BLM-iron was relatively inactive as a catalyst in the oxidation of AH^- (Figure 5b). Oxygen was essential for the catalytic reaction. SOD, catalase and hydroxyl radical scavengers (mannitol, t-BuOH, DMSO, 10 mM each) had no effect on the reaction rate in the presence of DNA.

In the presence of DNA the dependence of the oxidation rate on ionic strength was very marked. Although an increase in the ionic strength from 0.002 to 0.1, in the absence of DNA, lowered the oxidation rate, high I increased the oxidative activity of the ternary complex DNA-BLM-Fe(III) (Figure 5b).

At low ionic strength and in the absence of H_2O_2, the AH^- oxidation catalyzed by the DNA-BLM-iron complex was biphasic. A sigmoidal reaction curve was observed in which a very slow reaction rate (lag phase) was followed by a fast oxidation phase resembling the sigmoidal curves observed with the DNA-$(OP)_2Cu(II)$ ternary complex. The duration of the lag phase increased with [DNA]. At very high [DNA] (e.g. [DNA]/[BLM-iron] > 10), when the reaction was exceedingly slow, and upon increase of the ionic strength to 0.1, when the reaction was fast, AH^- oxidation curves seemed to be non-sigmoidal, as was the case with DNA-$(OP)_2Cu(II)$.

Ascorbate oxidation and DNA degradation

The effect of metal chelates on the formation of DNA double strand breaks (dsb), which accompanies the oxidation of AH^-, was studied by electrophoresis of the DNA on agarose gels.

AH^- oxidation catalyzed by Cu(II) ions, OP-Fe(II) and EDTA-Fe(III) was accompanied by degradation of the DNA present in the reaction mixture. Both copper ions and EDTA-Fe(III) catalyzed the formation of dsb, provided H_2O_2 was added. However, these dsb were not observed when the DNA/metal molar ratio exceeded 10:1 and most of the metal ions in the reaction mixture were in complex with DNA (Figure 1). In contrast, both OP-copper and BLM-iron, in concentrations at which the metal was predominantly present as a ternary complex and AH^- oxidation was very slow, caused extensive cleavage of DNA. This indicates that the DNA complexes of Cu(II) or EDTA-Fe(III) are inactive, both as catalysts of AH^- oxidation and in the formation of the active species which cause DNA damage.

Figure 6 shows time and component concentration effects on DNA scission catalyzed by $(OP)_2Cu(II)$. With BLM-Fe(III), as with $(OP)_2Cu(II)$ the formation of dsb was dependent on the oxidation of ascorbate. In the absence of ascorbate or under anoxic conditions where no ascorbate oxidation took place, no DNA cleavage was observed. The rate of DNA cleavage was proportional to [AH^-] and to [OP-copper] or [BLM-iron]. In the case of BLM-iron, however, increasing AH^- beyond 2 mM

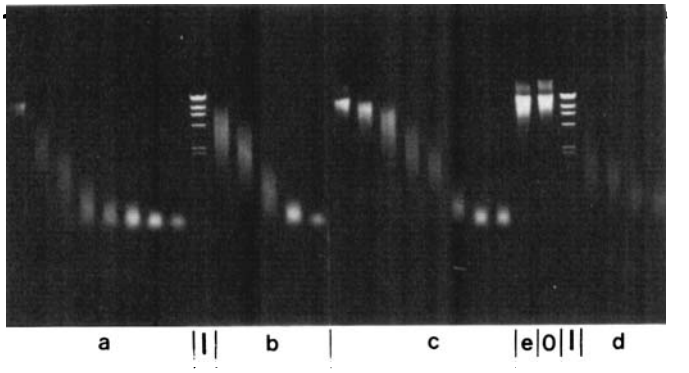

FIGURE 6 DNA scission by $(OP)_2Cu(II)$; time and component concentration effects. Ascorbic acid 1 mM; (OP 5 μM, $CuSO_4$ 1 μM), H_2O_2 0.5 mM; NaCl 10 mM; phosphate buffer pH 7.4 1 mM, calf thymus DNA 0.25 mM. Incubation time: 1 min, at 37°C. a. Time response – incubation time at 37°C; o, 0.25, 0.5, 1, 1.5, 2, 3, 4 min. b. Response to $[(OP)_2Cu(II)]$: 0.125, 0.25, 0.5, 1, 2 μM. c. Response to $[H_2O_2]$: 0, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2 mM. d. Response to [ascorbate]: 0.06, 0.125, 0.25, 0.5 mM. e. As in c with 2 mM H_2O_2 but without $(OP)_2Cu(II)$. l. Molecular weight standard: lambda DNA Hind III digest 1 μg. o. Untreated DNA 1 μg.

