BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 54–58 (1997)
ARTICLE NO. RC976277

Synthesis of Cr(IV)-GSH, Its Identification and Its Free Hydroxyl Radical Generation: A Model Compound for Cr(VI) Carcinogenicity

Ke Jian Liu,* Xianglin Shi,†,[1] and Nar S. Dalal‡,[2]

**Department of Radiology, Dartmouth Medical School, Hanover, New Hampshire 03755; †Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505; and ‡Department of Chemistry and High Magnetic Field Laboratory, Florida State University, Tallahassee, Florida 32306*

Received January 23, 1997

Current models of Cr(VI) carcinogenesis suggest an important role for Cr(IV) as an intermediate, toxic, carcinogenic species, but direct chemical evidence has been lacking. This is because Cr(IV) is a highly reactive oxidation state of Cr and few Cr(IV)-based compounds are known that can be used as a model compound containing a biological ligand. This study reports the isolation of such a stable Cr(IV) complex. The Cr(IV)-GSH complex has been synthesized through the reaction of Cr(VI) with GSH. Its electron paramagnetic resonance (EPR) spectrum exhibits g = 1.9629 and a peak-to-peak line width of 480 G in aqueous medium as well as in the powder form. Magnetic susceptibility measurements showed that the compound has a magnetic moment of 2.53 Bohr magneton per Cr, establishing that the Cr ion has two unpaired electrons, hence its identity as Cr(IV). The Cr(IV)-GSH complex is able to generate hydroxyl (˙OH) radical in the presence of molecular oxygen in aqueous medium. Catalase inhibited the ˙OH radical generation while H_2O_2 enhanced it, indicating that the ˙OH radical was generated via a Fenton-like reaction, H_2O_2 being generated as an intermediate in the reduction of molecular oxygen. Metal ion chelators, deferoxamine and 1,10-phenanthroline, attenuated the generation of Cr(IV)-mediated ˙OH radical. In the case of deferoxamine, a deferoxamine-derived free radical was generated as shown by EPR measurements. The results imply that Cr(IV) may play an important role in the mechanism of Cr(VI)-induced carcinogenesis and Cr(IV)-GSH can be used as a model compound to study the role of Cr(IV) in this mechanism.

Cr(VI)-containing compounds, such as the chromate, are established carcinogens (1, 2). Although the mechanism of Cr(VI)-induced carcinogenesis is still not well understood, it is generally thought that reduction of Cr(VI) to its lower oxidation states is an important step (3, 4). Jennette (5) demonstrated the formation of Cr(V) species in the reduction of Cr(VI) by NADPH/microsomes. Due to the reactive nature of Cr(V) species, she suggested that Cr(V) intermediates are likely candidates for the "ultimate" carcinogenic forms of carcinogenic chromium compounds (5). This suggestion led to an intensive study on the one-electron reduction of Cr(VI) by a variety of cellular reductants. Very recent studies have also demonstrated that in vivo reduction of Cr(VI) in mice generates Cr(V) species (6, 7). It has been established that Cr(V) is capable of generating ˙OH radicals from H_2O_2 via a Fenton-like mechanism (8, 9). Both Cr(V) and ˙OH are believed to be significantly involved in the mechanism of Cr(VI)-induced carcinogenesis (10-12).

In addition to Cr(V) species, recent studies have indicated that Cr(IV), another reactive intermediate generated in the reduction of Cr(VI), also may play an important role in the mechanism of Cr(VI)-induced carcinogenesis (13-17). In our recent studies, we have shown that Cr(IV) is able to generate ˙OH radicals via a Fenton-like reaction ($Cr(IV) + H_2O_2 \rightarrow Cr(V) + {}^{\cdot}OH + {}^{\cdot}OH^-$), with a reaction rate higher than that of the Cr(V)-mediated Fenton-like reaction (13-15). The ˙OH radicals generated by Cr(IV) from H_2O_2 are able to cause DNA strand breaks and hydroxylation of 2′-deoxyguanosine (dG) (13, 14). Very recently, we have synthesized a reactive Cr(IV) ester, Cr(IV)-2,4-dimethyl-2,4-pentanediol (14, 15). Using this Cr(IV) ester as a model Cr(IV) compound, we have shown that Cr(IV) is more potent in causing DNA strand breaks than Cr(V). While this chemically synthesized Cr(IV) ester can be conveniently used as a model Cr(IV) compound, it is not expected to form in cellular systems. Thus a more

[1,2] Authors to whom reprint requests should be addressed.

