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Electron Spin Resonance Evidence for Free Radical Generation in Copper-Treated Vitamin E- and Selenium-Deficient Rats: *In Vivo* Spin-Trapping Investigation

MARIA B. KADIISKA,[1] PHILLIP M. HANNA, SANDRA J. JORDAN, and RONALD P. MASON

National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

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SUMMARY

The ESR spin-trapping technique has been used to investigate free radical generation in copper-challenged rats deficient in vitamin E and/or selenium. Radical adduct excreted in the bile was detected only from copper-challenged rats deficient in both vitamin E and selenium. The phenyl-*N*-*t*-butylnitrone radical adduct has hyperfine coupling constants of a^N = 15.36 G and a_β^H = 2.50 G and arises from the trapping of a radical formed from an endogenous molecular species. The induction of this radical species *in vivo* may be important in the increased toxicity of copper in rats deficient in both vitamin E and selenium. These findings support the proposal that dietary selenium and vitamin E can protect against lipid peroxidation and copper toxicity. The results obtained suggest that the presence of only one of these nutrients in the diet is enough to prevent the formation of this radical adduct at ESR-detectable levels, and they provide the most direct ESR evidence yet obtained for the involvement of *in vivo* lipid peroxidation in the toxicity of copper.

Although copper is an essential micronutrient, it is toxic when high-level exposures occur. The information on clinical consequences of copper accumulation has been obtained from certain genetic metabolic diseases, carcinomas, and various forms of acute and chronic poisoning (1–5).

The manifestations of copper toxicosis include hepatic necrosis and intravascular hemolysis (6). Several mechanisms for the cellular injury and functional abnormalities caused by excess copper have been postulated. Excessive copper may inactivate essential enzymes by binding to protein cysteinyl thiol groups (7) or by labilizing cellular membranes (8, 9). In addition, Cu(II) ions have the highest affinity for nucleic acids among all essential trace elements found in living organisms (10).

Copper has been suggested to facilitate oxidative injuries through a free radical-mediated pathway analogous to the iron-mediated Haber-Weiss reaction. The hydroxyl radical formed through a series of redox reactions (11, 12) can then inactivate essential enzymes and initiate lipid peroxidation, processes that are harmful to cells and experimental animals (13–15). Accordingly, copper overload has been shown to be associated with increased generation of lipid peroxidation products and with a number of biochemical and pathological lesions in rats (16, 17). These studies are supported by *in vitro* studies, where ionic copper has been shown to initiate lipid peroxidation in erythrocytes (18, 19), liposomes (19), and microsomes (20, 21).

It is generally accepted that susceptibility to lipid peroxidation is influenced by the level of vitamin E and selenium in tissue (22–25). When both vitamin E and selenium are deficient in the diet, particular syndromes develop, including liver necrosis in rats (26), exudative diathesis in chicks (27), and white muscle disease in lambs (28). The lesions in these conditions are thought to result from unchecked oxidative processes that lead to membrane damage. Depletion of dietary vitamin E and selenium, for example, has been shown to be linked to the increased formation of lipid peroxidation products and increased hepatic peroxidative injury in different species, including rats (29–31). Therefore, the tissues of animals deficient in vitamin E and selenium have a greater tendency to undergo lipid peroxidation both *in vitro* (32) and *in vivo* (33) than do tissues of animals with a diet adequate in these nutrients.

Other studies also showed that rats whose diets were deficient in vitamin E and selenium were much more vulnerable to copper toxicity. Dougherty and Hoekstra (34) found that intraperitoneal administration of $CuSO_4$ to rats deficient in both vitamin E and selenium caused dose-dependent increases in expired ethane (an indirect index of lipid peroxidation) and acute mortality. Selenium and vitamin E supplementation of the diet prevented the increase of ethane production and mortality. Similarly, Dillard and Tappel (16) found increased pentane production and other products of lipid peroxidation in

[1] Permanent address: Institute of Physiology, Bulgarian Academy of Sciences, "Academician Georgy Bonchev" Street, Building 23, 1113 Sofia, Bulgaria.

