Free Rad. Res., Vol. 20, No. 4, pp. 205-218
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COPPER-LIGAND INTERACTIONS AND PHYSIOLOGICAL FREE RADICAL PROCESSES. PART 2. INFLUENCE OF Cu^{2+} IONS ON Cu^{+}-DRIVEN $^{\cdot}OH$ GENERATION AND COMPARISON WITH THEIR EFFECTS ON Fe^{2+}-DRIVEN $^{\cdot}OH$ PRODUCTION

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(Received August 2nd, 1993; in revised form October 18th, 1993)

In our search to establish a reference $^{\cdot}OH$ production system with respect to which the reactivity of copper(II) complexes could then be tested, the influence of free Cu^{2+} ions on the Cu^{+}/H_2O_2 reaction has been investigated.

This influence depends on the $C_{Cu^{2+}}/C_{Cu^{+}}$ ratio. At low Cu^{2+} concentrations, $^{\cdot}OH$ damage to various detector molecules decreases with increasing Cu^{2+} concentrations until $C_{Cu^{2+}}/C_{Cu^{+}}$ reaches unity. Above this value, $^{\cdot}OH$ damage increases sharply until $C_{Cu^{2+}}/C_{Cu^{+}}$ becomes equal to 5 with salicylate and 2 with deoxyribose, ratios for which the protective effect of Cu^{2+} cancels. Finally, at higher concentrations, Cu^{2+} ions logically add their own $^{\cdot}OH$ production to that normally expected from Cu^{+} ions. The possible origin of this unprecedented alternate effect has been discussed.

The possible influence of Cu^{+} ions on the generation of $^{\cdot}OH$ radicals by water gamma radiolysis has also been tested and, as already established for Cu^{2+} in a previous work, shown to be nonexistent. This definitely confirms that either form of ionised copper cannot scavenge $^{\cdot}OH$ radicals in the absence of a ligand.

KEY WORDS: Hydroxyl radical, Fenton reaction, gamma radiolysis, Cu^{2+} ions, Cu^{+} ions, ESR.

INTRODUCTION

Except in the case of high energy radiation, the highly reactive hydroxyl radicals produced *in vivo* are presumed to be generated by Fenton-like reactions involving transition metal ions.[1] The most likely process leading to $^{\cdot}OH$ physiological production is considered to require hydrogen peroxide and a reduced iron complex.[2,3] Hydrogen peroxide has also been ascribed a reductant role versus oxidised iron in Fenton chemistry.[4,5] However, the direct reduction of ferric complexes by H_2O_2 has recently been questioned on the basis of its inhibition by superoxide dismutase (SOD),[1,2,6,7] the actual process involving $O_2^{\cdot-}$ as an intermediate.

Like ferrous iron, copper in the reduced state can generate free $^{\cdot}OH$ radicals by reacting with hydrogen peroxide,[8,9] and with a much greater rate constant.[8]

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Hydrated cupric ions in the presence of a reducing agent and hydrogen peroxide have also been shown to catalyse the production of $^{\cdot}OH$ radicals *in vitro*.[10] Cu^{2+} ions can trigger Fenton-like reactions even in the absence of a reducing agent, but to a much lesser extent[10] – though more effectively than Fe^{3+} ions.[11] As with iron,[2,7] the reduction step apparently attributable to H_2O_2 would be due to the superoxide anion in that case.[12] An interesting point concerning copper comparatively to iron is its highly site-specific activity as a Fenton catalyst.[10,11,13] Endogenous ligands like albumin and amino acids would thus prevent it from inducing "free" hydroxyl radical formation *in vivo*.[10,11]

In addition to its potential role in Fenton chemistry, copper is involved in various oxygen free radical reactions. In particular, the well documented pharmacologic activity of copper complexes[14] against diseases with an inflammatory component is thought to derive from the SOD-like properties of these compounds[15,16] whose small size would favour membrane diffusion.[15] In this connection, copper complexes have even been proposed as the active metabolites of nonsteroidal anti-inflammatory drugs (NSAIDs).[17] On the other hand, most NSAIDs quickly react with $^{\cdot}OH$ radicals,[18] and the fact that free radical trapping of salicylic acid to give dihydroxybenzoic acid is directly related to the amount of Cu^{2+} ions bound to the salicylate anion[19] suggests that copper NSAIDs complexes may also play a role with respect to $^{\cdot}OH$ radicals in inflammation.[20] It has recently been proposed that copper redox complexes of $^{\cdot}OH$-inactivating-ligands (OILs) are potentially "catalase-like" antiinflammatory agents acting as "lures" for the Fenton reaction *in vivo*.[21]

Testing the latter hypothesis requires in the first place that the predominant copper-NSAID complexes likely to form at therapeutic levels *in vivo* can be identified.[22,23] Moreover, reference standardised systems for the production of $^{\cdot}OH$ radicals *in vitro* must also be established with respect to which the reactivity of previously identified copper complexes could then be tested.