0006-291X/97 $25.00

biologically relevant Cr(IV) model compound is needed to study the role of Cr(IV) in the mechanism of Cr(VI)-induced carcinogenesis. It may be noted that reduction of Cr(VI) by GSH and ascorbate, which are considered to be the important cellular Cr(VI) reductants has been reported to generate Cr(IV) as an intermediate species (11, 13, 16, 18). In this communication, we report our study in which the reaction of Cr(VI) with GSH was used to synthesize a Cr(IV)-GSH compound. This newly synthesized Cr(IV) compound was identified by EPR and magnetic susceptibility measurements. This compound is able to generate $^{\bullet}OH$ radical in the presence of molecular oxygen in aqueous medium. Catalase inhibited the generation of $^{\bullet}OH$ while H_2O_2 enhanced it. Molecular oxygen was consumed during the generation of $^{\bullet}OH$. Deferoxamine, a metal chelator, inhibited the Cr(IV)-mediated generation of $^{\bullet}OH$ with a concomitant generation of a deferoxamine-derived free radical. Although additional studies are needed to establish the exact molecular structure of this Cr(IV) complex, the results imply that Cr(IV) may play an important role in the mechanism of Cr(VI)-induced carcinogenesis and Cr(IV)-GSH can be used as a Cr(IV) model compound.

MATERIALS AND METHODS

Sodium dichromate ($K_2Cr_2O_7$), deferoxamine, 1, 10-phenanthroline, glutathione (GSH) and H_2O_2 were purchased from Sigma (St. Louis, MO). The spin trap, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Aldrich (Milwaukee, WI). Catalase was obtained from Boehringer Mannheim (Indianapolis, IN). Chelex 100, a chelating resin, was purchased from Bio-Rad Laboratories (Richmond, CA). DMPO was purified by charcoal decolorization and vacuum distillation. The purified DMPO did not contain any EPR detectable impurities. The phosphate buffer (pH 7.4) was treated with Chelex 100 to remove transition metal ion contaminants.

The Cr(IV)-GSH complex was prepared by mixing 100 mM GSH and 25 mM $Na_2Cr_2O_7$ in water at room temperature. The mixture was stirred for 30 minutes. A brown color appeared immediately after mixing. A brown precipitate was collected 2 hours after mixing, and washed with water several times. The Cr(IV)-GSH complex thus formed in solid form is stable at least for a year as measured by EPR spectroscopy and magnetic susceptibility.

All EPR measurements were made using a Varian E4 EPR spectrometer and a flat cell assembly. Hyperfine couplings were measured using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picryhydrazyl (DPPH) as reference standards (19). Reactants were mixed in a test tube in a total final volume of 450 ml. The reaction mixture was then transferred to a flat cell for EPR measurements. All experiments were carried out at room temperature under ambient air except those specifically indicated.

Magnetic susceptibility measurements were carried out using a Quantum Design SQUID magnetometer.

Oxygen consumption rate was measured using EPR oximetry. This method is based on the interaction between paramagnetic materials and molecular oxygen, which broadens the EPR signals of those paramagnetic materials via Heisenberg spin-exchange in a concentration-dependent manner (20, 21). The advantages of this method include rapid response time and sensitivity over a broad range of oxygen concentrations. The paramagnetic probe used was 2,2,6,6-tetramethylpiperidine-d_{16}-1-^{15}N-1-oxyl-4-one (^{15}N-PDT). The oxygen dependence of the probe's EPR spectrum was quantitated by changes in the spectral line width, whose values were related to specific oxygen concentrations. 5 mg of the Cr(IV) compound was added to 200 ml phosphate buffer solution (pH 7.0) containing 0.1 mM ^{15}N-PDT. After agitation with a Vortex machine, the mixture was drawn into a glass capillary tubing (i.d., 1 mm), and sealed at both ends with no air bubble inside. The sealed tubing was transferred to a standard EPR quartz tube, and the EPR spectrum of ^{15}N-PDT was recorded immediately every 110 seconds for 50 minutes. The line width of the high field peak of each recorded spectrum was calculated using a computer simulation program (Ewvoigt), and then converted into oxygen concentration using a standard calibration curve for ^{15}N-PDT.

