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In Vivo Evidence of Hydroxyl Radical Formation after Acute Copper and Ascorbic Acid Intake: Electron Spin Resonance Spin-Trapping Investigation

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SUMMARY

Copper has been suggested to facilitate oxidative tissue injury through a free radical-mediated pathway analogous to the Fenton reaction. By applying the ESR spin-trapping technique, evidence for hydroxyl radical formation *in vivo* was obtained in rats treated simultaneously with copper and ascorbic acid. A secondary radical spin-trapping technique was used in which the hydroxyl radical formed the methyl radical upon reaction with dimethylsulfoxide. The methyl radical was then detected by ESR spectroscopy as its adduct with the spin trap phenyl-*N*-*t*-butylnitrone (PBN). Because copper excreted into the bile from treated animals is expected to be maintained in the Cu(I) state (by ascorbic acid or glutathione), a chelating agent that would redox-stabilize it in the Cu(I) state was used to prevent *ex vivo* redox chemistry. Bile samples were collected directly into solutions of bathocuproinedisulfonic acid, a Cu(I)-stabilizing agent, and 2,2′-dipyridyl, a Fe(II)-stabilizing agent. If these precautions were not taken, radical adducts were generated *ex vivo* and could be mistaken for radical adducts generated *in vivo* and excreted into the bile. Besides the PBN/·CH_3 adduct, three other radical adducts were produced *in vivo* and excreted in bile.

Copper, like other heavy metals, can be quite toxic when introduced into a living system in amounts that exceed the ability of the system to render the metal inactive through protein binding or other means. One illustration of the toxicity of copper is the use of copper salts as a fungicide, molluskicide, and algicide (1). Copper check-valves in dispensing machines, copper-lined containers used for food storage, or copper cooking utensils are but a few sources from which gram quantities of copper can be introduced into the body. Excessive copper accumulation can also occur by such means as chronic copper intoxication of water supplied by copper pipes, accidental or abusive ingestion of copper-contaminated materials, or environmental exposure (2).

When administered to an animal, copper is rapidly taken up into the liver by a mechanism that does not appear to be saturable (3). Copper is then excreted into bile (3) or integrated into ceruloplasmin and secreted into plasma (4). The excretion of copper into bile has been shown to increase with increasing dosage in the rat up to 1 mg/kg but saturates at 3 mg/kg (5).

Hypercupremia (positive copper balance) is also observed in certain inherited metabolic diseases and carcinomas (1, 6). Inherited copper toxicosis is prevalent in Bedlington terriers (7). Hypercupremia in humans occurs in Wilson's disease (hepatolenticular degeneration) and Indian childhood cirrhosis (1). Maldistribution of body copper is observed in Menke's kinky hair disease (8).

More work has been conducted on the hepatic disposition of copper than on that of most other metals because of the attempts to characterize the molecular basis of these disorders. In the late 1970s the catalytic role of transition metals, particularly iron and copper, was implicated in free radical reactions. In biological systems there may be concentrations of redox-active copper sufficient to catalyze free radical reactions and account for various deleterious processes. It has been shown that traces of soluble copper or iron can catalyze the transformation of a superoxide radical anion ($\cdot O_2^-$) to the highly reactive hydroxyl radical ($\cdot OH$) via the metal-catalyzed Haber-Weiss reaction:

$$\cdot O_2^- + M^{n+} \rightarrow O_2 + M^{(n-1)+}$$

$$M = Cu\ (n = 2)\ \text{or}\ Fe\ (n = 3)$$

$$M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + OH^- + \cdot OH$$

Alternatively, a metal-mediated site-specific mechanism for free radical-induced biological damage has also been proposed

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ABBREVIATIONS: DMSO, dimethylsulfoxide; PBN, phenyl-*N*-*t*-butylnitrone; DP, 2,2′-dipyridyl; BC, bathocuproinedisulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

(9–11), where ·OH is formed site-specifically in the vicinity of the target molecule (Biol) and reacts at the site of its production.

$$\text{Biol} + M^{n+} \rightarrow \text{Biol-}M^{n+}$$

$$\text{Biol-}M^{n+} + {}^{\cdot}O_2^- \rightarrow \text{Biol-}M^{(n-1)+} + O_2$$

$$\text{Biol-}M^{(n-1)+} + H_2O_2 \rightarrow (\text{Biol-}M^{n+}\ldots {}^{\cdot}OH) + OH^-$$

$$(\text{Biol-}M^{n+}\ldots {}^{\cdot}OH) \rightarrow \text{damage}$$

Other reductants present in cells (such as ascorbate or glutathione) might be able to replace superoxide in the Haber-Weiss cycle and, therefore, promote the toxicity of a metal/hydrogen peroxide system. It has been previously demonstrated that the combination of ascorbate and Cu(II) causes damage to macromolecular structures such as polysaccharides and proteins through generation of reactive oxygen species (12–15). Studies *in vitro* showed that when albumin, for example, is exposed to copper and ascorbate it is site-specifically cleaved (15). Similarly, fibrinogen has been shown to coagulate as a result of its reaction with vitamin C and copper (16). Ascorbate has also been extensively used in combination with copper and copper complexes to cause enzyme inactivation and to generate strand breakage in DNA (14, 17, 18).

Although the antagonistic effects due to ascorbic acid supplementation include inhibition of copper absorption (19), signs of copper deficiency (20), and lowered tissue copper and ceruloplasmin levels (21, 22), the cytotoxicity of copper and ascorbate was attributed to ·OH radicals repeatedly formed by recycling Cu(II) back to Cu(I) (18, 23). However, under the same experimental conditions, investigators have noted both pro- and antioxidant effects of ascorbate with lower and higher concentrations of ascorbate, respectively (24, 25). Because ascorbic acid is the single most heavily consumed nutrient supplement, it is important that more information be gathered on the effects of ascorbic acid intake on copper bioavailability and utilization.