With this in view, a preliminary study of the influence of Cu^{2+} ions on $^{\cdot}OH$ superoxide-independent generation through water gamma radiolysis and Fe^{2+}-driven Fenton assays has recently been performed.[24] Without any effect on $^{\cdot}OH$ radicals selectively produced by radiolysis, Cu^{2+} ions have, however, been found to influence $^{\cdot}OH$ generation through Fenton reactions, but in an alternate manner depending on copper concentration. The Fe^{2+}-driven Fenton reaction was thus not considered the best system to test copper complex reactivities.[24]

As an alternative to the above system, the possibility of using the reaction of Cu^{+} ions with hydrogen peroxide to produce $^{\cdot}OH$ radicals under standardised conditions is examined in the present paper. In particular, variations of the amount of hydroxyl radicals generated in the presence of different concentrations of Cu^{2+} ions has been studied, with reference to the effect of the same ions on the yield of the Fe^{2+}-driven Fenton reaction.

MATERIALS AND METHODS

Reagents

CuCl (99.99% pure) was obtained from Aldrich; the absence of contaminating iron was checked by atomic absorption spectrophotometry. $CuSO_4.5H_2O$ and $FeSO_4.7H_2O$ were Normapur Prolabo reagents.

1,10-orthophenanthroline was a Prolabo pro analysis product. Salicylic acid (SLA), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Aldrich. Pyrocatechol was a Rectapur Prolabo reagent. 2-deoxy-D-ribose and 2-thiobarbituric acid (TBA) were from Sigma, hydrogen peroxide from Gifrer or Prolabo.

Each stock solution (1 mg/ml) of single- and double-stranded calf thymus DNA was treated with Chelex resin to remove contaminating metal ions and centrifuged to remove the resin before use. The amount and purity of the DNA contained in each sample was estimated from the absorbances at 260 and 280 nm (A_{260} = 1.000 corresponding to 50 μg of double-stranded DNA/ml).

All solutions were prepared from triply deionised and freshly deaerated water (pH 5-6). 3% (w/v)(10 vol) H_2O_2 stock solutions were prepared from 30% (w/v) (110 vol) parent solutions and regularly titrated against $KMnO_4$. Sodium stannate was used as a H_2O_2 stabiliser.

0.8 × 4 cm Poly-Prep columns from Bio-Rad filled with AG 50 W-X8 cation exchange resin were used to remove metal ions from samples before TBA test.[25]

Technical Equipment

Continuous radiolysis experiments were carried out on a ^{60}Co gamma source at the Claudius Regaud Centre in Toulouse.

The Philips HPLC equipment used to analyse hydroxylated aromatic derivates consisted of a PU 4100 solvent delivery pump, a 7125 Rheodyne valve fitted with a 20 μl injection loop, and a PU 4110 UV-detector. A reverse-phase column Bischoff 250 × 4 mm Nucleosil C_{18} (5 μm) was used for separation.

The colorimetric tests were carried out on a Perkin-Elmer Lambda 5 UV-Vis spectrophotometer.

ESR spectra were recorded at room temperature on a Brücker spectrometer Model ER200TT (X-band, 100 kHz field modulation). The samples were introduced into the ESR cavity in Vitrex 50 μl pre-calibrated pipettes filled by capillarity.

$^{\cdot}OH$ Radical Production

Fenton assays Cuprous chloride solutions were prepared under purified nitrogen by dissolving CuCl in freshly deaerated and acidified water, or directly in the presence of the detector molecule; the solution obtained was then ultrasonicated.

Continuous gamma-radiolysis Radiolysis experiments were performed on a ^{60}Co gamma source at a dose rate of 1.2 Gy.min^{-1}. The delivered dose ranged from 60 to 80 Gy as measured by ionisation dosimetry; the values found were in agreement with the results obtained using the $(op)_2$-Cu^{2+} dosimeter, assuming the yield of $(op)_2$-Cu^{+} to be G = 6.05 and ε = 6770 M^{-1}.cm^{-1} at 435 nm.[26,27]

The dimensions of the irradiation field were chosen to make scattering effects negligible (< 1%). Six glass tubes with a tight-fitting screw cap were placed on a plastic rack and dipped into a water bath at room temperature before exposition to the gamma-beam. The results were compared to appropriate non-irradiated blanks.

Experimental conditions were specifically chosen so that $^{\cdot}OH$ species were largely predominant among all the radicals produced, and adapted to each method of detection, i.e.:

- in the salicylate hydroxylation assay, the solutions were saturated with ultrahigh-purity N_2O,
- in the TBA test, deoxyribose or DNA solutions were purged of O_2 with N_2.