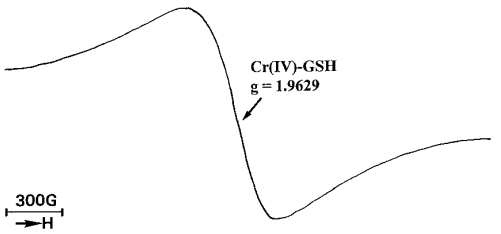


FIG. 1. EPR spectrum of an aqueous solution of Cr(IV)-GSH. The spectrometer settings were: receiver gain 3.2×10^3; time constant, 0.3 sec; incident microwave power, 20 mW. modulation amplitude, 4.0 G; center field, 3520 G and sweep width, 4,000 G.

RESULTS

Characterization Studies

The brown precipitate isolated from the reaction of $Na_2Cr_2O_7$ and GSH in a molar ratio of 1:2 (Cr:GSH) was characterized mainly by two techniques that are specific to the oxidation state of the metal center. These were (a) EPR spectroscopy and (b) magnetic susceptibility, as summarized below.

Figure 1 shows the EPR spectrum obtained from an aqueous solution (50 mg/ml) of the isolated powder. The spectrum is centered at g = 1.9629 $\pm$ 0.0005, with a peak-to-peak line width of 480 gauss. The intensity of the spectra increased in proportion to the concentration of the dissolved powder. The experimental procedure and the g-factor were consistent with a metal center of Cr, in an oxidation state as Cr(III), Cr(IV) or Cr(V). We could rule out Cr(V) because the line width of all known Cr(V) compounds in solution is only about 5 gauss (8, 9, 13, 22). The line width remained about the same when the spectrum was measured for the powder sample (data not shown). Cr(V) solids exhibit line width in the order of only 20-30 gauss (22), since there is just one unpaired electron on Cr(V). One expects a much larger line width for Cr(IV) and Cr(III), because the former has two unpaired electrons whereas the later has three and the electronic dipole-dipole interaction causes a broadening of the EPR lines for these ions. Our earlier studies on a Cr(IV)-ester demonstrated that one can expect a line width of about 400 gauss for a Cr(IV) complex (14, 15). However, neither the line width nor the g-factor provides a clear distinction between Cr(III) and Cr(IV). Therefore we used magnetic susceptibility measurements for a

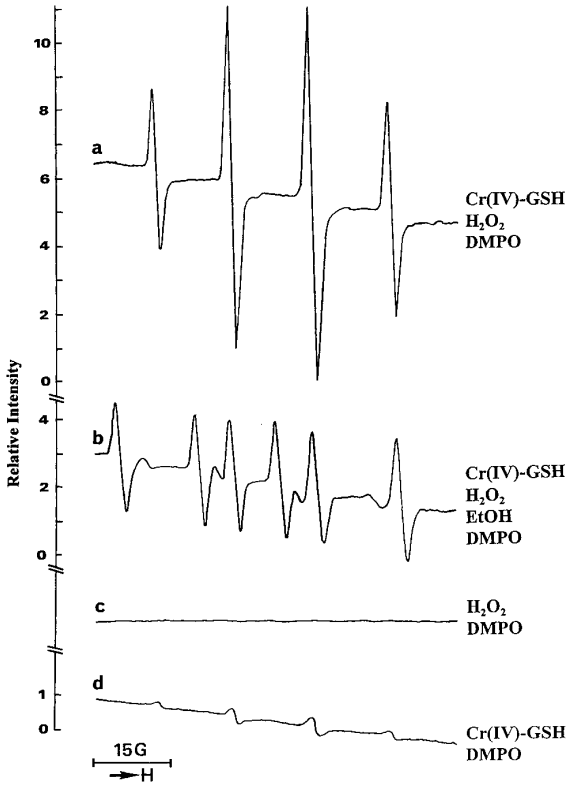
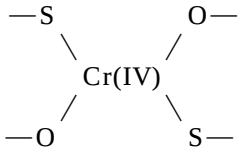


