Free Rad. Res. Comms., Vol. 15, No. 6, pp. 305–317
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COPPER-LIGAND INTERACTIONS AND PHYSIOLOGICAL FREE RADICAL PROCESSES. pH-DEPENDENT INFLUENCE OF Cu^{2+} IONS ON Fe^{2+}-DRIVEN $OH^{\cdot}$ GENERATION

PHILIPPE MAESTRE[1], LUC LAMBS[2], JEAN-PAUL THOUVENOT[1] and GUY BERTHON[2]†

[1]*Laboratoire de Biochimie, Hôpital Purpan, 31059 Toulouse, France.*
[2]*INSERM U305, Equipe "Bioréactifs: Spéciation et Biodisponibilité", Université Paul Sabatier, 38 rue des Trente-six Ponts, 31400 Toulouse, France*

(*Received June 13, 1991; in final form July 23, 1991*)

Prior to comparative studies on the reactivity of various copper complexes with respect to $OH^{\cdot}$ radicals, the influence of free Cu^{2+} ions on the superoxide-independent generation of $OH^{\cdot}$ radicals through Fenton assays and water gamma radiolysis has been tested in the present work.

Cu^{2+} ions have been shown to behave in a distinct manner towards each of these two production systems. As was logically expected from the noninvolvement of copper in $OH^{\cdot}$ radical production through gamma radiolysis, no influence of Cu^{2+} ions has been observed on the amount of radicals detected in that case. In contrast, Cu^{2+} ions do influence $OH^{\cdot}$ radical generation through iron-driven Fenton reactions, but differently depending on copper concentration.

When present in high concentrations, Cu^{2+} ions significantly contribute to $OH^{\cdot}$ radical production, which confirms previous observations on the reactivity of these in the presence of hydrogen peroxide. At lower levels corresponding to copper/iron ratios below unity on the contrary, Cu^{2+} ions behave as inhibitors of the $OH^{\cdot}$ production in a pH-dependent manner over the 1–6 range investigated: the lower the pH, the greater the inhibition.

The possible origin of this previously unreported inhibitory effect is discussed.

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

INTRODUCTION

Superoxide dismutase (SOD, EC 1.15.1.1) is believed to provide defense against cellular damage from active oxygen species.[1] The discovery of one copper atom per subunit active site of the protein has drawn attention to the possible SOD-like activity of copper complexes.[2]

In this connection, a number of copper complexes have been shown to scavenge superoxide radicals with greater efficiency than SOD in respect of direct free radical production through pulse radiolysis.[3] However, this effect has not been confirmed in indirect assays involving the xanthine–xanthine oxidase system *in vitro*.[3] Hypotheses have been put forward regarding the possible mechanisms involving copper ions or complexes in these processes,[4,5] but no certainty exists as to the nature of the actual chemical forms of the metal which interact with radical-generating systems *in vivo*. In

†Author to whom correspondence should be addressed.

particular, whether the copper complexes administered simply serve as carriers of Cu^{2+} ions towards the target site or are themselves involved as independent chemical species in the antiradical action still remains to be determined.

In addition to the above-mentioned activity of copper with respect to superoxide radicals, a possible action of complexes of this metal has recently been suggested against the more reactive $OH^{\cdot}$ species. In particular, it has been lately contended that the well documented scavenging effect of salicylic acid versus $OH^{\cdot}$ radicals is directly related to the amount of copper bound to this substance.[6]

Clearly, copper-ligand interactions must play a critical role in the activity of Cu^{2+} and Cu^{+} ions in free radical chemistry. In this respect, copper speciation studies are advisable to discriminate among all possible complexes those which are the most likely to be involved in the corresponding processes.[7] However, comparative studies on the reactivity of specifically selected copper complexes require that reference standardised systems of free radical generation under reproducible conditions be established beforehand.

In this context, the objective of the present paper was to investigate the effect of free Cu^{2+} ions on the superoxide-independent $OH^{\cdot}$ generation in two standardised models, namely Fenton assays and gamma radiolysis of water. Several metal-compatible detection techniques have been used, including salicylate hydroxylation assay, deoxyribose damage by thiobarbituric acid (TBA) assay, and ESR spin-trapping with 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO).

MATERIALS AND METHODS

Reagents

$CuSO_4$, $5H_2O$, $FeSO_4$, $7H_2O$, $CuNO_3$, $3H_2O$, $CuCl_2$ and Na_2WO_4, $2H_2O$ were Prolabo products of Normapur quality (France).