ABBREVIATIONS: DMSO, dimethylsulfoxide; BC, bathocuproinedisulfonic acid; DP, 2,2′-dipyridyl; PBN, phenyl-*N*-*tert*-butylnitrone.

copper-treated, vitamin E-deficient rats. Smaller increases of the same lipid peroxidation products were found in copper-treated rats fed a vitamin E-supplemented diet. Despite all the evidence for free radical-mediated lipid peroxidation from copper intoxication, the free radicals themselves have not been detected *in vivo*.

We have already reported the detection of *in vivo* hydroxyl radical adduct formation in an animal model of acute copper poisoning dependent on ascorbic acid intake (35). Because it has been observed that the toxicity of copper is considerably higher in vitamin E- and/or selenium-deficient rats, the present experiments were designed to determine whether copper overload *in vivo* promotes radical generation in vitamin E- and/or selenium-deficient rats and whether the radical species generated are the same as in our previous findings (35). With the use of ESR and a radical spin-trapping technique, we detected *in vivo* formation of endogenous, possibly lipid-derived, radicals in copper-challenged rats deficient in both vitamin E and selenium.

Materials and Methods

$CuSO_4 \cdot 5H_2O$, DMSO, and PBN were purchased from Aldrich Chemical Co. (Milwaukee, WI). BC and DP were obtained from Sigma Chemical Co. (St. Louis, MO), and [^{13}C]DMSO (minimum of 99 atom % ^{13}C) was from Isotec Inc. (Miamisburg, OH). Partially deuterated PBN (d_{14}-PBN) was purchased from MSD Isotopes (Merck Frosst Canada Inc., Montreal, Canada).

Male Sprague-Dawley rats obtained from Charles River Laboratories (Raleigh, NC) were used in all experiments. Twenty-one-day-old rats were fed purified diets (ICN Biochemicals, Cleveland, OH) containing the following vitamin E and selenium concentrations: the vitamin E- and selenium-deficient diet contained no significant amounts of either vitamin E (α-tocopherol) derivatives or selenium; the selenium-deficient diet was the same diet but was supplemented with 121 IU of α-tocopherol/kg of food; the vitamin E-deficient diet was the same diet as the doubly deficient diet but was supplemented with 0.22 mg of sodium selenite/kg of food; the vitamin E- and selenium-adequate (control) diet was the same diet as the doubly deficient diet but was supplemented with both vitamin E and selenium. All rats were maintained on their respective diets, with access to an unlimited quantity of the diet and water, for 30 days, at which time the rats weighed 165 ± 30 g. Each dietary group consisted of eight to 10 rats.

For *in vivo* experiments, bile was collected from anesthetized rats (Nembutal, 50 mg/kg, intraperitoneally). Bile ducts were cannulated with a segment of PE-10 tubing. Where indicated, rats were given an intragastric injection of 1 M $CuSO_4$ (1 mmol/kg)[2] dissolved in distilled water and an intraperitoneal injection of the nitrone spin trap PBN (250 mg/kg) dissolved in DMSO (1 ml/kg). Twenty-minute bile samples were collected for 2 hr, into plastic Eppendorf tubes containing 300 mM BC and 30 mM DP (25 μl/100 g of body weight). Samples were frozen immediately on dry ice and stored at −70° until ESR analysis. After thawing, bile samples were transferred to a flat cell and spectra were recorded. All spectra shown are from bile collected 2 hr after injection of $CuSO_4$ and PBN.

The concentrations of copper and iron excreted into the bile of copper-injected deficient and control animals were estimated by a modified method of Felsenfeld (36), as described previously (35). Glutathione peroxidase activity in the liver cytosol from rats fed deficient and control diets was measured according to the procedure of Lawrence and Burk (37). Protein was measured by the method of Lowry *et al.* (38).

For *in vitro* measurements, bile samples from control and vitamin E- and selenium-deficient rats were used. PBN, DMSO, DP, BC, and Cu(I) or Cu(II) were added to each bile sample and scans were taken 5 min after mixing.