FIG. 2. EPR spectra recorded from phosphate buffer solution (pH 7.4) of various combinations of 1.5 mg/ml Cr(IV)-GSH, 2 mM H_2O_2, 2.5% ethanol, and 100 mM DMPO. The spectrometer settings were: receiver gain 4.0×10^3; time constant, 0.3 sec; modulation amplitude, 0.8 G; incident microwave power, 20 mW; center field, 3520 G and sweep width, 200 G.

more definitive characterization of the oxidation state of the metal.

Magnetic susceptibility (D.C.) measurements on a 10 mg powder sample showed that the isolated complex had a magnetic moment of 2.53 Bohr magnetons per Cr at room temperature, indicating that the Cr ion has two unpaired electrons. Together with the EPR data, the magnetic susceptibility measurements identify the ion center as Cr(IV).

While additional information on the molecular structure must await a detailed X-ray diffraction study, we tentatively assign the following structure to the isolated Cr(IV)-GSH complex.



We feel that for our present purpose, the detailed crystal structure is not necessary, because our focus is on the oxidation state, and the capability of this complex to generate free radical. We hope that this study will arose the interest of others to pursue such crystal structure determinations.

Cr(IV)-Mediated ˙OH Radical Generation

Figure 2a shows the EPR spectrum obtained from a mixture of 1.5 mg/ml Cr(IV)-GSH, 2 mM H_2O_2 and 100 mM DMPO in pH 7.4 phosphate buffer. The spectrum consists of a 1:2:2:1 quartet with splittings of $a_H = a_N = 14.9$ G. Based on these splitting constants and the spectral lineshape, the spectrum was assigned to a DMPO/˙OH adduct. As supporting evidence for the ˙OH radical formation, EPR spin trapping measurements were made with 2.5% ethanol added as a secondary trap. As shown in Figure 2b, addition of ethanol generated a new spin adduct signal with measured hyperfine splittings of $a_N = 15.8$ G and $a_H = 22.8$ G. These splittings are typical of those of the DMPO/˙$CHOHCH_3$ adduct, showing that the DMPO/˙OH adduct is indeed the result of trapping of the ˙OH radical formed during the reaction. No detectable EPR signal was observed if Cr(IV) was omitted from the reaction mixture (Figure 2c). It may be noted that Cr(IV) alone, i.e., without the addition of H_2O_2, also generated an EPR signal of the DMPO/˙OH adduct, albeit much weaker in spectral intensity (Figure 2d). Figure 3a shows the EPR signal of DMPO/˙OH adduct from a Cr(IV)-GSH solution at a relatively higher concentration (2.5 mg/ml) and different spectrometer settings. A 1:2:2:1 quartet corresponding to DMPO/˙OH was observed, showing that Cr(IV) alone is able to generate ˙OH radical. Addition of catalase suppressed the ˙OH generation (Figure 3b), showing the involvement of H_2O_2 in the mechanism of ˙OH generation.

Since the Cr(IV) solution alone is able to generate ˙OH radical and H_2O_2 is involved, it is very likely that molecular oxygen is being converted to H_2O_2 and

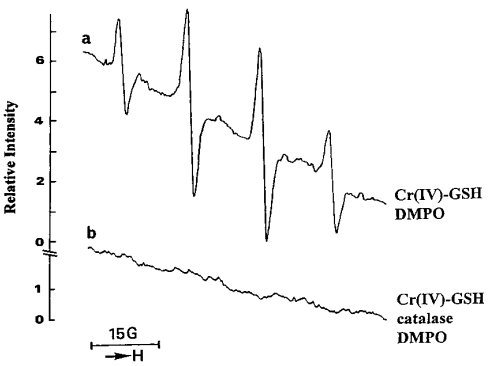


FIG. 3. (a) EPR spectrum recorded from pH 7.4 phosphate buffer solution of 2.5 mg/ml Cr(IV)-GSH and 100 mM DMPO; (b) same as (a) but with the addition of 10,000 units/ml catalase. The spectrometer settings were: receiver gain 6.3×10^3; time constant, 0.3 sec; modulation amplitude, 0.8 G; incident microwave power, 20 mW; center field, 3520 and sweep width, 200 G.