Salicylic acid (SLA), 2,3-dihydroxybenzoic acid (2,3-DHB), 2,5-dihydroxybenzoic acid (2,5-DHB) and 5,5′-dimethyl-1-pyrroline-N-oxide (DMPO) were supplied by Aldrich Chimie (France), whereas catechol (pyrocatechol) was a Rectapur Prolabo reagent (France).

2-deoxy-D-ribose and 2-thiobarbituric acid (TBA) were purchased from Sigma Chimie (France), and H_2O_2 from Gifrer or Prolabo (France).

All solutions, unbuffered except for HPLC determinations, 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 (Na_2SnO_3) was used as a H_2O_2 stabiliser.

0.8 × 4 cm poly-Prep columns from Bio-Rad filled with the AG 50 W–X8 Cation Exchange resin were used to remove metal ions from samples to be analysed through Cu-compatible TBA assay.[8]

Technical Equipment

Radiolysis experiments were performed with the ^{60}Co gamma source of the Centre Claudius Regaud (Toulouse).

The Philips HPLC equipment used to analyse hydroxylated salicylate derivatives obtained from the two radical-generating techniques consisted of a PU4100 solvent delivery pump, a 7125 Rheodyne valve fitted with a 20 μl injection loop, and a PU4110

UV-detector. A reverse-phase column Bischoff 250 × 4 mm Nucleosil C_{18} (5 μm) was used for separation.

The colorimetric test also used to detect the ortho-dihydroxylated salicylate derivatives was carried out with 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 held in the cavity by means of Vitrex 50 μl disposable pre-calibrated pipettes.

Linear voltammetry experiments required to check the Fe^{2+}/Fe^{3+} ratio of the ferrous sulfate solutions used to generate $OH^{\cdot}$ radicals in Fenton assays were carried out on a Dacfamov 05 potentiostat equipped with a platinum rotating disk electrode.

$OH^{\cdot}$ Radical Production

Fenton assays. Ferrous sulfate solutions were prepared by dissolving $FeSO_4$, $7H_2O$ crystals in freshly deaerated water under an atmosphere of purified nitrogen. The Fe^{2+}/Fe^{3+} ratio in these solutions was regularly checked by polarography. Any appearance of a negative current revealing the production of Fe^{3+} led to the renewal of the solution.

Gamma radiolysis. Irradiations were carried out using a ^{60}Co gamma source, at a dose rate of 1.2 Gy · min^{-1}. The delivered dose ranged from 30 to 80 Gy as measured by ionisation chamber. These values were in good agreement with the results obtained using the 0.8 N H_2SO_4 Fricke dosimeter without adding NaCl, assuming $G(Fe^{3+}) = 15.8$ Fe^{3+} ions × $(100\,eV)^{-1}$. Dimensions of the irradiation field were chosen so that measured scattering effects were negligible (< 1%).

Six glass tubes were exposed to the gamma beam in a water bath at room temperature (25°C); three of these contained the test solutions, the other three reference samples. Corrections were made with appropriate nonirradiated blanks.

Specific experimental conditions were designed to match the two detection techniques used:

– for the salicylate hydroxylation assay, salicylic acid solutions were saturated with ultrahigh-purity N_2O before irradiation so that $OH^{\cdot}$ species constituted more than 90% of all the radicals produced;

– for the TBA assay, deoxyribose solutions were purged of oxygen by bubbling with purified nitrogen.

$OH^{\cdot}$ Radical Detection

Salicylate hydroxylation assay

(a) *Fenton assays.* Reaction mixtures of 1.6 ml contained the following reagents introduced in the order: salicylic acid (5 mM), $CuSO_4$ ($0 < C_{Cu^{2+}} < 10$ mM), $FeSO_4$ ($0.5 < C_{Fe^{2+}} < 2.5$ mM) up to final concentrations in parentheses.

The reaction was initiated by adding H_2O_2 up to a concentration of 1 mM. After 20 min incubation in the dark at room temperature, the reaction was stopped by adding 100 μl of H_2SO_4 10 M.

Two methods were used to detect all the dihydroxy products generated by the addition of $OH^{\cdot}$ radicals to the salicylate ring:[9,10]

Colorimetric test

The colorimetric test described by Richmond *et al.*[11] was used to detect the ortho-dihydroxylated products, the absorbance at 510 nm being measured against appropriate blanks.

Averages of determinations reproducible within 5% for each concentration tested were expressed as percentages of the amount of dihydroxylated products generated in the absence of copper.

The absence of copper in the organic phase was checked by atomic absorption spectrometry following acidification.