ESR spectra were obtained with a Varian E-109 spectrometer operating at 20-mW microwave power and 9.33 GHz, with 100-kHz modulation frequency. Spectral simulations were performed on an IBM PC computer using a program written by D. R. Duling.[3]

Results

Fig. 1A shows the radical adduct signal in bile from rats fed for 30 days with a vitamin E- and selenium-deficient diet, as measured 2 hr after the rats were challenged with $CuSO_4$ (2 mmol/kg, intragastrically). A solution of the spin trap PBN (250 mg/kg) in DMSO (1 ml/kg, intraperitoneally) was also administered. The intensity of the signals was dose dependent with respect to both $CuSO_4$ and PBN (data not shown). In the absence of copper (Fig. 1B) or PBN (Fig. 1C), no significant amount of radical adduct was detected. The small doublet

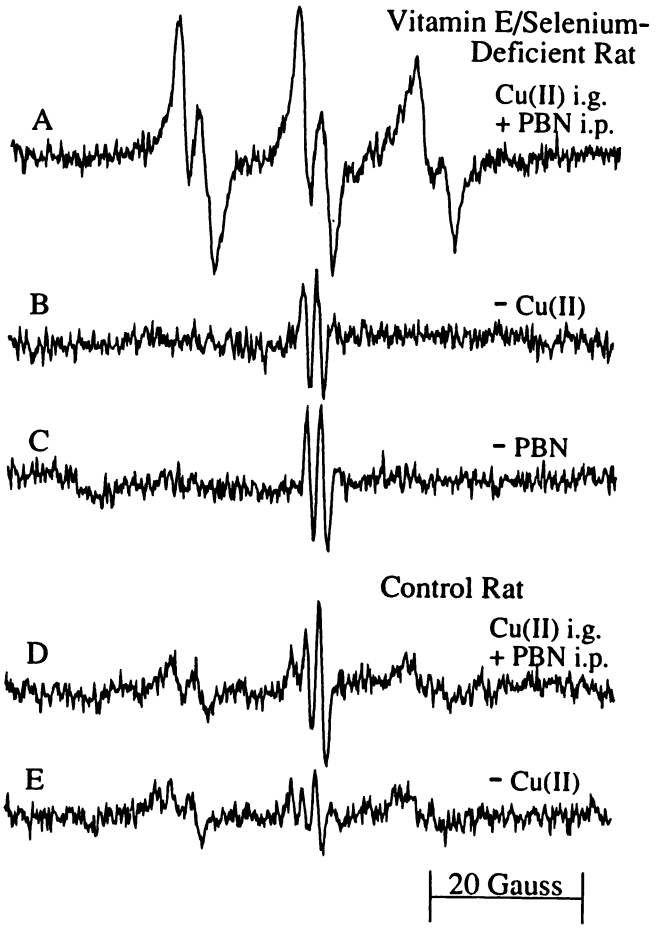

Fig. 1. ESR detection of a free radical formed *in vivo* and spin-trapped with PBN, from a copper-challenged rat deficient in both vitamin E and selenium. All spectra are from bile samples collected 2 hr after administration of copper. A, A vitamin E- and selenium-deficient rat was administered 2 mmol/kg $CuSO_4$, intragastrically, and 250 mg/kg PBN as a DMSO solution (1 ml/kg), intraperitoneally. B, As in A, but without administration of $CuSO_4$. C, As in A, but without administration of PBN. D, As in A, but the experiment was done with a rat fed a vitamin E- and selenium-adequate diet as a control. E, As in D, but without administration of $CuSO_4$. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 1 sec; scan rate, 5 G/min.

[2] The oral LD_{50} for $CuSO_4 \cdot 5H_2O$ in rats is 300 mg/kg (1.2 mmol/kg) (51).

[3] D. R. Duling. Simulation of multiple isotropic spin rap EPR spectra. Submitted for publication.

observed is due to the ascorbate radical anion (a^H = 1.7 G) formed from the one-electron oxidation of endogenous ascorbate. When the experiment was repeated with control rats fed a vitamin E- and selenium-supplemented diet, only a residual PBN radical adduct signal was detectable in either copper-treated (Fig. 1D) or nontreated (Fig. 1E) rats.