In the present study we show *in vivo* formation of hydroxyl radicals produced in rats during acute copper overload and ascorbic acid intake. We used a secondary trapping technique (26) in which the hydroxyl radical forms the methyl radical upon reaction with DMSO. The methyl radical is then detected by ESR spectroscopy as its adduct with the spin trap PBN. The *in vivo* detection of the hydroxyl radical adduct of PBN is extremely unlikely, due to its rapid decay (27). This is the first report of ESR evidence for hydroxyl radical generation by copper and ascorbic acid *in vivo*.

Materials and Methods

Chemicals. PBN, DMSO, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and L-ascorbic acid (sodium salt) were purchased from Aldrich. DP, 2,2′-biquinoline, and BC were from Sigma. [$^{13}C_2$]DMSO (minimum 99 atom % ^{13}C) was obtained from Isotec Inc. (Miamisburg, OH).

***In vivo* animal treatment.** Studies used Sprague-Dawley male rats of 380–450 g (Charles River Breeding Laboratories, Raleigh, NC). Rats were fed a standard chow mix (NIH Open Formula; Zeigler Brothers, Gardner, PA) and were allowed free access to both food and water.

Rats were anesthetized with Nembutal (50 mg/kg intraperitoneally) and anesthesia was maintained throughout the experiments. Bile ducts were cannulated with a segment of PE 10 tubing. All animals were given an intraperitoneal injection of the spin trap PBN (100 or 250 mg/kg of body weight) dissolved in DMSO (1 ml/kg of body weight) and an intragastric injection of 1 M $CuSO_4 \cdot 5H_2O$ (3 or 1 ml/kg) dissolved in distilled water. A 1 M solution of ascorbate was administered intragastrically, where indicated, at a dose of 0.5 or 3 ml/kg body weight.

Bile samples. Twenty-minute bile samples were collected for 2 hr into plastic Eppendorf tubes containing 25 μl of a solution containing 300 mM BC and 30 mM DP per 100 g of rat body weight. Samples were immediately frozen on dry ice and stored at −70° until ESR analysis or determination of iron and copper content.

Iron and copper analyses. After the intragastric administration of 1 M $CuSO_4$ (3 ml/kg), copper and iron levels in thawed 20-min bile samples were determined by a modified method of Ref. 28. One hundred microliters of rat bile were placed in an Eppendorf tube to which were added 200 μl of 0.1 M MOPS buffer, 100 μl of 0.25 M ascorbic acid, and 900 μl of 0.5 mg/ml 2,2′-biquinoline in glacial acetic acid for the Cu(I) measurements or 900 μl of 0.5 mg/ml DP in glacial acetic acid for the Fe(II) measurements. Absorbances were measured at 546 nm for Cu(I) and at 522 nm for Fe(II), using a SLM-AMINCO DW-2C spectrophotometer. Each sample was measured against a reference prepared exactly as described above, except that bile was excluded. Values were then converted to corresponding concentrations by using standard curves.

***In vitro* experiments in bile.** For *in vitro* studies, bile from untreated rats was added to tubes containing various combinations of PBN, DMSO, DP, BC, and ascorbic acid. Cu(I), Cu(II), or Fe(II) was added last to initiate the reaction. Scans were taken 5 min after mixing.

***In vivo* bile experiments.** For ESR measurements, bile samples were thawed and transferred to flat quartz cells within 5 min. All spectra shown are from bile collected 2 hr after the administration of $CuSO_4$ and ascorbic acid.

ESR spectra were obtained using a Varian E-109 spectrometer equipped with a TM_{110} cavity and operating at 9.3 GHz, 20-mW power, and 100-kHz modulation frequency. Spectra were recorded on an IBM-compatible computer interfaced to the spectrometer. Hyperfine coupling constants were determined from a spectral simulation program, SIMEPR (written by D. R. Duling of NIEHS), which sequentially varies all parameters of each radical species until a minimum in the error surface is located. Goodness of fit was judged by both a minimum in the sum of the squared residuals and visual comparisons.

Student's *t* test was used for statistical analysis of copper and iron levels from groups of animals. Values of p of <0.05 were taken to indicate significant differences.

Results

After an intragastric dose of $CuSO_4$ and ascorbic acid and intraperitoneal administration of PBN in DMSO to rats, the ESR analysis of bile samples collected into aqueous solutions of BC and DP gave a six-line spectrum (Fig. 1A). The formation of this radical adduct was dependent on the administration of $CuSO_4$ (Fig. 1B), PBN/DMSO (Fig. 1C), and ascorbate (Fig. 1D) and was apparently due to the *in vivo* formation of the PBN-methyl adduct, PBN/·CH_3, from hydroxyl radical. The ESR spectrum of bile from untreated rats showed only the doublet of the ascorbate radical, which was variable in intensity (data not shown). We were not able to show *in vivo* hydroxyl radical generation from rats treated only with $CuSO_4$ and PBN/DMSO (Fig. 1D).

Because copper excreted into the bile from treated animals is expected to be maintained in the Cu(I) state (by ascorbic acid, glutathione, or other biological reductants), the chelating agent BC, which stabilizes copper in the Cu(I) state, was used. A chelating agent for redox-stabilizing Fe(II), DP, was also used. We found that, if these precautions were not taken, signals from radical adducts generated *ex vivo* might be mis-