HPLC

All dihydroxylated derivatives can be detected by HPLC.

In the present case, the extracted aromatic compounds were dissolved into 500 μl of mobile phase consisting of 30 mM citric acid and 27 mM acetic acid at pH 4.7 as recommended by Maskos *et al.*[9] They were then eluted from a Bischoff column at a flow rate of 1 ml min^{-1} and detected by UV-absorption at 260 or 280 nm.

(b) *Gamma radiolysis.* 8 ml of a solution of 10 mM salicylic acid were exposed to the gamma-beam ($30 < \text{Dose} < 80$ Gy) in the absence or presence of copper ($0 < C_{Cu^{2+}} < 1$ mM).

Colorimetric test

After concentration of the radiation-produced dihydroxyderivatives into diethyl ether and solvent evaporation under nitrogen, the residue was dissolved in 500 μl of water, and the above colorimetric test applied.

HPLC

The residue resulting from the above concentration and evaporation procedures was dissolved in 500 μl of citrate buffer, and analysed by HPLC as in Fenton assays.

TBA assay. Given its better sensitivity with respect to the salicylate hydroxylation assay, the use of deoxyribose degradation with the release of TBA-reactive material has recently gained wide acceptance for detecting $OH^{\cdot}$ radical damage.[12,13] It has thus been employed in the present studies, with particular protocols for Fenton assays and gamma radiolysis, respectively.

(a) *Fenton assays.* Reaction mixtures of 2 ml contained 2-deoxy-D-ribose (5 mM), $CuSO_4$ at varying concentrations ($0 < C_{Cu^{2+}} < 250\,\mu$M), and freshly prepared $FeSO_4$ (50 μM) introduced in this order up to final concentrations in parentheses. The reaction was started by adding H_2O_2 up to 0.1 mM. After 15 min incubation at 37°C, the reaction was stopped by adding 125 μl of H_2SO_4 10 M.

To render the test metal-compatible, interfering cations were removed from the sample by passing through a Poly-Prep column filled with cation exchange resin.[8]

As in the above colorimetric test, average determinations relative to duplicate samples reproducible within 5% were expressed as percentages of the TBA-reactive material released in the absence of copper.

(b) *Gamma radiolysis.* A solution of 8 ml of 5 mM deoxyribose containing concentrations of copper in the range $0 < C_{Cu^{2+}} < 150\,\mu$M was irradiated as described above.

Free malondialdehyde[14] (MDA or propanedi-al) formation was detected by UV-absorption at 270 nm, wavelength at which absorption of the enolate form of free MDA is maximum.[15]

Results were expressed as percentages as in Fenton assays (see above).

DMPO spin-trapping. All ESR spectra were recorded at room temperature. DMPO was purified on charcoal before use, as described by Floyd.[16]

For technical reasons, DMPO spin trapping was used to detect $OH^{\cdot}$ radicals generated by Fenton systems only. Reaction mixtures of 360 μl contained the following reactions introduced in the order: DMPO (100 mM), $CuSO_4$ ($0 < C_{Cu^{2+}} < 100\,\mu M$), $FeSO_4$ (50 μM) up to final concentrations in parentheses.

The reaction was initiated by the addition of H_2O_2 up to a concentration of 0.3 mM. Spectra were recorded after a constant incubation time of 1.5 min. The effect of increasing concentrations of Cu^{2+} ions on the signal intensity of the DMPO$^{\cdot}$–OH adduct was measured at different pH values on a percentage scale in which the signal height in the absence of copper was taken as a 100% reference.

Speciation Speciation studies appeared necessary to get a knowledge of the distribution of the copper complexes present in the solutions in which Fenton reactions took place. The calculations were run with the help of the SPE and SPEPLOT programs,[17] using stability constants taken from the literature.[18]

RESULTS

Fenton Assays

In each system, the effect of Cu^{2+} ions on Fenton-driven $OH^{\cdot}$ production has been expressed as percentages with respect to the 100% value obtained in the absence of copper.

It is worth noting at this stage that no significant $OH^{\cdot}$ formation could be detected in the absence of Fe^{2+} ions, which was considered as a criterion for the absence of impurities likely to interact with the reaction system.

Salicylate Hydroxylation Assay

(a) *Colorimetric test.* Figure 1 shows the influence of added cupric ions on the amount of the reaction products detected (all dihydroxy derivatives except for 2,5-DHB). Clearly, two distinct effects are observed depending on copper concentrations: in contrast with the expected oxidising effect of copper confirmed here for high Cu^{2+}/Fe^{2+} ratios,[19] Cu^{2+} ions at low concentrations behave as potent inhibitors of Fe^{2+}-stimulated $OH^{\cdot}$ production.