slowed down DNA scission. In fact, we took advantage of this feature in order to stop the BLM-iron catalyzed reaction (see Methods). The metal chelator DTPA effectively binds metal ions but does not sequester the iron from the activated BLM-iron chelate.[27] Therefore, any activated chelate would continue to cleave DNA even in the presence of DTPA. However if 2 mM AH^- or iodide were added together with DTPA, the activated complex would be reduced and DNA cleavage would be stopped.

Figure 7 relates AH^- oxidation with the time course of DNA degradation catalyzed by BLM-Fe(III). It can be seen that DNA cleavage was completed within the slow phase of ascorbate oxidation before an appreciable fraction of the ascorbate present has decayed. After DNA was cleaved to fragments smaller than 125 base pairs, ascorbate oxidation accelerated and continued at a much higher rate, indicating that

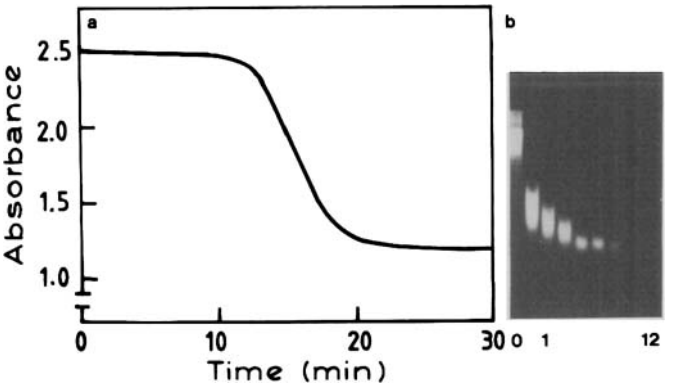

FIGURE 7 Correlation of AH^- oxidation and DNA scission catalyzed by BLM-Fe(III). Ascorbic acid 0.1 mM; BLM-Fe(III) 30 μM; calf thymus DNA 0.1 mM; phosphate buffer pH 7.4 1 mM at 37°C. Figure 7(a) AH^- oxidation curve. Figure 7(b) DNA scission: 0 – untreated DNA 1 μg, 1–12 DNA migration profiles at various incubation times: 0.25, 1, 4, 6, 8, 10, 12 min.

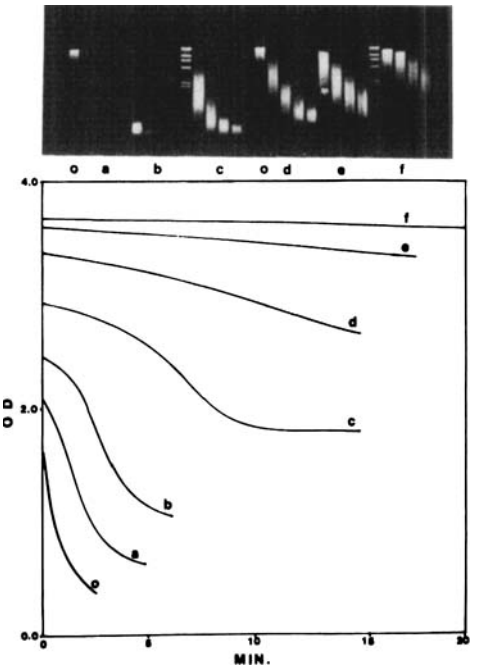

FIGURE 8 Ascorbate oxidation and DNA scission rates as a function of DNA concentration. Ascorbic acid 0.1 mM; H_2O_2 1 mM; (OP 10 μM, $CuSO_4$ 2 μM); phosphate buffer pH 7.4 1 mM at 37°C. [DNA] mM: 0 – no DNA; a – 0.05; b – 0.1; c – 0.2; d – 0.3; e – 0.4; f – 0.5. Electrophoretic migration profiles of reaction mixture samples containing equal amounts of DNA (1 μg). Incubation times: 1, 2, 3, 4 min. 0 – untreated DNA.