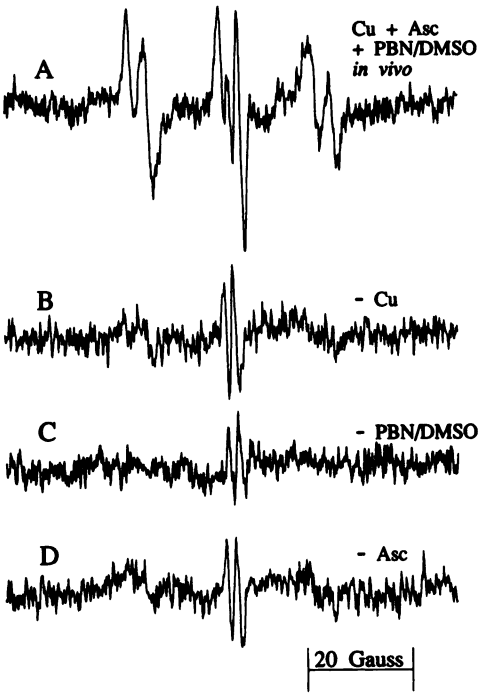

Fig. 1. A, ESR spectrum of radical adducts detected in the bile from rats 2 hr after $CuSO_4$ (1 mmol/kg) and ascorbic acid (*Asc*) (0.5 mmol/kg) were administered intragastrically and PBN (100 mg/kg) dissolved in DMSO (1 ml/kg) was administered intraperitoneally. Bile samples were collected into 25 μl of a solution containing 300 mM BC and 30 mM DP per 100 g of body weight. B, Same as in A, but rats were not given $CuSO_4$; C, same as in A, but rats were not given PBN/DMSO; D, same as in A, but rats were not given ascorbic acid. Spectrometer settings: modulation amplitude, 1.3 G; microwave power, 20 mW; scan time, 16 min; time constant, 1 sec.

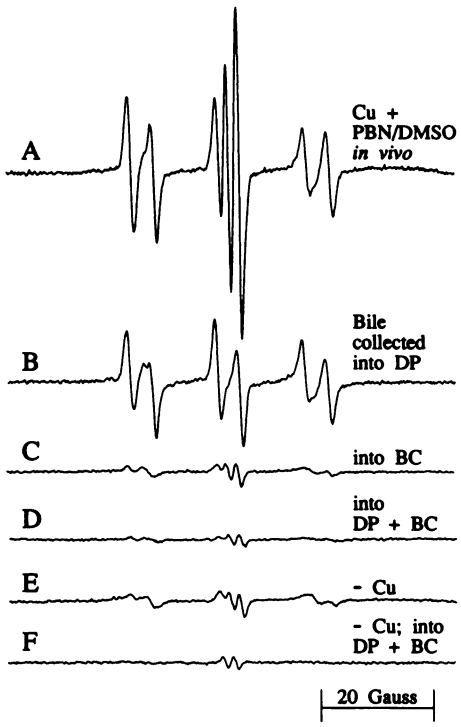

Fig. 2. A, ESR spectrum of radical adducts detected in the bile of rats 2 hr after administration of $CuSO_4$ (3 mmol/kg, intragastrically) and PBN/DMSO (250 mg/kg, intraperitoneally). B, Same as in A, except DP (25 μl of 30 mM per 100 g of body weight) was added to the collection vial; C, same as in A, except BC (25 μl of 300 mM per 100 g of body weight) was added to the collection vial; D, same as in A, except 25 μl of a solution containing BC (300 mM) and DP (30 mM) per 100 g of body weight were added to the collection vial; E, same as in A, but rats were not given $CuSO_4$; F, same as in A, but rats were not given $CuSO_4$, and BC and DP were added to the collection vial. Spectrometer settings: modulation amplitude, 1.3 G; microwave power, 20 mW; scan time, 8 min (A and B) or 16 min (C-F); time constant, 0.5 sec (A and B) or 1 sec (C-F).

taken for the adducts generated *in vivo* and excreted into the bile. For example, Fig. 2A shows the spectrum obtained from the bile of PBN/DMSO-treated rats during copper overload when no chelators were added to the bile. The radical adducts were still observed if only the Fe(II)-stabilizing agent DP (Fig. 2B) or the Cu(I)-stabilizing agent BC (Fig. 2C) was added to the bile-collecting tubes. When both chelating agents were used in the bile-collecting tube (Fig. 2D), no artifactual signals were detected. Under these conditions, the initial spectrum showed only the presence of the ascorbate radical. Fig. 2D shows the same experiment as Fig. 1D with a lower instrument gain. These findings suggest that, when bile was collected directly into solutions of Cu(I)- and Fe(II)-chelating agents, *ex vivo* hydroxyl radical generation was halted.

In rats treated with PBN and DMSO alone, a weak signal was detected in bile (Fig. 2E) but was completely suppressed by collection of bile into a solution of DP and BC or of DP alone. Apparently iron was present that also catalyzed *ex vivo* reactions (26). In an attempt to investigate this possibility, the biliary excretion of iron and copper from copper-treated rats was determined. Analyses of bile obtained after intragastric injection of $CuSO_4$ at a dose of 3 mmol/kg indicated that significant concentrations of both copper (Fig. 3) and iron (Fig. 4) were present, particularly in those samples collected during the second hour, compared with controls from nontreated rats.

In order to investigate under what conditions *ex vivo* reactions occur in the bile, a series of *in vitro* experiments were performed in which Cu(I), Fe(II), PBN, DMSO, BC, and DP were added to bile from nontreated rats. As shown in Fig. 5A, when Cu(I), PBN, and DMSO were added to bile in the presence of the Fe(II)-stabilizing agent DP, two prominent signals were detected from PBN/$\cdot CH_3$ (a^N = 16.30 G and a_β^H = 3.71 G) and PBN/$\cdot OCH_3$ (a^N = 15.08 G and a_β^H = 3.32 G). The formation of $\cdot OCH_3$ occurs subsequent to the reaction of $\cdot CH_3$ with O_2 (26, 29). When BC was included in the *in vitro* incubation, no radical adducts were detected (Fig. 5B). These findings are consistent with the known ability of BC to inhibit the oxidation of cuprous ion (30). When PBN, DMSO, and Fe(II) were added to bile, a prominent signal from two species was also detected (Fig. 5C) that was inhibited by DP (26). The addition of Cu(I) and Fe(II) to bile in the presence of both chelating agents did not give any detectable radical adducts (Fig. 5D).