This inhibitory effect, observed over a large range of Fe^{2+} concentrations ($0.5 < C_{Fe^{2+}} < 10$ mM), is maximum at a Cu^{2+}/Fe^{2+} ratio near 1 for the two lower salicylate concentrations. It is not affected by the nature of the anion of the copper salt (SO_4^{2-} can be replaced by NO_3^- or Cl^- without changing the result); nor is it by variations in the ionic strength in the range $0 < I < 0.15$ M with NaCl as a background salt. However, it progressively weakens as the pH is raised, as shown in Figure 2.

In this connection, it may be of interest to note that pH values above 6 could not be tested due to the oxidation of ferrous iron. This restriction has only limited

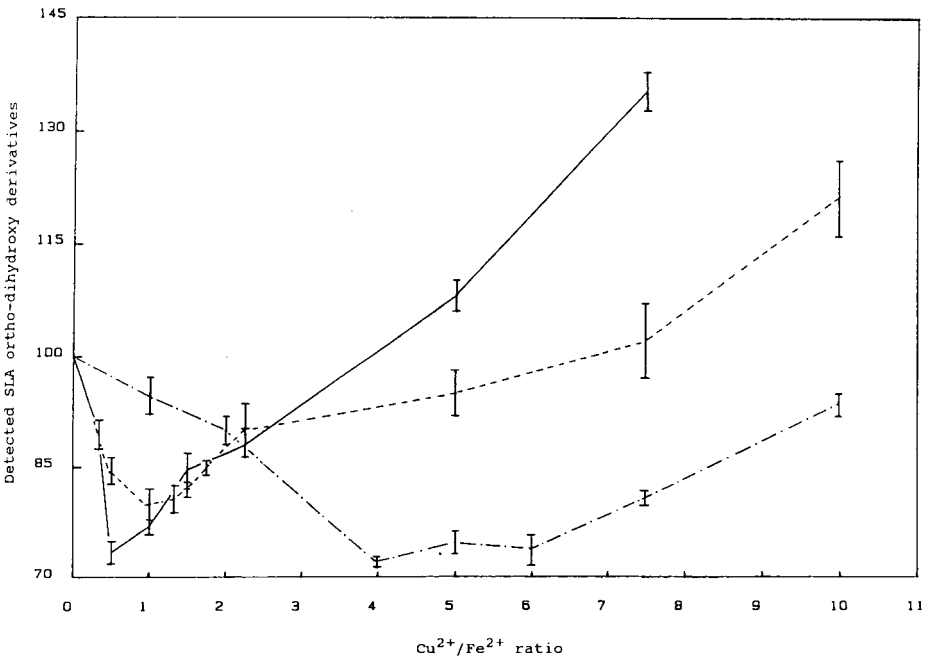

FIGURE 1 Relative amount of salicylate (SLA) ortho-dihydroxy derivatives detected by tungstate colorimetric assay as a function of the $C_{Cu^{2+}}/C_{Fe^{2+}}$ ratio with $C_{Fe^{2+}} = 0.5\,mM$ and $C_{SLA} = 0.5\,mM$ (——), 1 mM (– – –) and 5 mM (–·–·–) at pH 3.

consequences in the present case since pH values pertaining to pathological situations in which radical damages occur are situated in the range investigated. For instance, a pH of 5 has been reported in close proximity to the membrane of the activated macrophage.[20]

(b) *HPLC analysis.* The HPLC analysis was carried out under the same experimental conditions as the colorimetric test, so as to ensure that the inhibitory effect observed in this test did not simply portray a change in the distribution of dihydroxy derivatives (to the expense of ortho products), but did effectively reflect a global reduction in the $OH^{\cdot}$ radical production.

A separation test of 2,3-DHB, 2,5-DHB, catechol and salicylate from a standard mixture containing equivalent concentrations of these four reactants was first performed as a reference. Typical HPLC chromatograms of the salicylate hydroxylation products from the Fenton reaction in the absence and in the presence of copper are reported in Figure 3.

Figure 3 does effectively show that the total amount of the dihydroxylated products is substantially influenced. Moreover, the 2,3-DHB/2,5-DHB ratio has been observed to be independent of the $C_{Cu^{2+}}/C_{Fe^{2+}}$ ratio.