·OH Radical Detection

Salicylic Acid Hydroxylation Assay

(a) Fenton assays To new clean glass tubes were added the following reagents in the order stated:

- 0.8 ml salicylic acid (final concentration 5 mM),
- 0.2 ml of distilled water or 0.10 M phosphate-saline buffer in 0.15 M NaCl,
- and, under a N_2 blanket, CuCl ($0.05 < C_{Cu^+} < 1$ mM) or $FeSO_4$ ($0.1 < C_{Fe^{2+}} < 1$ mM).

Where indicated, 0.2 ml of specific reagent was added instead of distilled water or buffer. The reaction was initiated by adding H_2O_2 (final concentration 0.88 mM). After incubation in the dark at room temperature for 20 min, the reaction was stopped by adding 100 µl of H_2SO_4 10 N.

Two methods were used to detect the di-hydroxy products generated by the addition of hydroxyl radicals onto the aromatic ring:

(i) Colorimetric test
The test described by Richmond *et al.*[28] was used to detect the ortho-dihydroxylated products (2,3-DHBA and catechol). All the results shown are means of duplicates which differed by less than 5% after subtraction of appropriate blanks. They are expressed as control percentages, the 100% reference corresponding to the amount of dihydroxylated products generated with 0.1 mM $FeSO_4$.

(ii) HPLC
Both ortho (2,3-DHBA + catechol) and para (2,5-DHBA) dihydroxylated derivates can be detected by HPLC. The aromatic compounds, generated in the same experimental conditions as in the colorimetric test, were determined as recommended by Maskos *et al.*[29]

(b) Gamma-radiolysis 8 ml of a solution of 10 mM Na^+-salicylate ($4.5 < pH < 6.5$) were exposed to the gamma-beam (60 Gy < delivered dose < 80 Gy) in the absence or presence of copper ($C_{Cu^+} < 0.5$ mM) or iron ($C_{Fe^{2+}} < 0.5$ mM). The products were detected exactly as in the Fenton assays.

Degradation of Deoxyribose and DNA

(a) Fenton assays

(i) Degradation of deoxyribose
The following reagents were added to new clean glass tubes in the order stated:

- 1 ml deoxyribose (final concentration 5 mM),
- 0.5 ml of distilled water or phosphate buffer,
- and cuprous chloride ($C_{Cu^+} < 0.2$ mM) or iron sulfate ($C_{Fe^{2+}} < 0.2$ mM).

To make the test metal-compatible, interfering cations were removed by passage through a Poly-Prep column filled with cation exchange resin.[25]

TBA-reactivity was then measured as follows: 0.5 ml of 2.8% (w/v) trichloroacetic acid and 0.5 ml of 1% (w/v) thiobarbituric acid in 0.050 N NaOH were added to each glass tube (1.8 × 18 cm) before heating at 100°C for 5 minutes.

The resulting pink chromogen was assessed against appropriate blanks:

- by electronic absorption spectrophotometry at 532 nm in the deoxyribose degradation test,
- or for low levels of TBA-reactivity, by fluorimetry (excitation 515 nm, emission 553 nm).

(ii) Degradation of DNA

The most extensively investigated modifications of DNA by oxygen free radicals are mutagenic single-base substitutions. We have chosen to focus our attention on the damage to the sugar moiety. Single- or double-stranded DNA was substituted for deoxyribose and the maximum concentrations of metal lowered to 0.100 mM.

The reaction conditions were as above except that 28% trichloroactic acid was substituted for 2.8% w/v and low levels of fluorescence were measured at 532 nm excitation and 553 nm emission.[25,30] Determinations relative to duplicate samples were reproducible within 5%, the results being expressed as percentages of the TBA-reactive material released in the presence of iron.

In both deoxyribose and DNA degradation assays, the reaction was started by addition of hydrogen peroxide at a final concentration of 0.88 mM, and resulting mixtures were incubated in the dark at 37°C in a shaking waterbath for 30 min.

(b) Gamma-radiolysis

(i) Deoxyribose degradation

8 ml of 5 mM deoxyribose with or without varying concentrations of Cu^{+} ($C_{Cu^{+}} < 0.100$ mM) or Fe^{2+} ($C_{Fe^{2+}} < 0.100$ mM) were irradiated as described above. The formation of free malondialdehyde (MDA or propanedi-al) was detected by UV-absorption at 270 nm.[30]

(ii) DNA degradation

8 ml of double- or single-stranded DNA (0.25 and 0.5 $mg.ml^{-1}$ respectively) with or without added metal ions: Cu^{+} ($C_{Cu^{+}} < 0.030$ mM) or Fe^{2+} ($C_{Fe^{2+}} < 0.030$ mM) were gamma-irradiated (60 Gy < delivered dose < 80 Gy).

In both tests, the TBA-reactivity was measured and expressed exactly as described in the Fenton assays.