We demonstrated previously (35) that samples must be collected directly into a solution of the Cu(I)-stabilizing agent BC and the Fe(II)-stabilizing agent DP. This procedure prevents excreted copper and iron from becoming *ex vivo* Fenton catalysts and generating radical adducts during sample collection. Similar to previous results (35), excretion of both copper and iron (to a much lesser extent) was elevated in the bile of rats given an intragastric dose of $CuSO_4$ (data not shown). When 0.3 mM Cu(II) or Cu(I) was added *in vitro* to the bile from nontreated, vitamin E- and selenium-deficient rats containing 75 mM BC, 7.5 mM DP, 1.5 mM PBN, and 0.3 mM DMSO, no signals were detected from PBN adducts. Similar results were obtained with bile from rats fed a control diet (data not shown). These results indicate that the detectable radical adduct shown in Fig. 1A was formed and trapped *in vivo*, presumably in the liver, rather than *ex vivo*. Therefore, in the present experiments bile was collected into a solution of BC and DP to ensure that the ESR spectrum shown in Fig. 1A is from radical adducts formed *in vivo* and excreted in the bile.

To investigate further the individual roles of vitamin E and selenium in protecting against radical generation in copper-challenged rats, additional experiments were carried out. If rats were fed diets adequate in vitamin E and/or selenium, little or no radical adduct could be detected (Fig. 2).

Glutathione peroxidase is a cytosolic, selenium-dependent enzyme that protects the cell by reducing cellular peroxides without formation of free radical intermediates (39). Table 1 shows the glutathione peroxidase activities determined in liver cytosol from rats fed diets of various combinations of vitamin E and selenium deficiencies. The glutathione peroxidase activity was significantly decreased in rats fed a selenium-deficient or a combined vitamin E- and selenium-deficient diet, but was moderately increased in the group fed the vitamin E-deficient diet, compared with the control group. Because glutathione peroxidase activity is used as a means of assessing nutritional or functional selenium status, we interpret the absence of detectable radical adduct formation by vitamin E-deficient or selenium-deficient rats (Fig. 2) as evidence for the functional interrelationship between vitamin E and selenium (23, 30, 31, 40, 41).

In an effort to identify the radical adduct(s) in Fig. 1A, the experiment was repeated with the use of d_{14}-PBN. The advantage of using d_{14}-PBN is in the removal of any unresolved hyperfine splittings from the protons of the PBN spin trap, which can cause inhomogeneous broadening of the ESR signal. Thus, unresolved hydrogen couplings from hydrogens of the trapped radical or an underlying second radical adduct with overlapping hyperfine coupling constants may be resolved.

The result of the experiment with d_{14}-PBN is shown in Fig. 3A. Although the linewidth was decreased, as expected, no additional hyperfine couplings were resolved. The best simulation of this ESR signal is shown in Fig. 3B and required parameters of a^N = 15.36 G, a_β^H = 2.50 G, and 90% Lorentzian lineshape. An ESR signal with 100% Lorentzian lineshape is indicative of a single magnetic species, because this lineshape