Iron-Catalysed Degradation of Deoxyribose

Advantage was taken of the greater sensitivity of the TBA assay to use a Cu-compatible

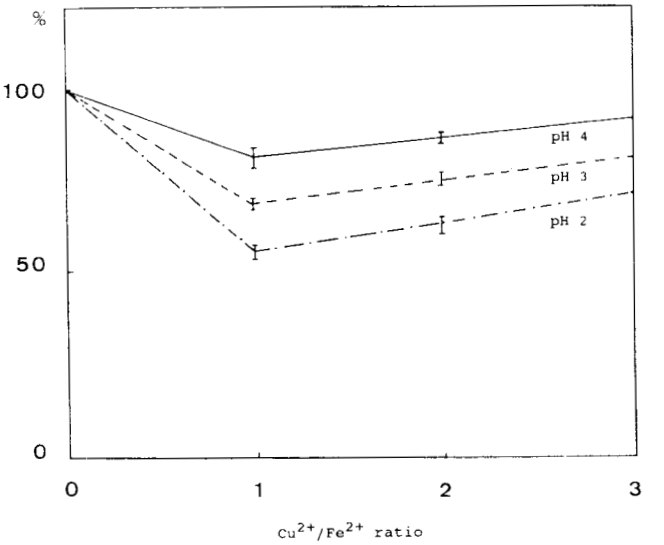

FIGURE 2 Influence of pH on the variation of the amount of salicylate ortho-dihydroxy derivatives detected by tungstate colorimetric assay as a function of the $C_{Cu^{2+}}/C_{Fe^{2+}}$ ratio ($C_{Fe^{2+}} = C_{SLA} = 0.5\,mM$).

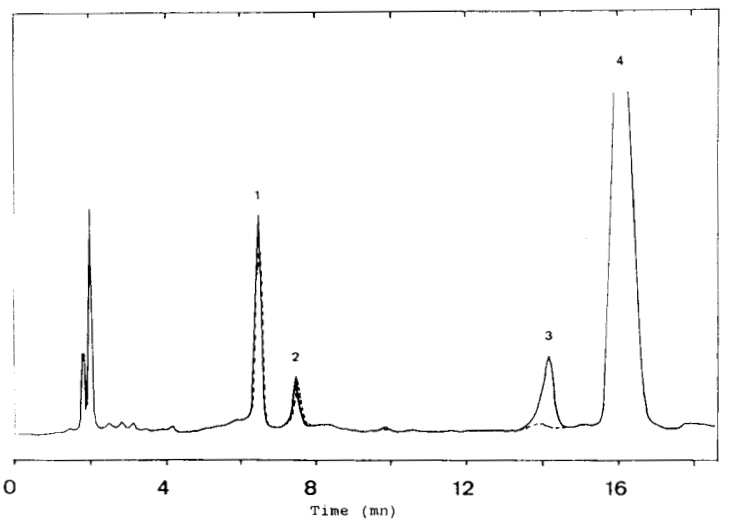

FIGURE 3 HPLC chromatograms showing separation of isomeric dihydroxybenzoates at 260 nm in a Fenton assay solution containing SLA 20 μM in the absence (——), and in the presence (– – –) of copper (peak identification: 1 = 2,3-DHB, 2 = 2,5-DHB, 3 = pyrocatechol, 4 = SLA).