It was suspected that DP and BC might not be able to prevent the occurrence of *ex vivo* radical reactions if copper and ascorbic acid were both injected into the rat, so additional *in vitro* control experiments were performed to examine this possibility. When both DP and BC were included in incubation mixtures, only the ascorbate radical was detected, regardless of which metals were present (data not shown). An additional *in vivo* experiment was performed where bile samples from animals treated with ascorbate and PBN/DMSO, but not $CuSO_4$ (Fig. 1B), were collected into aqueous solution of BC and DP con-

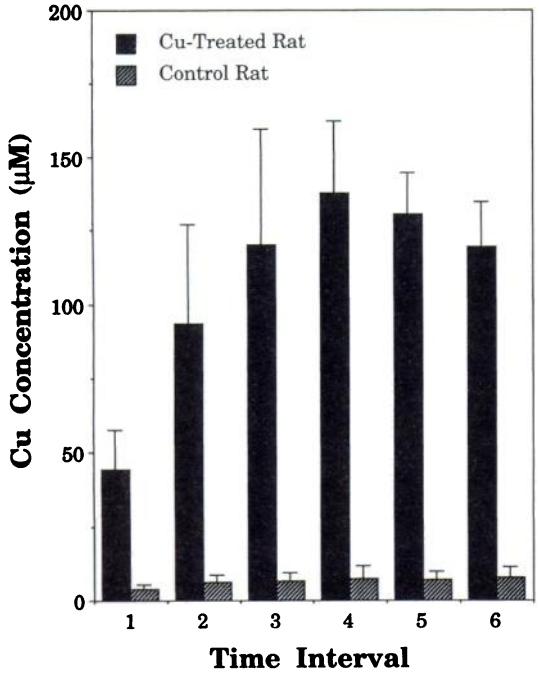

Fig. 3. Concentrations of copper excreted in the bile of rats after administration of an intragastric dose of 3 mmol/kg $CuSO_4$. Concentration values are an average for bile from five rats (four for the controls); *error bars*, standard errors. One time interval corresponds to the collection of bile over a 20-min period, such that the fourth time interval corresponds to the bile sample collected for 20 min beginning 1 hr after $CuSO_4$ injection.

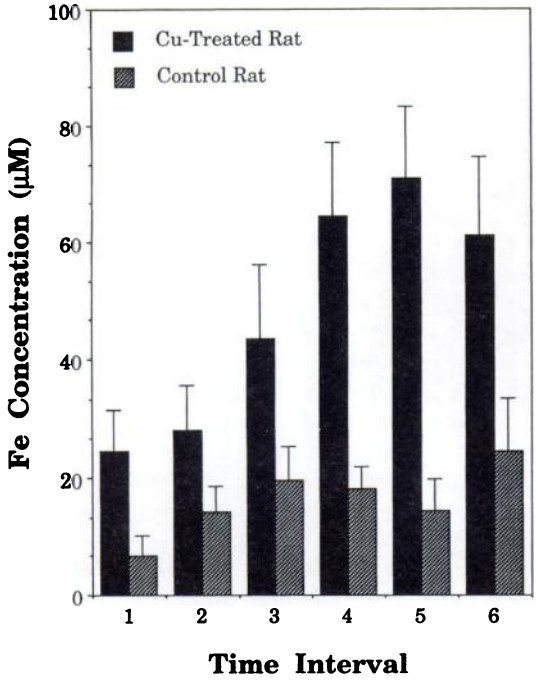

Fig. 4. Concentrations of iron excreted in the bile of rats after an intragastric dose of 3 mmol/kg $CuSO_4$ was administered. Concentration values are an average for bile from five rats (four for the controls); *error bars*, standard errors. The time intervals correspond to those in Fig. 3 and the analyses for iron were performed on the same samples as for copper.

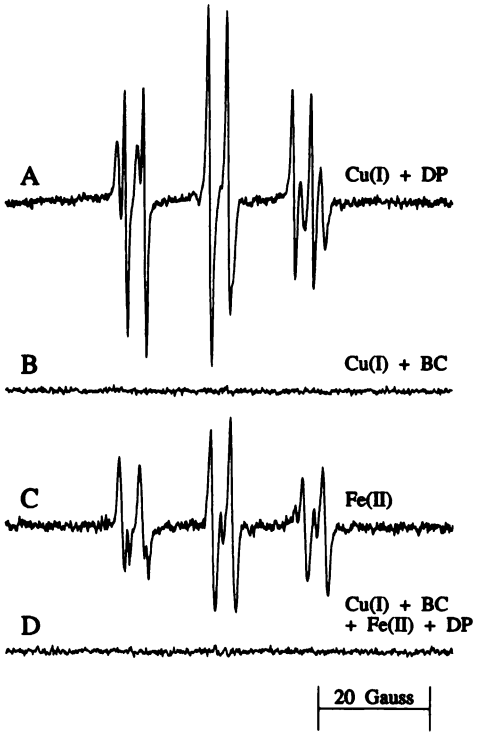

Fig. 5. Inhibition of redox chemistry *in vitro* from Cu(I) and Fe(II) by BC and DP in a 95:5 bile DMSO mixture containing 15 mM PBN. A, 0.25 mM Cu(I) (dissolved anaerobically in acetonitrile) (45) and 2.5 mM DP; B, 0.25 mM Cu(I) and 2.5 mM BC; C, 0.25 mM Fe(II); D, 0.25 mM Cu(I), 0.25 mM Fe(II), 2.5 mM BC, and 2.5 mM DP. Spectrometer settings: modulation amplitude, 0.25 G; microwave power, 20 mW; scan time, 8 min; time constant, 0.64 sec.

taining 70 μM CuCl (final concentration). At this concentration of CuCl, which is comparable with that excreted in bile (Fig. 3), no detectable radical adduct formation occurred (data not shown).