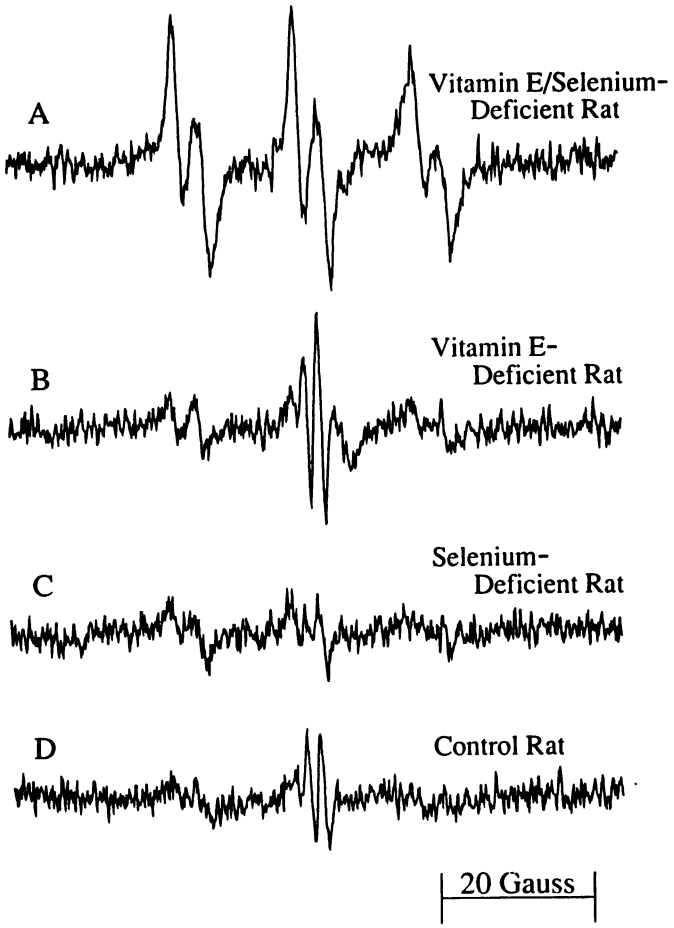

Fig. 2. ESR detection of a free radical formed *in vivo* and spin-trapped with PBN in copper-challenged rats with various combinations of vitamin E and selenium deficiencies. All spectra are from bile samples collected 2 hr after administration of copper. A, A vitamin E- and selenium-deficient rat was administered 2 mmol/kg $CuSO_4$, intragastrically, and 250 mg/kg PBN as a DMSO solution (1 ml/kg), intraperitoneally. B, As in A, but with a vitamin E-deficient rat. C, As in A, but with a selenium-deficient rat. D, As in A, but the experiment was done with a rat fed a vitamin E- and selenium-adequate diet as a control. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 1 sec; scan rate, 5 G/min.

TABLE 1

Liver cytosolic glutathione peroxidase activity in rats fed various combinations of vitamin E- and selenium-deficient diets for 30 days

Assay was according to the procedure of Lawrence and Burk (37). Values are mean ± standard error.

Rat status	Group size (*n*)	Glutathione peroxidase activity[a]
		μmol/min/mg of protein
Vitamin E- and selenium-adequate (controls)	8	124.9 ± 9.6
Vitamin E-deficient	8	167.6 ± 11.9[b]
Selenium-deficient	10	19.5 ± 1.3[c]
Vitamin E- and selenium-deficient	5	30.9 ± 2.5[c]

[a] Reported as μmol of NADPH oxidized/min/mg of protein.
[b] $p < 0.014$ with respect to control group, according to unpaired *t* test.
[c] $p < 0.001$ with respect to all groups, according to unpaired *t* test.