DMPO spin trapping All ESR spectra were recorded at room temperature. The spin trap DMPO was purified on charcoal before use, as described by Floyd.[31]

Reaction mixtures (total volume 0.360 ml) contained 100 mM DMPO, CuCl ($C_{Cu^{+}} < 0.100$ mM) or $FeSO_4$ ($C_{Fe^{2+}} < 0.050$ mM). The reaction was initiated by the addition of H_2O_2 up to a concentration of 0.3 mM. Experimental specifications were as described previously.[24]

The signal intensity of the spin adduct DMPO$^{\cdot}$-OH was measured at different concentrations of Cu^{2+} ions and expressed in percentages, the signal height in the absence of copper ($C_{Fe^{2+}} = 0.025$ mM) being taken as a 100% reference.

Statistical Analysis

Student's unpaired *t*-test was adopted for comparisons between groups. All data are expressed as mean ± SEM.

RESULTS

Fenton Assays

Salicylic acid hydroxylation assay The products of the reaction of Cu^+ with H_2O_2 were investigated and compared with those obtained under similar conditions via the reaction of Fe^{2+} with H_2O_2, which is known to lead to the formation of $^{\cdot}OH$ radicals.

(a) Colorimetric test Salicylic acid is hydroxylated by Cu^+ plus H_2O_2 in the presence or absence of O_2. The influence of pH and Cu^{2+} on its hydroxylation was investigated with Cu^+ and Fe^{2+}, successively.

Figure 1 shows the pH-dependent amounts of ortho di-hydroxy products obtained with each redox metal. With increasing pH, the product yield drops off at a lower pH in the case of copper(I). Figure 2 shows the effects of increasing Cu^{2+} on the metal-induced hydroxylation of salicylate. In both cases, the results are expressed in percentages, the sample to which no copper(II) was added being taken as a 100% reference.

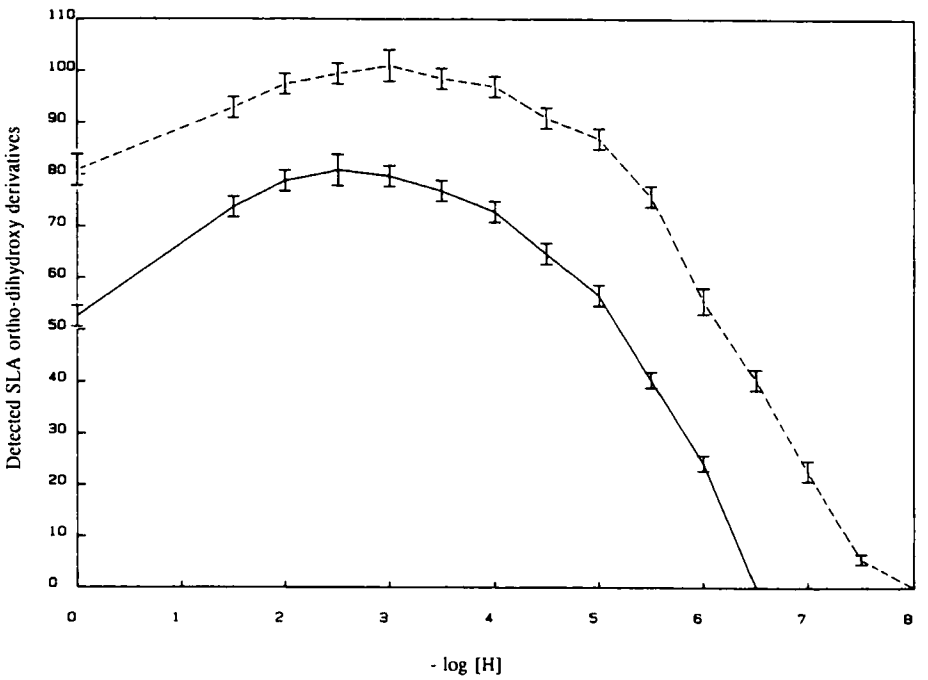

FIGURE 1 Influence of pH on the relative variation of the amount of dihydroxy-benzoates from reactions of equimolar (0.50 mM) Fe^{2+} (---) and Cu^+ (—) ions with 0.88 mM H_2O_2.