Having demonstrated *in vivo* copper- and ascorbic acid-dependent free radical formation, we next sought to identify the radical adducts detected. Isotope substitution techniques, such as the incorporation of a magnetic nucleus (typically ^{13}C) into a radical adduct, can permit identification of a variety of radicals trapped in complex biological systems (31). Using this approach, experiments were performed with ^{13}C-substituted DMSO, which were expected to confirm the formation of PBN/$\cdot{}^{13}CH_3$ *in vivo*. The ESR signal detected in the bile of rats given an intraperitoneal dose of [$^{13}C_2$]DMSO/PBN and an intragastric dose of $CuSO_4$ and ascorbic acid is shown in Fig. 6A. This spectrum clearly contains a signal from the PBN/$\cdot{}^{13}CH_3$ radical adduct, which is the dominant species and is characterized by the hyperfine coupling constants of $a^N = 16.30$ G, $a_\beta^H = 3.63$ G, and a_β^C (^{13}C) = 3.81 G (Table 1; Figs. 6B and 7B).

Four other radical species were present in bile from copper- and ascorbic acid-treated rats (Fig. 7); however, their assignments are complicated by the heterogeneity of the bile. Although an acceptable simulation could not be obtained unless a minimum of five separate adducts were included in the simulations, only the assignments for the PBN/$\cdot{}^{13}CH_3$ adduct (due to the additional splitting of the ^{13}C nucleus from [$^{13}C_2$] DMSO) and the ascorbate free radical can be considered unique. The final values reported in Table 1 were consistently obtained from several computer simulations in which various reasonable initial values were used.

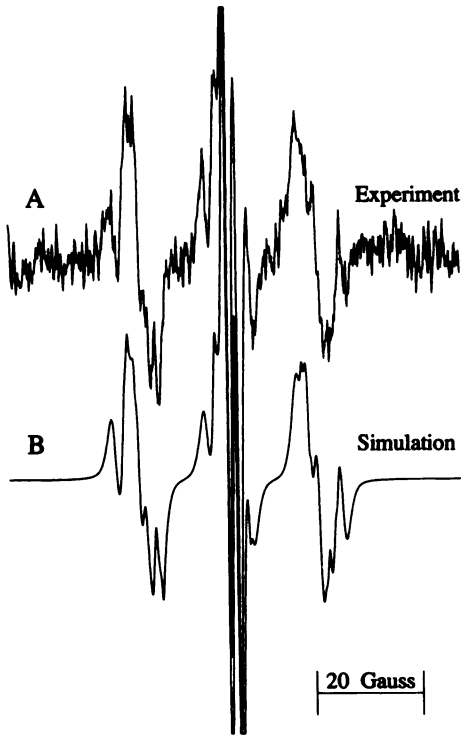

Fig. 6. A, ESR spectrum of radical adducts detected in the bile from rats 2 hr after $CuSO_4$ (3 mmol/kg, intragastrically), ascorbic acid (0.5 mmol/kg, intragastrically) and PBN (250 mg/kg) dissolved in [$^{13}C_2$]DMSO (1 ml/kg, intraperitoneally) were administered. Bile samples were collected into 25 μl of a solution containing 300 mM BC and 30 mM DP per 100 g of body weight. Spectrometer settings: modulation amplitude, 1.3 G; microwave power, 20 mW; scan time, 1 hr; time constant, 4 sec. B, Computer simulation of spectrum in A. Hyperfine coupling constants are given in Table 1.

The adduct labeled as PBN/·Y in Fig. 7C has not been identified but has also been shown to be present in bile from experiments of iron overload.[2] Only a nitrogen hyperfine coupling constant of 15.01 G was used to simulate the spectral contribution from this nitroxide, but the corresponding linewidth used (1.3 G) was relatively large, suggesting that unresolved hyperfine coupling constants may be present. A similar three-line signal (a^N = 15.92 G) was reported for a PBN-dependent adduct that was formed during ischemia/reperfusion-induced injury to gerbil brain and extracted into chloroform (32). A PBN-derived nitroxide may lack a β-hydrogen coupling as the result of abstraction of this hydrogen in a double-trapping reaction sequence (33).

The hyperfine coupling constants for the adduct shown in Fig. 7D suggest that this adduct may be due to an aliphatic carbon-centered radical, especially from its a^N/a_β^H ratio (34); however, the possibility of an alkoxyl radical adduct, PBN/·OR, cannot be dismissed. The carbon dioxide anion radical adduct, PBN/·CO_2^- (Fig. 7E), has been identified from its distinctive coupling constants and is possibly a result of the oxidation of endogenous formate anion by hydroxyl radical (35). In fact, the latter three radical adducts all appear to be products of spin-trapping of free radicals formed from endogenous molecules, because they all lack hyperfine coupling from ^{13}C; however, the adduct in Fig. 7D may be due to the methoxy radical formed from the reaction of ·$^{13}CH_3$ with O_2 (26). The ascorbate free radical was observed in all bile samples from copper-treated rats (Fig. 7F).

Discussion

One broadly accepted explanation for the cytotoxicity of copper stems from its redox activity and its potential to catalyze the repeated production of highly active oxygen-derived species (9, 30, 36). ESR evidence for hydroxyl radical formation *in vivo* due to the combination of copper overload and ascorbic acid intake is provided by spin-trapping of the methyl radical resulting from the scavenging of hydroxyl radical by DMSO. Although alkoxyl radicals have also been shown to react with DMSO to form the methyl radical (37), their rate of rearrangement through a 1,2-hydrogen atom shift is on the order of 10^5–10^6 sec^{-1} (38, 39) and $>10^6$ sec^{-1} for decomposition through β-scission (40). In contrast, the rate of reaction of DMSO with alkoxyl radicals to form the methyl radical is $<1.3 \times 10^3$ $M^{-1}sec^{-1}$ (37); therefore, it seems unlikely that alkoxyl radicals that may have formed during copper overload could be responsible for the DMSO-dependent methyl radical formation. Fatty acid alkoxyl radicals react internally with double bonds to form epoxides even faster (41). On the other hand, the reaction rate of hydroxyl radical with DMSO is $>10^9$ $M^{-1}sec^{-1}$ (42), and this is, therefore, the species most likely responsible for the DMSO-dependent methyl radical formation *in vivo*.