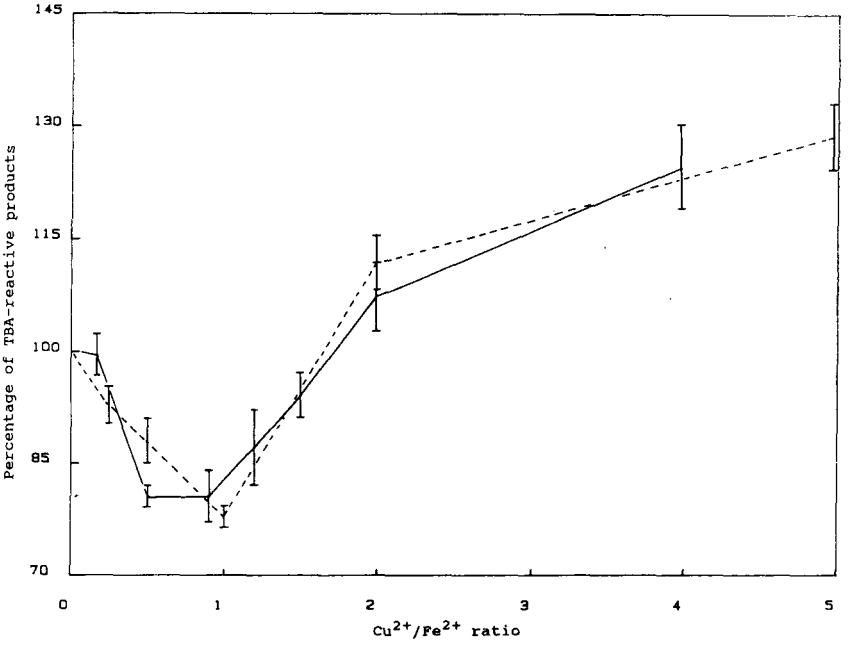

FIGURE 4 Relative amount of TBA-reactive products released as a function of the C_{Cu2+}/C_{Fe2+} ratio with $C_{Fe2+} = 50\,\mu M$ and $C_{deoxyribose} = 50\,\mu M$ (——) and $100\,\mu M$ (– – –).

form of this technique to test concentrations of copper in the range of those occurring *in vivo*.

In comparison with the salicylate hydroxylation assay, the addition of Cu^{2+} ions caused a lesser change in the global amount of reaction products obtained. Only a slight – though significant – decrease was indeed observed in the amount of TBA-reactive material (MDA) released. Data corresponding to 25 experiments are summarized in Figure 4.

At the low iron concentrations tested, addition of excess EDTA prior to the heating step decreased the formation of MDA by 45 ± 7%, the presence of Cu^{2+} ions at the above-mentioned concentrations inducing the same inhibitory effect (23 ± 5%) as in the absence of iron chelator.

DMPO Spin-Trapping

ESR DMPO spin trapping was also used to detect the production of $OH^{\cdot}$ radicals, so as to test that generation of other oxygen radicals like $O_2^{\cdot -}$ could not derive from the possible interference of copper with the iron-driven Fenton system.

Even after times of incubation inferior to the half-life of the $O_2^{\cdot -}$-derived spin adduct DMPO$^{\cdot}$ – OOH, no sextuplet signal was observed. The unique spectrum detected as from the usual reaction time of 1.5 min was the 1 : 2 : 2 : 1 quartet relative to DMPO$^{\cdot}$ – OH, resulting from equivalent nitrogen and β-proton coupling constants (see insert in Figure 5).

Variations in intensity of the DMPO$^{\cdot}$ – OH signal due to the presence of different concentrations of copper can be seen in Figure 5.

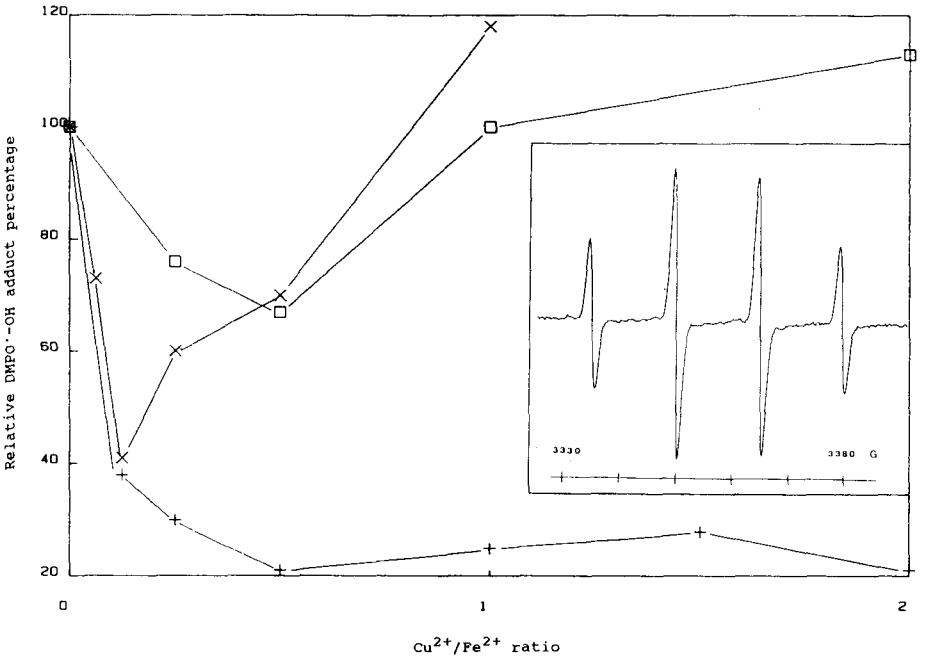

FIGURE 5 Relative DMPO$^{\cdot}$-OH adduct signals expressed as percentages of that obtained in the absence of copper, as a function of the C_{Cu2+}/C_{Fe2+} ratio for pH = 2.5 (+), pH = 3.5 (×), pH = 5.5 (□). Insert: ESR detected DMPO$^{\cdot}$-OH adduct signal with $A_N = A_H = 14.9$ G after $t = 2$ min at room temperature. $C_{DMPO} = 100$ mM, $C_{Fe2+} = 50\,\mu$M and $C_{H_2O_2} = 3$ mM; pH = 5.75; $G = 2 \times 10^5$.