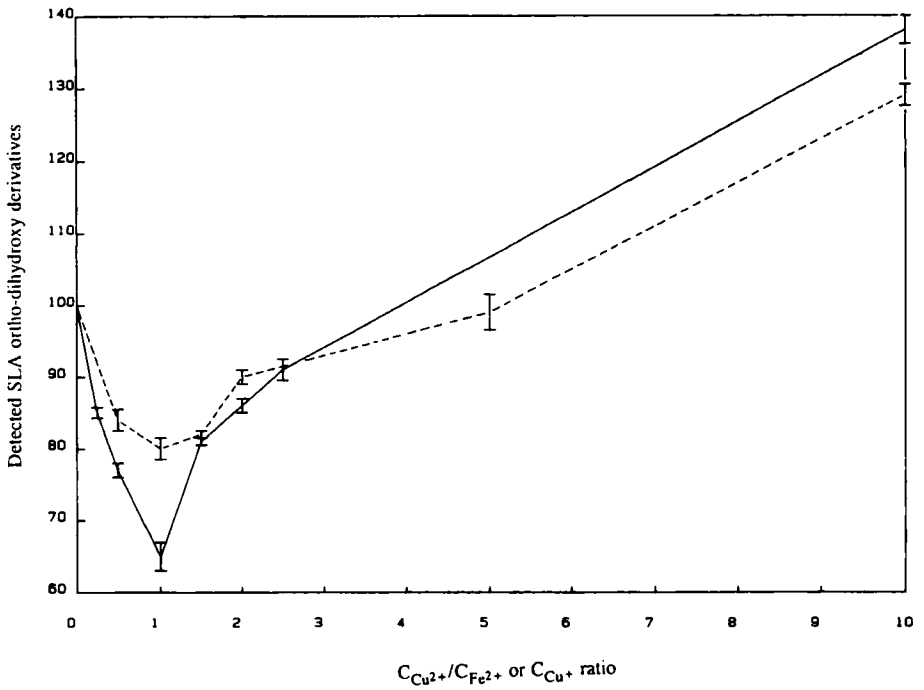

FIGURE 2 Effect of Cu^{2+} on the hydroxylation reaction of salicylate promoted by equimolar concentrations (0.50 mM) of Fe^{2+} (---) or Cu^{+} (—).

We have recently reported a pH-dependent inhibitory effect of copper(II) on the iron-driven Fenton reaction when the $C_{Cu^{2+}}/C_{Fe^{2+}}$ ratio equals one.[24] A similar phenomenon is observed here. With increasing Cu^{2+}, the product yield decreases, reaches a minimum when the $C_{Cu^{2+}}/C_{Cu^{+}}$ ratio equals 1 and then increases linearly.

(b) HPLC In order to check that products of iron- and copper-driven Fenton reactions on salicylate are identical, we compared the total hydroxylation yield and the isomer distributions obtained in the presence of H_2O_2 and Cu^{+} or Fe^{2+}, successively. Care was taken to minimize the fluctuations in the isomer distributions depending on the reaction conditions, including pH, dioxygen, ionic strength and the presence of adventitious redox metal ions.

A comparison of the copper results shown in Figure 3 with those obtained with iron[24] as well as by radiolysis (not shown here) confirmed the identity of the salicylate hydroxylation products in all cases.

Formation of TBA-reactive Material

(a) 2-deoxy-D-ribose degradation Lower concentrations of copper ($25 < C_{Cu^{+}} < 250\,\mu M$) could be tested by a metal-compatible method. TBA-reactive material was efficiently induced by the treatment with Cu^{+} plus H_2O_2 ($0.75 < C_{H_2O_2} < 0.950$ mM). The product yield reached a plateau with increasing Cu^{+} or deoxyribose. As in the iron-driven Fenton reaction, the test was positive over a large pH range ($2.5 < pH < 7.5$).

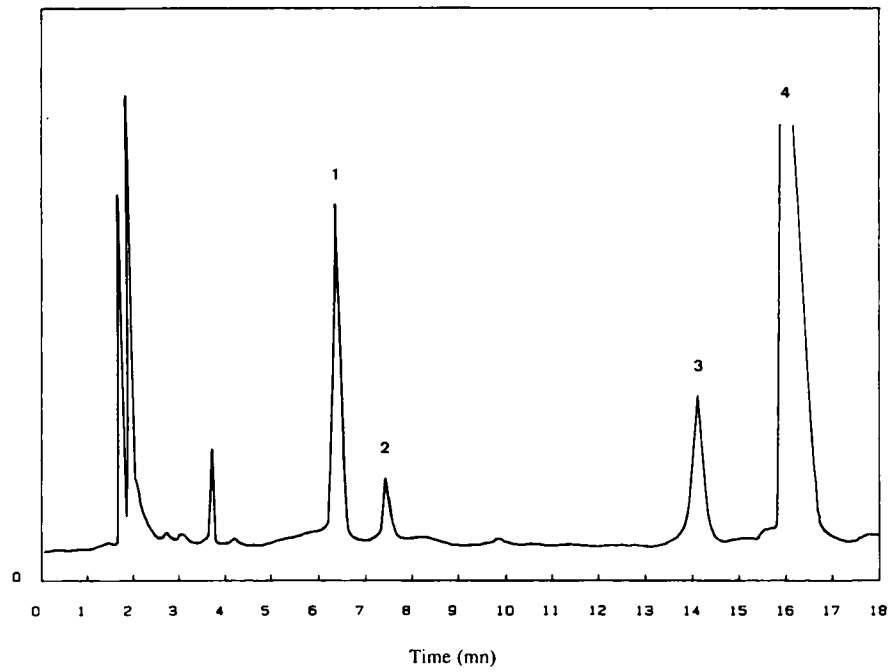

FIGURE 3 HPLC chromatograms showing separation of isomeric dihydroxy benzoates at 260 nm (peak identification: 1 = 2,3-DHBA, 2 = 2,5-DHBA, 3 = pyrocatechol, 4 = SLA).