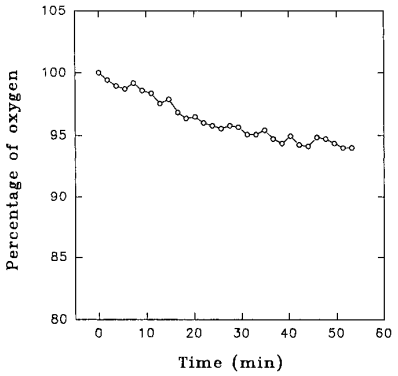


FIG. 4. Oxygen consumption by Cr(IV)-GSH in aqueous solution. The EPR oximetry technique was used to measure the concentration of oxygen in the aqueous solution containing Cr(IV)-GSH (5 mg/ml) in a sealed capillary tubing. The measured oxygen concentration was converted to the percentage of the concentration at the start of the reaction.

eventually to ˙OH radical. The EPR oximetry technique (20, 21) was used to measure the concentration of oxygen in the aqueous solution containing Cr(IV)-GSH (5 mg/ml) in a sealed glass capillary tubing. The measured oxygen concentration was converted to the percentage of the concentration at the start of the reaction. As shown in Figure 4, the Cr(IV) solution is indeed able to consume molecular oxygen. About 5% of oxygen was consumed in about 50 minutes after initiation of reaction.

Figure 5 shows the spectrum obtained from 1.5 mg/mg Cr(IV)-GSH, 2 mM H_2O_2, 2 mM deferoxamine, and 200 mM DMPO. A computer simulation analysis of this signal yielded hyperfine splittings of a_N = 7.7 G and a_H = 6.2 G. This is a typical deferoxamine nitroxide radical signal (23). 1,10-Phenanthroline also attenuated the generation of ˙OH from the mixture of Cr(IV)-GSH and H_2O_2 but did not generate an EPR detectable, 1,10-phenanthroline-derived radical.

DISCUSSION

A solid Cr(IV)-GSH compound has been synthesized using the reaction of Cr(VI) with GSH. This chemically synthesized compound is quite stable at least over a period of one year when stored in a refrigerator. As to the possible chemical structure of this complex, we can state that the ratio of Cr to GSH is 1:2 since the compound is formed best under these conditions. The Cr(IV) may be bound in a square-pyramidal coordination, so that there is a free site to which H_2O_2 can bind, promoting bond homolysis and ˙OH generation. Since GSH is considered as an important Cr(VI) reductant, the Cr(IV)-GSH complex can be used as a model Cr(IV) compound to study the role of Cr(IV) in the mechanism of Cr(VI) carcinogenesis.

The results obtained from the present study show that Cr(VI)-GSH is able to generate ˙OH radicals upon reaction with H_2O_2. The ˙OH radicals are able to react with ethanol to generate ethanol-derived free radicals, indicating that the ˙OH radicals generated in the reaction are "free" and are not tightly associated with Cr(IV)-GSH complex. It can be noted that ˙OH radicals generated in the reaction of H_2O_2 with certain other metal ions, such as nickel, copper-, or zinc-containing superoxide dismutase, exhibit only limited reactivity (24, 25). The ˙OH radicals generated by these systems are considered to be within the domain of certain macromolecules and not "free" to exhibit significant activity. Since the results obtained from the present study show that ˙OH radicals generated by Cr(IV) from H_2O_2 are able to react with ethanol, they may be able to cause damage to DNA and other cellular targets.

This study has demonstrated that Cr(IV)-GSH alone without any exogenous H_2O_2 is capable of generating ˙OH radical. The mechanism of ˙OH generation also involves reaction of Cr(IV) with H_2O_2 as demonstrated by the inhibition of ˙OH radical generation by catalase. Since no H_2O_2 is added to the reaction mixture, H_2O_2 has to be generated by the reduction of molecular oxygen. This appears to be the case, since EPR oximetry measurements show that an aqueous solution of Cr(IV)-GSH complex consumed molecular oxygen.

