ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 293, No. 2, March, pp. 281–286, 1992

Deferoxamine Inhibition of Cr(V)-Mediated Radical Generation and Deoxyguanine Hydroxylation: ESR and HPLC Evidence[1]

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Received August 8, 1991, and in revised form October 28, 1991

Electron spin resonance (ESR) and high-performance liquid chromatography (HPLC) techniques were utilized to investigate the effect of deferoxamine on free radical generation in the reaction of Cr(V) with H_2O_2 and organic hydroperoxides. ESR measurements demonstrated that deferoxamine can efficiently reduce the concentration of the Cr(V) intermediate as formed in the reduction of Cr(VI) by NAD(P)H or a flavoenzyme glutathione reductase/NADH. ESR spin trapping studies showed that deferoxamine also inhibits Cr(V)-mediated ·OH radical generation from H_2O_2, as well as Cr(V)-mediated alkyl and alkoxy radical formation from *t*-butyl hydroperoxide and cumene hydroperoxide. HPLC measurements showed that ·OH radicals generated by the Cr(VI)/flavoenzyme/NAD(P)H enzymatic system react with 2′-deoxyguanine to form 8-hydroxy-2′-deoxyguanine (8-OHdG), a DNA damage marker. Deferoxamine effectly inhibited the formation of 8-OHdG also.

Chromates are known to induce tumors (1, 2), mutations (3), and DNA damage (4–6). However, the underlying biochemical mechanism(s) is (are) not fully understood (6). Recent studies have indicated that hydroxyl (·OH) radical may play an important role (7–11). The mechanistic studies of Cr(VI)-related ·OH radical generation in biochemical systems have shown the reaction of a Cr(V) species with hydrogen peroxide (H_2O_2) through a Fenton-like mechanism to be one of the important steps (9–11):

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + \cdot OH + OH^- . \quad [1]$$

In biological systems the Cr(V) species are generated via the reduction of Cr(VI) by a variety of components, such as microsomes (12), mitochondria (13), mitochondrial electron transfer complexes (14), and at least three flavoenzymes (glutathione reductase (GSSG-R),[3] lipoyl dehydrogenase, and ferredoxin–$NADP^+$ oxidoreductase) (9, 15, 16). In the case of flavoenzymes, Cr(V) formation is accompanied by the reduction of molecular oxygen (O_2) to H_2O_2. The Cr(V) species then reduces H_2O_2 to generate ·OH radicals and Cr(VI) (16).

To understand the mechanism by which Cr(V)-generated ·OH radicals cause cytotoxicity, Aiyar *et al.* (8) investigated the reaction of Cr(VI) and H_2O_2 to generate ·OH radical and its reaction with 2′-deoxyguanine (dG) to form 8-hydroxy-2′-deoxyguanine (8-OHdG):

$$dG + \cdot OH \rightarrow 8\text{-OHdG}. \quad [2]$$

The formation of 8-OHdG was measured (8) using high-performance liquid chromatography (HPLC). The formation of 8-OHdG is considered a marker of DNA damage by the ·OH generating species (17). Thus Aiyar *et al.* (8) suggested that Cr(VI) toxicity might be a result of the DNA damage from Cr(VI)-related ·OH radical generation. These results prompted us to investigate some possible way of attenuating Cr(V)-related ·OH radical generation in biological systems. Following earlier reports on Fe^{2+} and V^{4+} (18, 19), we examined the effectiveness of several metal ion chelators and found that deferoxamine can effectly block Cr(V)-mediated ·OH radical generation, as well as Cr(V)-related 8-OHdG generation. Additionally, to explore the generality of the effectiveness of deferoxamine in inhibiting Cr(V)-related radical for-

[1] This research has been supported by the Department of the Interior's Mineral Institute program administered by the Bureau of Mines through the Generic Mineral Technology Center for Respirable Dust under Grant G1135142 and by NIOSH Grant U60-CCU306149-01-1.

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[3] Abbreviations used: GSSG-R, glutathione reductase; dG, 2′-deoxyguanine; 8-OHdG, 8-hydroxy-2′-deoxyguanine; DMSO, dimethyl sulfoxide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ESR, electron spin resonance.

0003-9861/92 $3.00

mation, we examined Cr(V)-induced decomposition of simple organic hydroperoxides. We observed that Cr(V)-induced decomposition of organic hydroperoxides does generate alkyl and alkoxy radicals and that deferoxamine inhibits their formation also.