To produce the highly reactive hydroxyl radical, Cu(II) must be reduced to Cu(I). It is assumed that Cu(I) is oxidized by H_2O_2 to yield ·OH, although the reaction between Cu(I) and H_2O_2 may not be a simple one-electron oxidation of Cu(I) to Cu(II) with concomitant formation of the free hydroxyl radical (10, 43–45). We found that, in ascorbic acid-treated rats, hydroxyl radical (or a closely related species such as a high-

[2] M. J. Burkitt, M. B. Kadiiska, P. M. Hanna, S. J. Jordan, and R. P. Mason. An ESR spin-trapping investigation into the effects of paraquat and desferrioxamine on hydroxyl-radical generation during acute iron-poisoning. Submitted for publication.

TABLE 1

Hyperfine coupling constants obtained from the simulation of Fig. 6A

The five radical species used to simulate the experimental spectrum are shown in Fig. 7, B–F and are presented at the relative concentrations determined from the composite spectra in Figs. 6B and 7A. The radical assignments listed below are discussed in the text.

Radical detected	Hyperfine coupling constants in Gauss				a^N/a_β^H	Relative concentration	Source
	a^N	a_β^H	a_β^C (^{13}C)	a^H			
PBN/·$^{13}CH_3$	16.30	3.63	3.81		4.49	49	Fig. 7B
PBN/·$^{13}CH_3$	16.18	3.38	3.96		4.79		Footnote 2
PBN/·Y	15.01					28	Fig. 7C
PBN/·C	16.01	3.59			4.46	11	Fig. 7D
PBN/·CO_2^-	15.42	4.88			3.16	2	Fig. 7E
·Asc$^-$				1.74		10	Fig. 7F

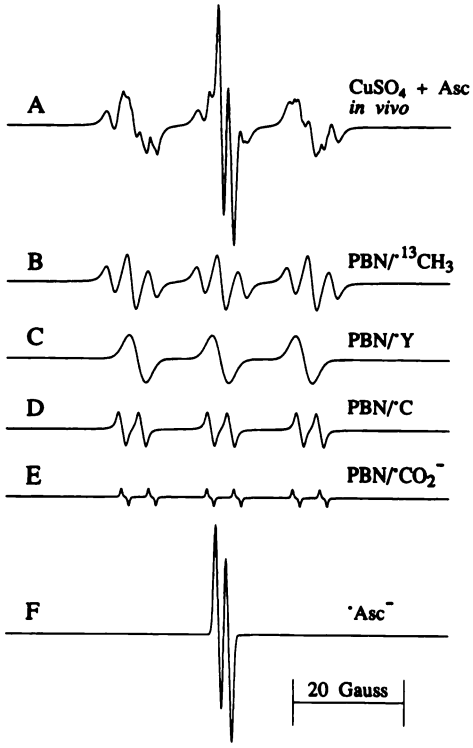

Fig. 7. Relative contributions of the assigned radical adducts from computer simulation of the experimental spectrum shown in Fig. 6A. A, Complete simulation; B, simulation of PBN/·$^{13}CH_3$ (linewidth, 0.96 G; lineshape, 86% Lorentzian 14% Gaussian); C, simulation of PBN/·Y (linewidth, 1.3 G; lineshape, 100% Gaussian); D, simulation of PBN/·C (linewidth, 0.50 G; lineshape, 97% Lorentzian 3% Gaussian); E, simulation of PBN/·CO_2^- (linewidth, 0.16 G; lineshape, 44% Lorentzian 56% Gaussian); F, simulation of ascorbate radical (linewidth, 0.29 G; lineshape, 100% Gaussian). Simulated linewidths are corrected for experimental modulation amplitude contributions. The hyperfine coupling constants obtained from these simulations are given in Table 1.

valence metal species) is generated during acute copper overload. The suggested mechanism involves the oxidation of ascorbate to the ascorbate radical, the reduction of Cu(II) (either spuriously protein bound or non-protein bound) to Cu(I), and the production of superoxide, hydrogen peroxide, and hydroxyl radical.

$$\text{Ascorbate} + Cu^{2+} \rightarrow \text{Ascorbate radical} + Cu^{+} + H^{+}$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + \cdot O_2^{-}$$

$$2\cdot O_2^{-} \rightarrow H_2O_2 + O_2$$

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

The results in this study support a free radical mechanism that attributes an important role to ascorbic acid and copper *in vivo* in the induction of biological damage already demonstrated in numerous *in vitro* experiments (9, 12, 14, 18, 23, 46). From our studies here it is clear that generation of detectable ·OH during acute copper poisoning of rats is dependent on high intragastric concentrations of ascorbic acid. Conceivably, radical production may occur in the digestive tract, where the spin-trapped adduct is absorbed and transported directly to the liver via the portal vein. However, we could not detect any free radical in blood samples drawn from the portal vein (data not shown). Other mechanisms have been discussed in which ascorbate promotes the release of iron and copper from metal storage and metal transport proteins and the release of copper from ceruloplasmin (47).

In cellular systems, glutathione is well suited to participate as a copper ligand by virtue of its millimolar concentration in many cells and the thermodynamic strength of the copper-thiolate bond. The importance of glutathione in metal detoxification is also supported by its role as a substrate for GSH peroxidases and its reaction with reactive oxygen species. In an *in vitro* study, Hanna and Mason (45) provided direct evidence for the inhibition of free radical formation from Cu(I) and H_2O_2 by glutathione and other thiols. No radicals were detected when these ligands were present in stoichiometric excess, whereas Fe(II) remained redox active and generated both GS· and ·OH radicals in the presence of GSH and H_2O_2. The inhibition by glutathione of free radical formation from Cu(I) and H_2O_2 may also have implications *in vivo*. Although we were not able to detect ·OH from copper-treated rats even after depletion of glutathione (data not shown), the role of glutathione is still presumed to be significant because, even under these conditions, the glutathione concentration is still in large excess over the copper. Furthermore, the lack of an observed adduct from copper treatment alone was not a result of reduction by GSH of the radical adduct formed *in vivo*, because radical adducts are easily detected *in vitro* in the presence of GSH (45) and *in vivo* from iron overload experiments (26).