The observed capacities of Cu^+ and Fe^{2+} to catalyse the sugar degradation were compared: Fe^{2+} was found more damaging than Cu^+ by 125 ± 4% ($p < 0.05$).

The effect of Cu^{2+} was tested: as shown in Figure 4 where results are expressed as percentages with respect to the 100% control value obtained in the absence of Cu^{2+}, a slight but significant ($p < 0.05$) inhibitory effect was observed at low $C_{Cu^{2+}}/C_{Cu^+}$ ratios, especially near unity.

(b) Damage to the sugar in DNA The reaction of H_2O_2 ($0.17 < C_{H_2O_2} < 0.24$ mM) with Cu^+ ($15 < C_{Cu^+} < 30\,\mu$M) was studied in the presence of single- or double-stranded DNA (1.0 and 0.5 mg.ml^{-1}, respectively). Neither Cu^+ nor hydrogen peroxide alone produced detectible amounts of TBA-reactive material. A Cu^+-specific chelating agent, orthophenanthroline, inhibited by 65 ± 5% the formation of the TBA-reactive material promoted by Cu^+ plus H_2O_2.

The activities of equimolar Cu^+ and Fe^{2+} in causing MDA formation were compared: in the concentration range $20 < C_{metal\ ion} < 100$ mM, Fe^{2+} proved to be more damaging to DNA than Cu^+: the amount of TBA-reactive material was increased by 125 ± 5%.

DMPO hydroxylation Figure 5a shows an ESR spectrum of the radical adduct of DMPO observed when H_2O_2 was added to a mixture solution of Cu^+ ($C_{Cu^+} <$ 0.100 mM) and DMPO.

The unique 1:2:2:1 signal observed was strictly identical with that obtained via the Fe^{2+}-driven reaction. The quartet with hyperfine splitting constants $a_N = a_H =$

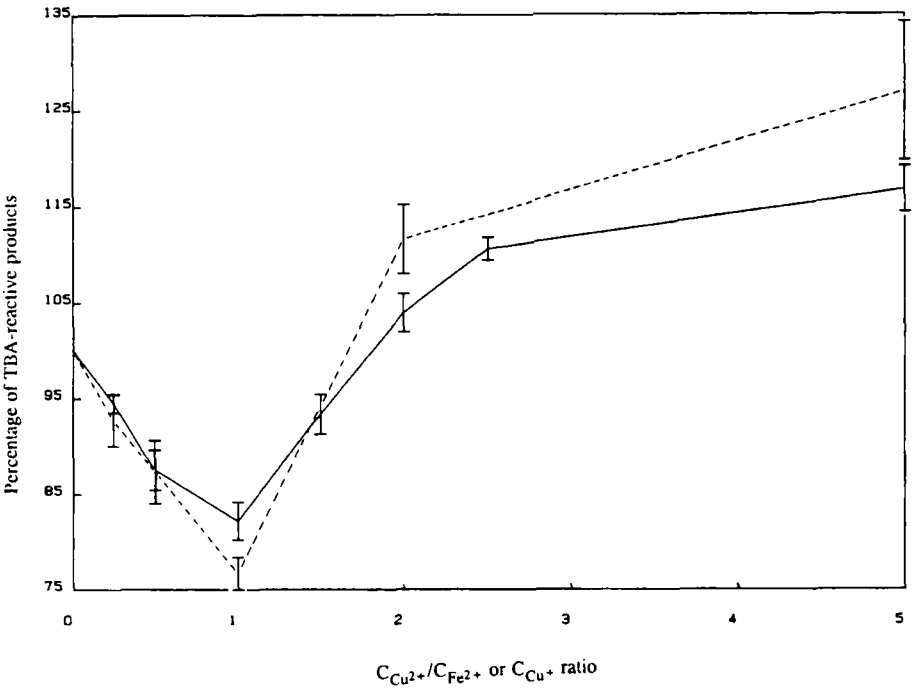

FIGURE 4 Effect of Cu^{2+} on deoxyribose degradation by Fe^{2+} (---) or Cu^{+} (—) ions (0.050 mM).

14.8 G, respectively due to the nitroxide nitrogen atom and the β-hydrogen, can be assigned to the hydroxyl radical adduct DMPO$^{\cdot}$-OH. This was verified by adding ethanol, with which $^{\cdot}OH$ radicals react to produce a signal due to the spin-trapping of α-hydroxyethyl radicals, as shown in Figure 5b.