As the copper concentration is raised, the ESR DMPO$^{\cdot}$ – OH signal decreases to reach a minimum at a Cu^{2+}/Fe^{2+} ratio near 0.5 for the two lower pH values. Further addition of cupric ions increases the amount of spin adduct formed, suggesting that the well documented spin trap decomposition occurring at high levels of copper[22] does not take place at these concentrations ($C_{Cu2+} < 500\,\mu$M).

In addition, other experiments not reported here have shown that the inhibitory effect of Cu^{2+} ions on $OH^{\cdot}$ production is pH dependent: the lower the pH, the greater the inhibition.

Gamma Radiolysis

Radiolysis of water is a convenient way of producing standardized amounts of $OH^{\cdot}$ radicals in the absence of iron: the noninfluence of Cu^{2+} ions on this system under the experimental conditions of the Fenton assays was tested.

Following gamma-irradiation, the $OH^{\cdot}$ radicals formed in the absence or in the presence of varying concentrations of Cu^{2+} ions were detected with the same methods as in Fenton assays, i.e. aromatic hydroxylation and deoxyribose degradation. Irradiation times were designed so that the $OH^{\cdot}$ radical yields were approximately the same as with the Fenton systems (± 5%).

Salicylate Hydroxylation Assay

Over the range of experimental conditions investigated ($0 < C_{Cu^{2+}} < 1\,mM$), the production of ortho-dihydroxylated derivatives detected by the colorimetric test as well as the overall yield of dihydroxylated derivatives measured by HPLC was found totally independent of the copper concentration.

Deoxyribose Degradation

At the low copper concentrations tested, no influence of the metal was noted on the amplitude of the absorption peak of the irradiated solution corresponding to the generation of free MDA, or, after TBA test, in the amount of TBA reactive products released.

DISCUSSION

The first objective of the present work was to test the influence of free Cu^{2+} ions on the superoxide-independent generation of $OH^{\cdot}$ radicals through Fenton assays and water gamma radiolysis.

A distinct behaviour has been characterised for Cu^{2+} ions towards each of these production systems. Whereas no influence of Cu^{2+} ions has been noted on the amount of $OH^{\cdot}$ radicals generated through gamma radiolysis – as was logically expected from the noninvolvement of the metal in the generation process in that case – two opposite effects have been observed on the $OH^{\cdot}$ radicals detected in Fenton assays, depending on copper concentrations. At high concentration, Cu^{2+} ions have been shown to significantly contribute to $OH^{\cdot}$ radical production in this case, which confirms previous observations on the reactivity of these in the presence of hydrogen peroxide.[19] At lower levels corresponding to copper to iron ratios less than unity, however, Cu^{2+} ions inhibit $OH^{\cdot}$ production in a pH-dependent manner over the 1–6 range investigated: the lower the pH, the greater the inhibition.

Concerning the origin of this previously unreported inhibitory effect, several hypotheses may be put forward:

(i) as mentioned above, cupric ions can, like their reduced counterparts, generate hydroxyl radicals in the presence of hydrogen peroxide, but with a considerably less efficiency.[19] This must also be the case with respect to ferrous ions. Since the production of $OH^{\cdot}$ radicals is reduced for $C_{Cu^{2+}}/C_{Fe^{2+}}$ ratios less than unity while it keeps growing above, it is reasonable to assume that a competition exists between the two metal ions for the same reactant of the radical generation process so that, on account of its lesser efficiency in this process, copper needs to reach much higher concentrations than iron to induce the same radical production. In this case, Cu^{2+} ions at low concentrations would substitute for the more efficient Fe^{2+} ones on the presumed reactive site so that $OH^{\cdot}$ production would be globally lessened.