Clearly, in our system of copper-intoxicated rats copper alone does not cause the formation of detectable PBN/·CH_3, unlike what had been demonstrated for iron (26). Either the binding of copper to its physiological ligands prevents it from catalyzing ·OH formation (45, 48, 49) or ·OH participates in site-specific reactions of the bound copper and does not have the opportunity to react with DMSO to form the methyl radical in detectable concentrations (11, 23, 43, 46). Free radical production from copper *in vivo*, however, can be stimulated by sufficient quantities of ascorbic acid to produce radical adducts similar to those observed from iron overload.[2]

Finally, we showed that, if bile samples from copper-treated rats were not collected directly into solutions of chelating agents that would redox-stabilize copper and traces of iron, signals from radical adducts generated *ex vivo* might be mistaken for adducts generated *in vivo* and then excreted into the bile. In summary, the findings presented here indicate that radicals detected in the bile are formed *in vivo* due to the combination of acute copper and ascorbic acid intake.

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References

1. Scheinberg, I. H., and I. Sternlieb. Copper toxicity and Wilson's disease, in *Trace Elements in Human Health and Disease* (A. S. Prasad, ed.). Academic Press, New York, 415–438 (1976).
2. Harris, E. D. Copper in human and animal health, in *Trace Elements in Health* (J. Rose, ed.). Butterworth and Co. Publishers Ltd., London, 44–73 (1983).
3. Owen, C. A., Jr. Absorption and excretion of ^{64}Cu-labeled copper by the rat. *Am. J. Physiol.* **207**:1203–1206 (1964).
4. Owen, C. A., Jr. and A. L. Orvis. Release of copper by rat liver. *Am. J. Physiol.* **218**:88–91 (1970).
5. Klaassen, C. D. Effect of alteration in body temperature on the biliary excretion of copper. *Proc. Soc. Exp. Biol. Med.* **144**:8–12 (1973).
6. Kobayashi, S., and J. Sayato-Suzuki. Zinc and copper accumulation and isometallothionein induction in mouse ascites sarcoma S180A cells. *Biochem. J.* **249**:69–75 (1988).
7. Su, L.-C., S. Ravanshad, C. A. Owen, Jr., J. T. McCall, P. E. Zollman, and R. M. Hardy. A comparison of copper loading disease in Bedlington terriers and Wilson's disease in humans. *Am. J. Physiol.* **243**:226–239 (1982).