Radiolysis Studies

Salicylic acid hydroxylation Addition of Cu^{+} ($0.25 < C_{Cu^{+}} < 1$ mM) to the reaction mixture did not induce significant changes in the hydroxylation yield ($p < 0.050$). Moreover, the inhibitory effects of various scavengers were found correlated with the rate constants of their reactions with $^{\cdot}OH$, irrespective of the presence of Cu^{+} under the present conditions (results not shown here).

Formation of TBA-reactive Material

(a) 2-deoxy-D-ribose degradation test After addition of Cu^{+} ($75 < C_{Cu^{+}} < 150\,\mu$M) to the reaction mixture, the formation of TBA-reactive material was not significantly modified ($p < 0.050$) and the ability of $^{\cdot}OH$ scavengers to protect against gamma-induced damage remained similar to that observed in the absence of Cu^{+}.

(b) Damage to the sugar in DNA No enhancing effect of Cu^{+} ($15 < C_{Cu^{+}} < 25\,\mu$M) versus DNA damage was noted under our experimental conditions ($p <$

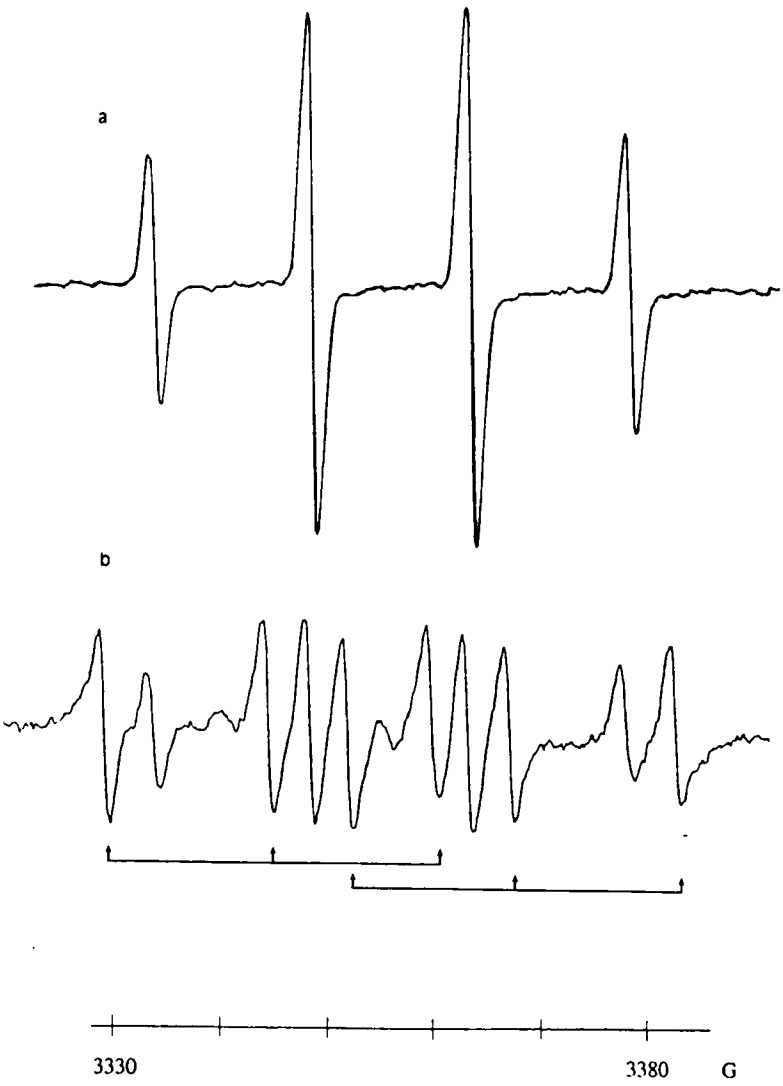

FIGURE 5 a) ESR detected DMPO˙-OH signal with $a_H = a_N = 14.9$ G at $t = 2$ min. $C_{DMPO} = 100$ mM, $C_{CuCl} = 0.025$ mM and $C_{H_2O_2} = 0.88$ mM at room temperature, pH = 4.5, G = 2 10^5. b) Effect of ethanol 20 mM ($a_H = 15.6$ G and $a_N = 22.9$).

0.01), and the inhibitory effects of various ˙OH scavengers were not correlated with the rate constants of their reactions with ˙OH in aqueous solutions (results not shown here).

DISCUSSION

The reality of the oxidation of copper(I) salts by H_2O_2 in Fenton-like reactions in aqueous solution has long been debated.[9, 32-34] In particular, the possible involvement of copper(III) in the production of ˙OH radicals was evoked.[32] This problem

now seems to have been definitely settled.[9] However, the present work which for the first time to our knowledge makes direct use of free Cu^{+} ions to generate hydroxyl radicals may shed further light on the role of copper in free radical chemistry.

Two factors are relevant in this respect: (i) the influence of pH, and (ii) the effect of Cu^{2+} on the Cu^{+}-driven reaction.