The results obtained in the present study also show that metal chelators, deferoxamine and 1,10-phenanthroline, inhibited Cr(IV)-mediated ˙OH generation from H_2O_2. Deferoxamine is used for prevention and treatment of iron overload (26, 27) as well as for combating toxic effect of vanadium (28). The formation of deferoxamine nitroxide radical with a concomitant disappearance of DMPO/˙OH, as found in this study, indicates that deferoxamine site-specifically reacts with ˙OH radical to generate deferoxamine nitroxide radical. With regard to 1,10-phenanthroline, it is a membrane-permeable chelating agent, which has been reported to inhibit H_2O_2-induced DNA damage (29, 30), mutation and transformation (31). This che-

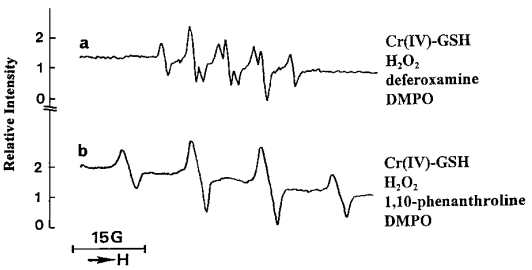


FIG. 5. (a) EPR spectrum recorded from pH 7.4 phosphate buffer solution of 1.5 mg/ml Cr(VI)-GSH, 2 mM H_2O_2, 2 mM deferoxamine, and 200 mM DMPO; (b) same as (a) but using 2 mM 1,10-phenanthroline instead of deferoxamine. The spectrometer settings were: receiver gain 4.0×10^3; time constant, 0.3 sec; modulation amplitude, 0.5 G; incident microwave power, 20 mW; center field, 3520 G and sweep width, 200 G.

lator is also able to form complexes with transition metal ions, such as iron to block $^{\bullet}OH$ generation (29-32). This chelator has been reported to protect cells from either Cr(VI)-induced alkali-labile sites or the combination of alkali-labile sites plus DNA damage (33). The results obtained from the present study show that proper chelation may be used to inhibit Cr(IV)-mediated $^{\bullet}OH$ generation and to prevent or attenuate Cr(VI)-mediated cellular damage.

In conclusion, this study demonstrates that reaction of Cr(VI) with GSH generates an isolatable Cr(IV)-GSH complex, which is stable for at least a year when stored in a refrigerator. This Cr(IV)-GSH complex is able to generate $^{\bullet}OH$ radical in the presence of oxygen in aqueous medium. Molecular oxygen was reduced to H_2O_2, which reacts with Cr(IV) to generate $^{\bullet}OH$ radicals via a Fenton-like reaction. Metal chelators, deferoxamine and 1,10- phenanthroline, attenuated the Cr(IV)-mediated $^{\bullet}OH$ generation. This Cr(IV)-GSH may be useful as a Cr(IV) model compound to study the role of Cr(IV) in the mechanism of Cr(VI)-induced carcinogenesis. It is expected that this study will arouse new interest in the crystal growth and structural determination of this and related compounds.

ACKNOWLEDGMENTS

We wish to thank Dr. Alex Smirnov, University of Illinois, for performing the magnetic susceptibility measurements. NSD thanks Florida State University for a partial support of this work. This research used the facilities of the EPR Center for the Study of Viable Systems at Dartmouth supported by the National Institute of Health Grant P41 RR11602-01A1.