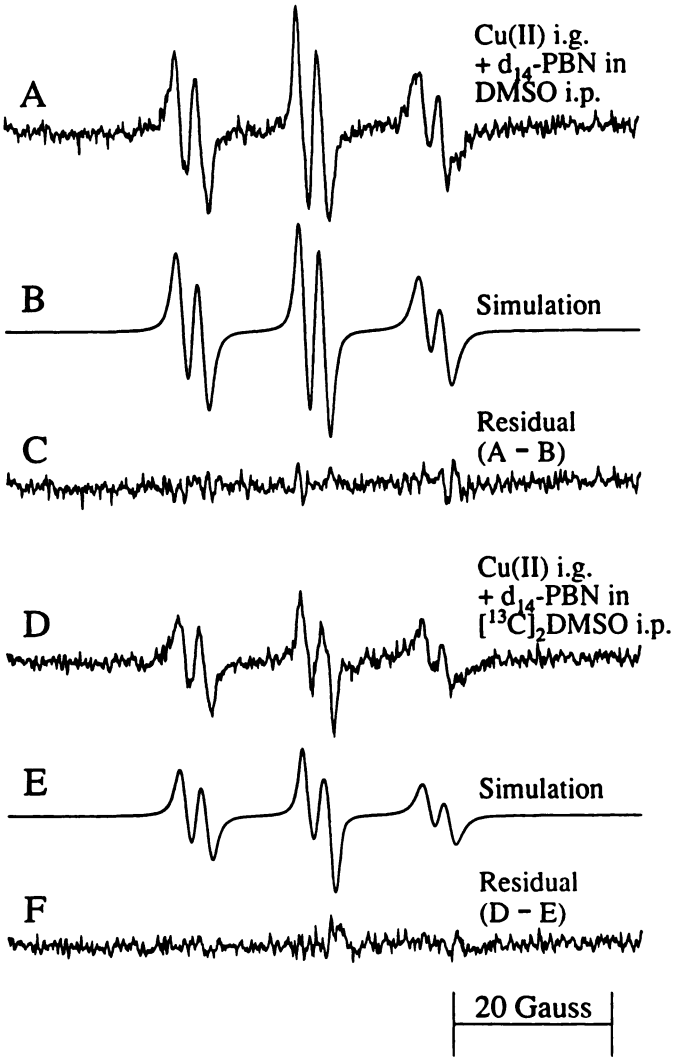

Fig. 3. Effect of isotope substitutions on the ESR spectra of the PBN radical adduct formed *in vivo* in copper-challenged rats deficient in both vitamin E and selenium. Spectra are from bile samples collected 2 hr after administration of copper. A, A vitamin E- and selenium-deficient rat was administered 2 mmol/kg $CuSO_4$, intragastrically, and 250 mg/kg d_{14}-PBN in a DMSO solution (1 ml/kg), intraperitoneally. B, Computer simulation of A with a^N = 15.36 G, a^H_β = 2.50 G, and a 90% Lorentzian lineshape. C, Residual of A minus B, indicating the precision of the simulated spectrum. D, A vitamin E- and selenium-deficient rat was administered 2 mmol/kg $CuSO_4$, intragastrically, and 250 mg/kg d_{14}-PBN in a [^{13}C]DMSO solution (1 ml/kg), intraperitoneally. Additional carbon-13 (I = 1/2) hyperfine splittings are not observed. E, Computer simulation of D with a^N = 15.40 G, a^H_β = 2.54 G, and a 100% Lorentzian lineshape. F, Residual of D minus E, indicating the precision of the simulated spectrum. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 0.67 G; time constant, 4 sec; scan rate, 1.33 G/min.

is the theoretical ideal. The residual signal in Fig. 3C is the difference between the experimental spectrum in Fig. 3A and the computer-generated simulation in Fig. 3B. From these results, it is clear that only one major species is responsible for the ESR signal observed in Fig. 3A; however, a small contribution from a second minor radical adduct cannot be excluded.

Although a positive identification of radical adducts formed *in vivo* is very difficult, tentative assignments can be made through isotope substitutions and careful comparisons with ESR parameters of known PBN adducts. If sufficient hydroxyl radical is formed *in vivo*, as is the case for rats administered both $CuSO_4$ and ascorbate (36), then the PBN-methyl radical adduct (PBN/$\cdot CH_3$) can be detected in bile. The methyl radical results from the reaction of hydroxyl radical with the administered DMSO. The ESR parameters of the radical adduct detected here do not correspond to those of PBN/$\cdot CH_3$ (a^N = 16.38 G and a^H_β = 3.64 G) or the related PBN-methoxy radical adduct (PBN/$\cdot OCH_3$) (a^N = 15.18 G and a^H_β = 3.50 G), as measured when these species were generated *ex vivo* by a Fenton system in bile from vitamin E- and selenium-deficient rats. PBN/$\cdot OCH_3$ is formed by trapping of the $\cdot OCH_3$ that results from the decomposition of $\cdot OOCH_3$, which is formed from the reaction of $\cdot CH_3$ with O_2 (42).