the DNA-BLM-Fe(III) ternary complex is a much less efficient catalyst for the oxidation of AH^- than are BLM-iron and the ternary complex of BLM-iron with small fragments of DNA. The same time relation between DNA degradation and the onset of the rapid phase of AH^- oxidation was found with DNA-$(OP)_2Cu(II)$. These observations suggest that the sigmoidal ascorbate oxidation curves result from the very low activity of the ternary complexes as catalysts of AH^- oxidation, in comparison with the DNA-free metal chelates, and the relatively high efficiency of the ternary complexes as DNA cleaving agents. At high [DNA], but without deliberate addition of H_2O_2, both AH^- oxidation and DNA scission with $(OP)_2Cu(II)$ are very slow (Figures 2, 6 and 10), resulting in non-sigmoidal curves of AH^- oxidation (Figure 2).

DNA scission through complexes of DNA with $(OP)_2Cu(II)$ or BLM-Fe(III)

The rate of DNA scission with $(OP)_2Cu(II)$ or BLM-iron was about 100 fold higher than the rate of scission with unchelated copper ions or with EDTA-Fe(III) respectively, whereas the rate of AH^- oxidation was increased only about two fold (Table I). Moreover, BLM-Fe(III) and EDTA-Fe(III), at concentrations which exerted similar rates of ascorbate oxidation, catalyzed DNA scission to different degrees. This differential effect on AH^- oxidation versus DNA cleavage, together with the lack of effect of hydroxyl radical scavengers on both AH^- oxidation (Table II) and DNA degradation (Figures 10 and 11), suggested both a site specific reaction and that

cleavage of DNA through the ternary complex is more efficient than cleavage through free metal ions or other metal chelates. In order to test this assumption, we measured the rates of DNA scission and AH^- oxidation as a function of DNA concentration (Figure 8).

It can be seen that although the concentration of DNA-$(OP)_2Cu(II)$ does not change significantly in the range of 0.3–0.5 mM DNA, the rates of oxidation and DNA scission continue to decline with the increase in [DNA] and concomitant decrease in [$(OP)_2Cu(II)$]. This supports the suggestion that the ternary complex is reduced through reactions (1) and (2). DNA double strand breaks are produced through the reaction of $(OP)_2Cu(I)$ or DNA-$(OP)_2Cu(I)$ with H_2O_2, and we have shown that these reactions proceed at a similar rate.[16] Assuming that these two reactions have the same proficiency in producing dsb, no effect of [DNA] on the rate of dsb formation would be expected, contrary to the results shown in Figure 8. However, if the reaction of DNA-$(OP)_2Cu(I)$ with H_2O_2 were more efficient in producing dsb, one would expect the number of dsb per the amount of AH^- oxidized to increase with [DNA]. This was tested as shown in Figure 9. The number of dsb/μmole AH^- consumed at high [DNA] (0.4 mM) was clearly greater than the number of dsb produced at 0.05 mM DNA, where only about 80% of the $(OP)_2Cu(II)$ was bound to DNA. The increase in efficiency of dsb formation upon increase of [DNA] supports the assumption that DNA cleavage proceeds through DNA-$(OP)_2Cu(I)$, in agreement with proposed "site-specific" mechanisms.[28]

Factors affecting DNA scission

SOD and OH radical scavengers such as t-BuOH, formate and DMSO had no effect either on the rates of AH^- oxidation or DNA cleavage induced by $(OP)_2Cu(II)$ or BLM-Fe(III), although mannitol diminished DNA scission slightly (Figures 10 and 11). The two metal chelates exhibited similarities in their behavior as catalysts of ascorbate oxidation and DNA cleavage, but they differed in two respects:

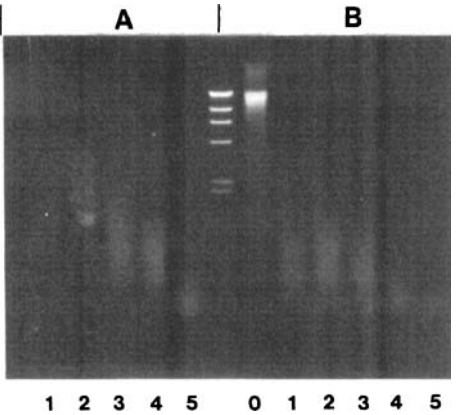

FIGURE 9 Effect of [DNA] on $(OP)_2Cu(II)$ catalyzed scission of DNA at various [DNA] and constant AH^- consumption. Ascorbic acid 0.1 mM; (OP 5 μM, $CuSO_4$ 1 μM); H_2O_2 1 mM; phosphate buffer pH 7.4; calf thymus [DNA] mM: 0 – untreated DNA. 1 – 0.05; 2 – 0.1; 3 – 0.2; 4 – 0.4; 5 – 0.5. Various times of incubation at 37°C: A – electrophoretic migration profiles after oxidation of 2 nmoles of ascorbate. B – electrophoretic profiles after oxidation of 4 nmoles of ascorbate.

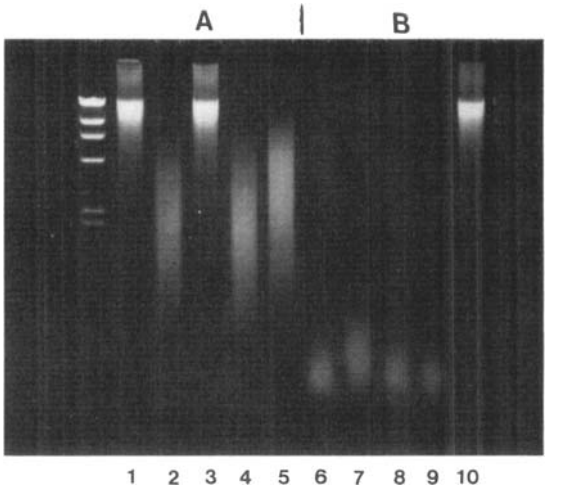

FIGURE 10 DNA scission by $(OP)_2Cu(II)$ – effects of H_2O_2, catalase, SOD and $OH^{\cdot}$ scavengers. Ascorbic acid 0.2 mM; (OP 5 μM, $CuSO_4$ 1 μM); H_2O_2 1 mM; NaCl 10 mM; phosphate buffer pH 7.4 1 mM; calf thymus DNA 0.25 mM; catalase 75 mg/l; SOD 100 mg/l; Na azide 1 mM; Mannitol and t-BuOH 10 mM at 37°C. A – Without added H_2O_2, incubation time 10 min. Additions were: 2 – no addition; 3 – catalase; 4 – catalase plus azide; 5 – SOD. B – With H_2O_2 added, incubation time 1 min. Additives were: 6 – no addition; 7 – mannitol; 8 – t-BuOH; 9 – SOD; 10 – as in 6 but without ascorbate; 1 – untreated DNA.

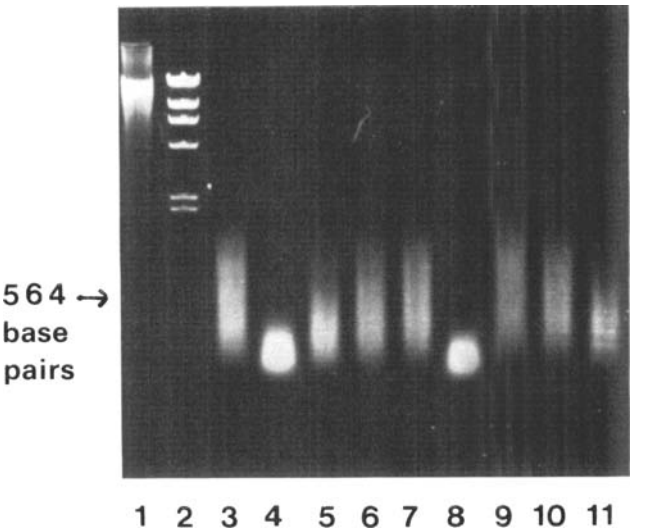

FIGURE 11 DNA scission by BLM-Fe(III) – effects of catalase, SOD and $OH^{\cdot}$ scavengers. 1. Control, untreated calf thymus DNA 1 μg. 2. Lambda DNA Hind III digest, molecular weight standard 1 μg. 3. Calf thymus DNA 0.25 mM; BLM-Fe(III) 1 μM; NaCl 100 mM; phosphate buffer pH 7.4 1 mM; incubation, 2 min at 37°C. 4. 3 + catalase 50 mg/l. 5. 4 + Na azide 1 mM. 6. 3 + SOD 100 mg/l. 7. 3 + autoclaved SOD 100 mg/l. 8. 4 + SOD 100 mg/l. 9. 3 + mannitol 10 mM. 10. 3 + t-BuOH 10 mM. 11. 3 + DMSO 10 mM.