(i) Considering the fact that the strictly pH-dependent reaction involving copper(III), i.e.

$$Cu^{+} + H_2O_2 \rightarrow [Cu(III)] \rightarrow Cu^{2+} + {}^{\cdot}OH + H_3O^{+},$$

is shifted to the right in acidic solutions[35], it can reasonably be assumed that the derivatives produced here in weakly acid and neutral media are exclusively generated by the Fenton-like reaction:

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + {}^{\cdot}OH + OH^{-},$$

which confirms Eberhardt *et al.*'s findings.[9]

(ii) The influence of Cu^{2+} on the production of $^{\cdot}OH$ radicals by Cu^{+} is twofold.

At high $C_{Cu^{2+}}/C_{Cu^{+}}$ ratios – above 5 with salicylate (Figure 2) and above 2 with deoxyribose (Figure 4) – there is an additional production of $^{\cdot}OH$ radicals. This effect, presumably due to the reduction of the Cu^{2+} added, is in line with previous observations that Cu^{2+} can react with H_2O_2 to initiate a Fenton-like reaction,[10] the superoxide anion acting as the reductant in that case.[12]

More surprisingly, low Cu^{2+} concentrations significantly inhibit $^{\cdot}OH$ formation, this effect being maximum for $C_{Cu^{2+}}/C_{Cu^{+}}$ ratios near unity (Figures 2 and 4). Although unprecedented for copper, this inhibitory action is reminiscent of that recently observed with iron under similar experimental conditions.[24]

This alternate influence of Cu^{2+} on the Cu^{+}-driven $^{\cdot}OH$ production deserves some comments:

- it has been suggested in our previous study relative to the Fe^{2+}-driven Fenton reaction that a competition of Cu^{2+} ions towards Fe^{2+} ions for the detector molecule would occur at the expense of the amount of $^{\cdot}OH$ radicals apparently produced. In contrast with that case,[24] Cu^{2+} and Cu^{+} ions involved here have very different coordinating capabilities. The "soft" character of Cu^{+} ions virtually prevents them from associating with oxygen-donor bearing ligands. However, it may be that the Cu^{2+} ions bound to the detector molecules somewhat decrease their detecting efficiency, the amount of $^{\cdot}OH$ detected being lessened until Cu^{2+} ions reach a sufficiently high concentration to take over the $^{\cdot}OH$ production process.
- a direct scavenging effect of Cu^{2+} on $^{\cdot}OH$ radicals is ruled out in view of the radiolysis experiments described above. The generation of other active dioxygen species might a priori be involved. However, no signal corresponding to the DMPO adduct of superoxide ($O_2^{\cdot-}$ or its protonated form, the hydroperoxyl radical $HO_2^{\cdot}$) could be detected in our ESR studies; moreover, active superoxide dismutase exerts no protective effect against oxidative damage to DNA due to copper plus hydrogen peroxide.[36] In the same way, the specific occurrence of the Cu^{+}-peroxide complex recently reported[33,34] to result from the reaction of Cu^{+} with H_2O_2 does not seem likely here because of the similarity with iron.[24]
- as was already advanced in the iron case,[24] the origin of the alternate influence of Cu^{2+} on the Cu^{+}-driven reaction may also lie in a favourable arrangement of redox potentials with respect to the $^{\cdot}OH$ production.

Our investigation of the possible influence of Cu^+ ions on the generation of $^{\cdot}OH$ radicals by water gamma radiolysis also deserves a short comment. It has recently been claimed that some copper(II) complexes displayed radioprotectant properties when administered to mice before[37] or even after[38] irradiation. As already established for Cu^{2+} in our previous work,[24] Cu^+ ions reveal to have no influence on the amount of $^{\cdot}OH$ radicals detected following water gamma irradiation. This demonstrates that no form of ionised copper can scavenge $^{\cdot}OH$ radicals in the absence of a ligand.

Turning back to our declared objective to establish a reference system of standardised $^{\cdot}OH$ production with respect to which particular copper(II) complexes could be tested, the present Cu^+-driven Fenton reaction offers a determining advantage, i.e. it involves a single metal. This greatly simplifies the speciation calculations necessary to ensure the predominance of a given copper(II) complex in solution before adding hydrogen peroxide. Moreover, as the +1 and +2 oxidation states of copper display markedly different Lewis acid characters, Cu^{2+} is virtually the only metal ion capable of coordinating with oxygen- and nitrogen-containing ligands present in solution. However, the necessity to keep all test solutions free of oxygen to prevent unwanted oxidation of Cu^+ before the addition of H_2O_2 is a heavy constraint to the experimentalist in routine experiments. For this reason, attempts are currently in progress to use copper in its oxidised form together with reductants in the same context.

Acknowledgements

We thank A. Mari (CNRS Toulouse) and P. François (Centre Claudius Regaud, Toulouse) for helpful assistance to ESR and radiolytic studies, respectively.

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Accepted by Professor J.M.C. Gutteridge