When [^{13}C]DMSO was used instead of [^{12}C]DMSO, no additional hyperfine splitting from ^{13}C (I = 1/2) was observed (Fig. 3D), again indicating that PBN/$\cdot CH_3$ was not present. Values of a^N = 15.40 G and a^H_β = 2.54 G, with a 100% Lorentzian lineshape, were obtained from the simulation shown in Fig. 3E. These simulated ESR parameters are nearly identical to those obtained from Fig. 3B. Fig. 3D also contains a small underlying signal from the ascorbate radical, which was included in the simulation shown in Fig. 3E. The absence of an isotope substitution effect on the ESR signal in Fig. 3D makes a unique assignment of this radical species problematic. Nevertheless, these results indicate that the radical species trapped by PBN *in vivo* in copper-challenged, vitamin E- and selenium-deficient rats was formed from an endogenous molecule such as a polyunsaturated fatty acid.

Discussion

The experiments described above provide ESR evidence that an endogenous radical was trapped and detected in copper-challenged, vitamin E- and selenium-deficient rats. In contrast, we were not able to detect significant concentrations of radical adduct from copper-treated rats fed a control diet or a diet deficient in only vitamin E or selenium. The model used in this study is one of a single, high dose of Cu(II) resulting in acute toxicity.[2]

It is known from other studies that Cu(II) increases the lipid peroxidation products formed in selenium- and vitamin E-deficient rats (16, 19, 34, 43). Therefore, the mechanism of copper toxicity apparently involves lipid peroxidation. According to the current hypothesis, transition metal ions are involved in lipid peroxidation by decomposing lipid peroxides into their peroxyl and alkoxyl radicals, which can then abstract hydrogen and perpetuate a chain reaction of lipid peroxidation (44, 45):

$$LOOH + M^{n+} \rightarrow LO^\bullet + M^{(n+1)+} + OH^-$$

$$LOOH + M^{(n+1)+} \rightarrow LOO^\bullet + M^{n+} + H^+$$

$$LO^\bullet + LH \rightarrow LOH + L^\bullet$$

$$LOO^\bullet + LH \rightarrow LOOH + L^\bullet$$

$$L^\bullet + O_2 \rightarrow LOO^\bullet$$

where L represents a lipid molecule and M represents a redox-active metal ion. The direct participation of transition metals in an initiating reaction by electron abstraction from unsaturated fatty acids has also been proposed (46).

$$M^{(n+1)+} + LH \rightarrow M^{n+} + L^\bullet + H^+$$

Other mechanisms have been proposed to describe cellular

copper toxicity based on its ability to catalyze the formation of toxic oxygen species (11, 12, 47). In a previous study we presented ESR evidence that hydroxyl radical was generated *in vivo* in rats treated simultaneously with Cu(II) and ascorbic acid (35). In this study, *in vivo* hydroxyl radical formation from a copper-challenged, vitamin E- and selenium-deficient rat could not be detected.

Although no direct evidence has been presented for the generation of lipid-derived radicals in our animal model, we provide conclusive evidence that the radical adduct is formed from an endogenous molecule. Had the generation of hydroxyl radical been the primary toxic action of copper in these experiments, then the DMSO-dependent PBN/$\cdot CH_3$ adduct should have been detected (35). From the hyperfine splitting constants obtained for the radical adduct shown in Figs. 1A and 2A and the lack of a carbon-13 isotope splitting when [^{13}C]DMSO was used, it is apparent that the radical adduct is not a product of hydroxyl radical scavenging by DMSO. It should be noted, however, that an inability to detect radical adducts in bile by ESR is not proof that it is not formed *in vivo*. A number of factors, such as the relative rates of radical adduct formation, destruction, and excretion, can all affect the detection of a radical adduct.

The fact that the radical adduct could be detected only in rats that were deficient in both vitamin E and selenium strongly suggests that its formation is associated with lipid peroxidation. In this respect, the primary role of vitamin E in preventing free radical-initiated peroxidative tissue damage is widely accepted, and the functional interrelation between vitamin E and selenium has been recognized (48). In a variety of experimental systems, studies have shown that selenium may complement the antioxidant function of vitamin E via selenoenzymes (49, 50). Indeed, in this role they are a part of a complex system of antioxidant protection *in vivo* that includes free radical scavengers and several families of enzymes. In conclusion, the present study provides the most direct ESR evidence yet of lipid peroxidation-associated free radical formation in the toxicity of copper.

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Send reprint requests to: Maria B. Kadiiska, Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.