1) With $(OP)_2Cu(II)$, H_2O_2 was essential for both AH^- oxidation and DNA scission and it replaced oxygen under anoxic conditions. Catalase inhibited both these reactions. With BLM-iron, H_2O_2 did not replace oxygen in anoxia; it enhanced AH^- oxidation but almost totally blocked DNA cleavage. Therefore, addition of catalase enhanced DNA degradation. The inhibition of DNA degradation by H_2O_2 could result from the known sensitivity of BLM-iron to inactivation by H_2O_2.

2) DNA cleavage and AH^- oxidation induced by the two catalysts were also oppositely affected by ionic strength. Increasing I enhanced AH^- oxidation by $(OP)_2Cu(II)$ but slightly inhibited the reaction by BLM-Fe(III). DNA scission was oppositely affected; increase in I up to 0.1 enhanced DNA cleavage by BLM-iron but inhibited cleavage by OP-copper. Higher ionic strength (> 0.5 M NaCl) inhibited DNA cleavage by BLM-Fe(III), as also observed by Sausville *et al.*[25] Figure 12 shows the effects of I on the degradation of lambda DNA by OP and BLM. High ionic strength produces a compact conformation of DNA. Since $(OP)_2Cu(II)$ binds to the bases of ds DNA, we suppose that at high I, when DNA is in the closed form, less DNA-$(OP)_2Cu(II)$ would be formed and more $(OP)_2Cu(II)$ would remain free. This is consistent with our observations that the AH^- oxidation rate is increased but DNA cleavage is relatively inhibited. In the case of BLM the form of binding is not well understood. The side chain is known to be involved in the binding to DNA, although

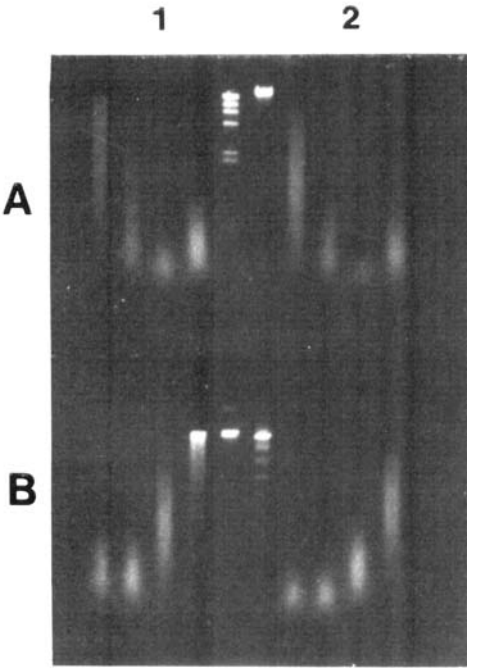

FIGURE 12 Effect of ionic strength on the degradation of lambda DNA with $(OP)_2Cu(II)$ and BLM-Fe(III). Ascorbic acid 1.mM; phosphate buffer pH 7.4 1.mM; lambda DNA 0.075 mM at 37°C. A – BLM-Fe(III) 1 μM, incubation time: 1–4 min, 2–8 min from left to right; in o, 10, 100 :and 500 mM NaCl, B – $(OP)_2Cu(II)$ 1 μM; H_2O_2 1 mM incubation time: 1, 0.5 min, 2, 1 min from left to right; in 0, 10, 100 and 500 mM NaCl.

there is some evidence suggesting that a part of the BLM molecule may intercalate between the DNA base pairs.[19] It is therefore difficult to attribute the increased DNA scission activity at high I to some specific conformational change in the DNA.