8. Ettinger, M. J., H. M. Darwish, and R. C. Schmitt. Mechanism of copper transport from plasma to hepatocytes. *Fed. Proc.* **45**:2800–2804 (1986).
9. Chevion, M. A site specific mechanism for free radical induced biological damage: the essential role of redox active transition metals. *Free Radicals Biol. Med.* **5**:27–37 (1988).
10. Sutton, H. C., and C. C. Winterbourn. On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Radicals Biol. Med.* **6**:53–60 (1989).
11. Goldstein, S., and G. Czapski. Transition metal ions and oxygen radicals. *Int. Rev. Exp. Pathol.* **31**:133–164 (1990).
12. Chiou, S.-H. DNA- and protein-scission activities of ascorbate in the presence of copper ion and a copper-peptide complex. *J. Biochem. (Tokyo)* **94**:1259–1267 (1983).
13. Uchida, K., and S. Kawakishi. Oxidative depolymerization of polysaccharides induced by the ascorbic acid-copper ion systems. *Agric. Biol. Chem.* **50**:2579–2583 (1986).
14. Shinar, E., T. Navok, and M. Chevion. The analogous mechanisms of enzymatic inactivation induced by ascorbate and superoxide in the presence of copper. *J. Biol. Chem.* **258**:14778–14783 (1983).
15. Marx, G., and M. Chevion. Site-specific modification of albumin by free radicals: reaction with copper(II) and ascorbate. *Biochem. J.* **236**:397–400 (1985).
16. Marx, G., and M. Chevion. Fibrinogen coagulation without thrombin: reaction with vitamin C and copper (II). *Thromb. Res.* **40**:11–18 (1985).
17. Aronovtich, J., D. Godinger, A. Samuni, and G. Czapski. Ascorbic acid oxidation and DNA scission catalyzed by iron and copper chelates. *Free Radical Res. Commun.* **2**:241–258 (1987).
18. Stoewe, R., and W. A. Prutz. Copper-catalyzed DNA damage by ascorbate and hydrogen peroxide: kinetics and yield. *J. Free Radicals Biol. Med.* **3**:97–105 (1987).
19. Van Campen, D., and E. Gross. Influence of ascorbic acid on the absorption of copper by rats. *J. Nutr.* **95**:617–622 (1968).
20. Hunt, C. E., W. W. Carlton, and P. M. Newberne. Interrelationships between copper deficiency and dietary ascorbic acid in the rabbit. *Br. J. Nutr.* **24**:61–69 (1970).
21. Smith, C. H., and W. R. Bidlack. Interrelationship of dietary ascorbic acid and iron on tissue distribution of ascorbic acid, iron and copper in female guinea pigs. *J. Nutr.* **110**:1398–1408 (1980).
22. Milne, D. B., S. T. Omaye, and W. H. Amos, Jr. Effect of ascorbic acid on copper and cholesterol in adult cynomolgus monkeys fed a diet marginal in copper. *Am. J. Clin. Nutr.* **34**:2389–2393 (1981).
23. Samuni, A., J. Aronovtich, D. Godinger, M. Chevion, and G. Czapski. On the cytotoxicity of vitamin C and metal ions: a site specific Fenton mechanism. *Eur. J. Biochem.* **137**:119–124 (1983).
24. Bendich, A., L. J. Machlin, O. Scandurra, G. W. Burton, and D. D. Wayner. The antioxidant role of vitamin C. *Adv. Free Radicals Biol. Med.* **2**:419–444 (1986).
25. Laudicina, D. C., and L. J. Marnett. Enhancement of hydroperoxide-dependent lipid peroxidation in rat liver microsomes by ascorbic acid. *Arch. Biochem. Biophys.* **278**:73–80 (1990).
26. Burkitt, M. J., and R. P. Mason. Direct evidence for *in vivo* hydroxyl-radical generation in experimental iron overload: an ESR spin-trapping investigation. *Proc. Natl. Acad. Sci. USA* **88**:8440–8444 (1991).
27. Kotake, Y., and E. G. Janzen. Decay and fate of the hydroxyl radical adduct of α-phenyl-*N*-*tert*-butylnitrone in aqueous media. *J. Am. Chem. Soc.* **113**:9503–9506 (1991).
28. Felsenfeld, G. The determination of cuprous ion in copper proteins. *Arch. Biochem. Biophys.* **87**:247–251 (1960).
29. Britigan, B. E., T. J. Coffman, and G. R. Buettner. Spin trapping evidence for the lack of significant hydroxyl radical production during the respiration burst of human phagocytes using a spin adduct resistant to superoxide-mediated destruction. *J. Biol. Chem.* **265**:2650–2656 (1990).
30. Goldstein, S., and G. Czapski. The role and mechanism of metal ions and their complexes in enhancing damage in biological systems or in protecting these systems from the toxicity of $O_2^{\cdot-}$. *J. Free Radicals Biol. Med.* **2**:3–11 (1986).
31. Mottley, C., and R. P. Mason. Nitroxide radical adducts in biology: chemistry, applications and pitfalls, in *Biological Magnetic Resonance* (L. J. Berliner and J. Reuben, eds.), Plenum Publishing Corp., New York, Vol. 8. 489–546 (1989).
32. Oliver, C. N., P. E. Starke-Reed, E. R. Stadtman, G. J. Liu, J. M. Carney, and R. A. Floyd. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. *Proc. Natl. Acad. Sci. USA* **87**:5144–5147 (1990).
33. Janzen, E. G. Spin trapping and associated vocabulary. *Free Radical Res. Commun.* **9**:163–167 (1990).
34. Li, A. S. W., and C. F. Chignell. The NoH value in EPR spin trapping: a new parameter for the identification of 5,5-dimethyl-1-pyrroline-*N*-oxide spin adducts. *J. Biochem. Biophys. Methods* **22**:83–87 (1991).
35. Aurian-Blajeni, B., M. Halmann, and J. Manassen. Radical generation during the illumination of aqueous suspensions of tungsten oxide in the presence of methanol, sodium formate and sodium bicarbonate: detection by spin trapping. *Photochem. Photobiol.* **35**:157–162 (1982).
36. Halliwell, B., and J. M. C. Gutteridge. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219**:1–14 (1984).
37. Gara, W. B., and B. P. Roberts. An electron spin resonance study of the reactions of alkoxyl and trimethylsiloxyl radicals with dialkyl sulfoxides. *J. Chem. Soc. Perkin Trans. II* 1708–1715 (1977).
38. Gilbert, B. C., R. G. G. Holmes, H. A. H. Laue, and R. O. C. Norman. Electron spin resonance studies. L. Reactions of alkoxy radicals generated from alkyl hydroperoxides and titanium(III) ion in aqueous solution. *J. Chem. Soc. Perkin Trans. II* 1047–1052 (1976).
39. Schuchmann, H.-P., and C. von Sonntag. Methyl peroxyl radicals: a study of the γ-radiolysis of methane in oxygenated aqueous solutions. *Z. Naturforsch. Teil B Anorg. Chem. Org. Chem.* **39**:217–221 (1984).
40. Gilbert, B. C., P. D. R. Marshall, R. O. C. Norman, N. Pineda, and P. S. Williams. Electron spin resonance studies. 61. The generation and reactions of the *tert*-butoxyl radical in aqueous solution. *J. Chem. Soc. Perkin Trans. II* 1392–1400 (1981).
41. Chamulitrat, W., M. F. Hughes, T. E. Eling, and R. P. Mason. Superoxide and peroxyl radical generation from the reduction of polyunsaturated fatty acid hydroperoxides by soybean lipoxygenase. *Arch. Biochem. Biophys.* **290**:153–159 (1991).
42. Buxton, G. V., C. L. Greenstock, W. P. Helman, and A. B. Ross. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($\cdot OH/\cdot O^-$) in aqueous solution. *J. Phys. Chem. Ref. Data* **17**:513–886 (1988).
43. Samuni, A., M. Chevion, and G. Czapski. Unusual copper-induced sensitization of the biological damage due to superoxide radicals. *J. Biol. Chem.* **256**:12632–12635 (1981).
44. Goldstein, S., and G. Czapski. Mechanism and reaction products of the oxidation of Cu(I)-phenantholine by H_2O_2. *J. Free Radicals Biol. Med.* **1**:373–380 (1985).
45. Hanna, P. M., and R. P. Mason. Direct evidence for inhibition of free radical formation from Cu(I) and hydrogen peroxide by glutathione and other potential ligands using the ESR spin-trapping technique. *Arch. Biochem. Biophys.* **295**:205–213 (1992).
46. Rowley, D. A., and B. Halliwell. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals in the presence of copper salts: a physiologically significant reaction? *Arch. Biochem. Biophys.* **225**:279–284 (1983).
47. Jacob, R. A., J. H. Skala, S. T. Omaye, and J. R. Turnlund. Effect of varying ascorbic acid intakes on copper absorption and ceruloplasmin levels of young men. *J. Nutr.* **117**:2109–2115 (1987).
48. Nakamura, M., and I. Yamazaki. One-electron transfer reactions in biochemical systems. VI. Changes in electron transfer mechanism of lipoamide dehydrogenase by modification of sulfhydryl groups. *Biochim. Biophys. Acta* **267**:249–257 (1972).
49. Freedman, J. H., M. R. Ciriolo, and J. Peisach. The role of glutathione in copper metabolism and toxicity. *J. Biol. Chem.* **264**:5598–5605 (1989).

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