Since it has been claimed that the amount of $OH^{\cdot}$ radicals detected in Fenton assays directly depends on the extent of the redox metal ion binding to the detector molecule,[6,19,21] the detectors used in the present experiments naturally come to mind as first candidates to the role of specific reactant suggested above. Speciation studies performed in respect of $Fe^{2+} - Cu^{2+}$–salicylate equilibria (Figure 6), which show that (i) Fe^{2+} salicylate complexes are formed to a much lesser extent than Cu^{2+} ones under

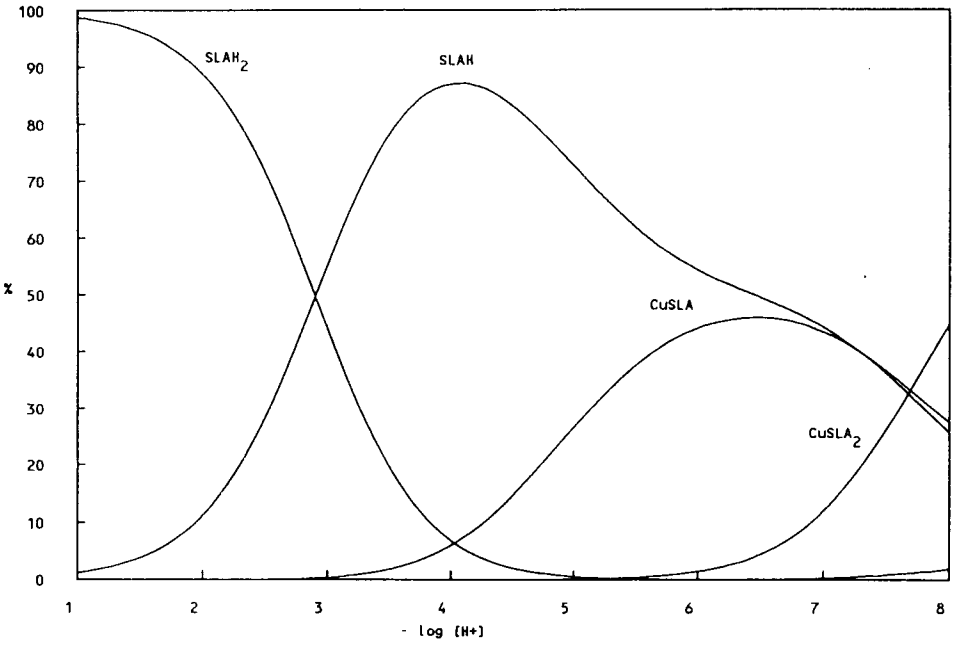

FIGURE 6 Simulated distribution of salicylate into its different complex species in the presence of Fe^{2+} and Cu^{2+} ions in a Fenton assay solution before the addition of H_2O_2 (C_{SLA} = 5 mM, C_{Fe2+} = 2.5 mM, C_{Cu2+} = 2.5 mM).

the experimental conditions investigated, (ii) copper complexation increases with the pH as from pH 3 – i.e. the more copper bound to salicylate, the less inhibitory effect – tend to support this view.

According to this hypothesis, the concentration-dependent alternate influence of Cu^{2+} ions would only be a new illustrative example of the notion of Fenton reaction specificity;[6]

(ii) in addition to $OH^{\cdot}$, other radicals may *a priori* result from possible deviations of the Fenton reaction in the presence of copper. In this respect, it is difficult to totally exclude a possible role for the superoxide radical that would result from the reduction of Cu^{2+} by H_2O_2,[19] even though no sextuplet characteristic of the $DMPO^{\cdot}$–OOH adduct was ever detected in ESR at high spin trap concentrations.

It has also been suggested that $OH^{\cdot}$ radicals are not the major damaging species formed at neutral pH where they would be superseded by derivatives of high iron oxidation states,[23–25] but the formation of such derivatives appears unlikely here since the inhibitory effect decreases as the pH is raised;

(iii) the observation of a maximum extent of the inhibitory effect at a specific $C_{Cu^{2+}}/C_{Fe^{2+}}$ ratio as well as its dependence on pH suggests that redox phenomena could also take place between two metal ions. Complementary investigations will be necessary to assess their real contribution to the above effect.

In conclusion, because of the concentration-dependent alternate influence of Cu^{2+} ions on the production of hydroxyl radicals in that case, the Fe^{2+}-driven Fenton

reaction does not appear as the best reference system with respect to which copper complex reactivities should be investigated.

Acknowledgements

We are grateful to A. Mari for his technical assistance in recording the ESR spectra, and to P. Francois for the access given to the ^{60}Co gamma source.

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Accepted by Dr J.M.C. Gutteridge