DNA degradation by copper chelates, H_2O_2 and radiolytically produced O_2^-

We compared the rates of DNA cleavage induced by copper ions, $(OP)_2Cu(II)$ and $(BPY)_2Cu(II)$ with H_2O_2 and radiolytically produced O_2^-. Air saturated solutions of 0.5 mM DNA were irradiated in the presence of 10–20 mM formate, H_2O_2, and copper chelates, using a series of pulses generated by a linear accelerator. Under these experimental conditions the predominant radical found in the system was the superoxide anion. Following irradiation, DNA was electrophoresed on agarose gel and the extent of DNA damage produced (ssb and dsb) was evaluated. Pulse radiolysis of aerated solutions of DNA in the absence of formate, conditions which produce about equal amounts of O_2^- and $OH^{\cdot}$, induced extensive nicking and many dsb. When formate was included in the irradiated solution no dsb was observed even in the presence of 1 mM H_2O_2. A few ssb, common to H_2O_2, were observed. This shows that O_2^-, as such, does not effectively cause dsb, whereas OH radicals induce dsb efficiently (Figure 13). When $(OP)_2Cu(II)$ and H_2O_2 were included in the irradiated solutions, the

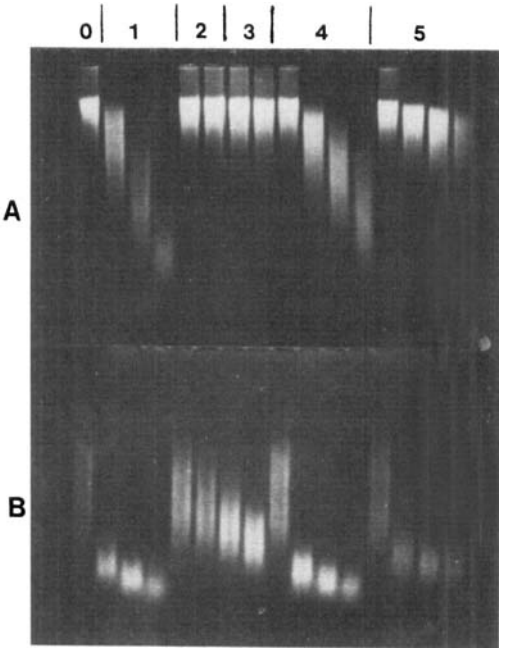

FIGURE 13 DNA damage induced by copper chelates, H_2O_2 and radiolytically produced O_2^-. Na formate 20 mM; (OP 20 μM, $CuSO_4$ 7.5 μM); (BPY 20 μM, $CuSO_4$ 7.5 μM); H_2O_2 1 mM; phosphate buffer pH 7.4 1 mM; calf thymus DNA 0.5 mM. In the absence of formate 1 pulse produced 8.5 μM $OH^{\cdot}$. In the presence of formate 1 pulse produced 20 μM O_2^-. Electrophoretic profile of irradiated DNA analyzed for: A – double strand breaks; B – single strand breaks. 0 – untreated DNA irradiated in the presence of: 1 – buffer; 1, 2 and 4 pulses, 2 – formate and OP; 2 and 4 pulses, 3 – formate, OP and H_2O_2; 2 and 4 pulses, 4 – formate, OP, $CuSO_4$ and H_2O_2; 0, 1, 2 and 4 pulses, 5 – formate, BPy, $CuSO_4$ and H_2O_2; 0, 1, 2 and 4 pulses.

formation of ssb and dsb, was markedly enhanced, showing that $(OP)_2Cu(II)$ catalyzes the formation of dsb in the presence of O_2^- and H_2O_2. Comparing the catalytic activities of 7.5 μM $(OP)_2Cu(II)$ and $(BPY)_2Cu(II)$ we found that both induced a roughly similar number of ssb, although OP-copper was somewhat more effective than $(BPY)_2Cu(II)$ (Figure 13(b)). However, $(OP)_2Cu(II)$ gave rise to more dsb than $(BPY)_2Cu(II)$ (Figure 13(a)). Under similar conditions, Que *et al.*[15] and Marshall *et al.*[13] fond that OP cleaved DNA while BPY did not. The difference between the two copper complexes was explained on kinetic grounds.[16,21] We calculated that the oxidation of Cu(I) proceeds in the case of OP through the reaction of DNA-$(OP)_2Cu(I)$ with H_2O_2 whereas with BPY the oxidation of Cu(I) by H_2O_2 proceeds mainly through $(BPY)_2Cu(II)$. Thus, with OP most of the $OH^{\cdot}$ radicals are formed at the binding site, while with BPY the majority of the $OH^{\cdot}$ radicals are formed in the bulk. This site specific reactivity explains the high efficiency by which $(OP)_2Cu(II)$ catalyzes the production of dsb in the presence of H_2O_2 and either O_2^- or ascorbate as reductants.

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