REFERENCES

1. Langard, S. (1988) *Sci. Total Environ.* **71,** 341–350.
2. De Flora, S., Bagnasco, M., Serra, D., and Zanacchi, P. (1990) *Mutat. Res.* **238,** 99–172.
3. Tsapakos, M. J., and Wetterhahn, K. E. (1983) *Chem. Biol. Interactions* **46,** 265–277.
4. Connett, P. H., and Wetterhahn, K. E. (1983) *Struct. Bonding* **54,** 93–124.
5. Jennette, K. E. (1982) *J. Am. Chem. Soc.* **104,** 874–875.
6. Liu, K. J., Jiang, J., Swartz, H. M., and Shi, X. (1994) *Arch. Biochem. Biophys.* **311,** 248–252.
7. Liu, K. J., Shi, X., Jiang, J., Goda, F., Dalal, N. S., and Swartz, H. M. *Arch. Biochem. Biophys.* **323,** 33–39.
8. Shi, X., and Dalal, N. S. (1989) *Biochem. Biophys. Res. Commun.* **163,** 627–634.
9. Shi, X., and Dalal, N. S. (1990) *Arch. Biochem. Biophys.* **277,** 342–350.
10. Klein, C. B., Frenkel, K., and Costa, M. (1991) *Chem. Res. Toxicol.* **4,** 592–604.
11. Standeven, A. M., and Wetterhahn, K. E. (1991) *Chem. Res. Toxicol.* **4,** 616–625.
12. Kasprzak, K. S. (1991) *Chem. Res. Toxicol.* **4,** 604–615.
13. Shi, X., Mao, Y., Knapton, A. D., Ding, M., Rojanasakul, R. J., Gannett, P. M., Dalal, N. S., and Liu, K. J. (1994) *Carcinogenesis* **15,** 2475–2478.
14. Luo, H., Lu, Y., Shi, X., Mao, Y., and Dalal, N. S. (1996) *Ann. Clin. Lab. Sci.* **26,** 185–191.
15. Luo, Y., Shi, X., Mao, Y., and Dalal, N. S. *J. Inorg. Biochem.* (In press).
16. Fresco, P., and Kortenkamp, A. (1994) *Carcinogenesis* **15,** 1773–1778.
17. Bose, R., Moghaddas, S., and Gelerinter, E. (1992) *Inorg. Chem.* **31,** 1987–1994.
18. Stearns, D. M., Courtney, K. D., Giangrande, P. H., Phieffer, L. S., and Wetterhahn, K. E. (1994). *Environ. Health. Perspect.* **102** (suppl.) 3, 21–25.
19. Dalal, N. S., Suryan, M. M., and Seehra, M. S. (1981) *Anal. Chem.* **55,** 953–957.
20. Swartz, H. M., Bacic, G., Friedman, B., Goda, F., Grinberg, O. Y., Hoopes, P. J., Jiang, J., Liu, K. J., Nakashima, T., O'Hara, J., Walczak, T. (1995) *in* Oxygen Transport to Tissue XVI (Hogan, M. C., *et al.*, Eds.), pp. 119–128, Plenum Press, New York.
21. Hyde, J., and Subczynski, W. K. (1989) *Biol. Magnetic Resonance* **8,** 399–425.
22. Shi, X., Dalal, N. S. (1988) *Biochem. Biophys. Res. Commun.* **156,** 137–142.
23. Hanna, P. M., Kadiiska, M. B., and Mason, R. P. (1992) *Chem. Res. Toxicol.* **5,** 109–115.
24. Inoue, S., and Kawanishi, S. (1989) *Biochem. Biophys. Res. Commun.* **159,** 445–451.
25. Yim, B. B., Chock, P. B., and Stadtman, E. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5006–5010.
26. Halliwell, B. (1989) *Free Radical Biol. Med.* **7,** 645–651.
27. McLaren, G. D., Muir, W. A., and Kellermeyer, R. W. (1983) *CRC Crit. Rev. Clin. Lab. Sci.* **19,** 205–265.
28. Keller, R. J., Rush, J. D., and Grover, T. A. (1991) *J. Inorg. Biochem.* **41,** 269–276.
29. Keys, S., and Tyrrel, R. M. (1990) *Carcinogenesis* **11,** 787–791.
30. Shacter, B., Lopez, R. L., Beecham, E. J., and Janz, S. (1989) *J. Biol. Chem.* **265,** 6693–6699.
31. Nassi Calo, L., Mello Filho, A. C., and Meneghini, R. (1989) *Carcinogenesis* **10,** 1055–1057.
32. Larramendy, M., Mello Filho, A. C., Lem Martins, E. A., and Meghini, R. (1987) *Mutat. Res.* **178,** 57–63.
33. Sugiyama, M., Tsuzuki, K., and Haramaki, N. (1993) *Arch. Biochem. Biophys.* **305,** 261–266.