MATERIALS AND METHODS

Phosphate buffer (pH 7.2), dimethyl sulfoxide (DMSO), and $K_2Cr_2O_7$ (as a source of Cr(VI)) were purchased from Fisher. GSSG-R from bovine intestinal mucosa, lipoyl dehydrogenase from clostridium, ferredoxin-$NADP^+$ oxidoreductase from spinach leaves, catalase from bovine liver, NAD(P)H, $NAD(P)^+$, H_2O_2, *N*-ethylmaleimide, and dG were all purchased from Sigma. Spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich and was purified by charcoal decolorization (20). This method consists of successively treating the DMPO with activated charcoal until all free radical impurities disappear as verified by electron spin resonance (ESR) spectroscopy.

ESR measurements were made with a Bruker ER 200D X-band (9.5 GHz) ESR spectrometer utilizing a rectangular (TE_{102}) microwave cavity and a flat cell, essentially as reported earlier (9–11). Hyperfine couplings were measured (to 0.1 G) directly from magenetic field separations. For accurate measurements of the *g*-values and hyperfine splittings, the magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, Model ER 035A) and the microwave frequency was measured with a digital frequency counter (Hewlett-Packard, Model 5340A). Care was taken to use the same flat cell and to maintain the same orientation of the flat cell in the ESR cavity.

The HPLC measurements were made with a Waters 710B HPLC system, employing electrochemical detection (0.6 V), essentially as described by Floyd *et al.* (21). The solvent used for elution was a mixture of 95% phosphate buffer at pH 6.8 and 5% methanol. The 8-OHdG standard was prepared from dG and purified by semipreparative HPLC (22).

The concentrations given in the figure legends are final concentrations. All experiments were carried out at room temperature.

RESULTS

A. *Effect of Deferoxamine on Cr(V) and · OH Formation*

Figure 1a shows a typical ESR spectrum obtained from an aqueous solution of $K_2Cr_2O_7$ (20 mM), NADPH (0.5 mM), and GSSG-R (0.25 mg/ml). The spectrum is centered at $g = 1.9792$, exhibits five partially resolved principal components with 0.84-G spacing, and was assigned to a Cr(V)–NADPH complex with oxygens at the metal coordination sites (11, 15, 16, 23). Addition of deferoxamine to the reaction mixture caused a significant reduction in the ESR signal intensity (Fig. 1b). Since the concentration of $K_2Cr_2O_7$ (~20 mM) was much higher than that of the deferoxamine (4 mM), it is unlikely that the Cr(V) signal attenuation was due to the chelation of Cr(VI) itself. To examine whether the enzyme (GSSG-R) played a significant role, we utilized the Cr(V)–NADPH complex as a nonenzymatic source of Cr(V). As reported earlier (11), reaction of Cr(VI) with NADPH at high concentrations (25 mM for both $K_2Cr_2O_7$ and NADPH) generates a long-lived Cr(V)–NADPH complex (Fig. 1c) (11). Addition of deferoxamine (4 mM) to such a reaction mixture also reduced the Cr(V) formation (Fig. 1d), indicating that the enzyme (GSSG-R) itself was not involved in the Cr(V) signal decrease.

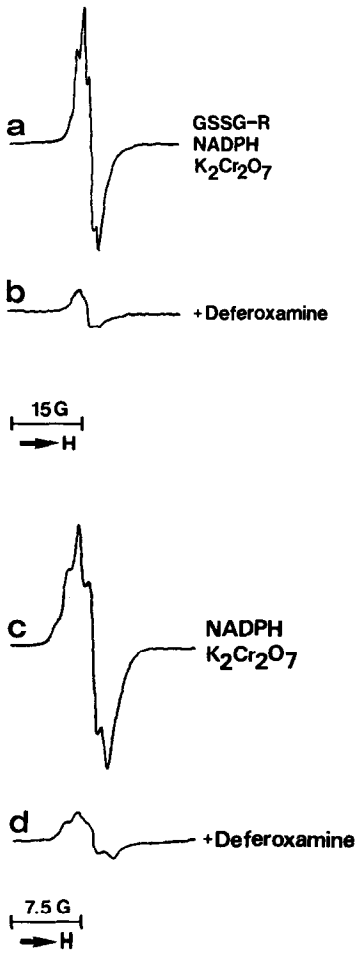


FIG. 1. (a) ESR spectrum recorded from a phosphate buffer solution (pH 7.2) of 0.5 mM NADPH, 20 mM $K_2Cr_2O_7$, and 0.2 mg/ml GSSG-R. (b) Same as (a) but with 4 mM deferoxamine added. (c) From a phosphate buffer solution (pH 7.2) of 20 mM NADPH and 25 mM $K_2Cr_2O_7$. (d) Same as (a) but with 4 mM deferoxamine added.

Using high spectrometer gain and modulation amplitude, we made an attempt to detect Cr(V)–deferoxamine complex formation, with a view to find clues to how deferoxamine attenuates Cr(V) formation, but did not succeed. Our failure here might be due to at least two causes: (a) the ESR spectrum of the Cr(V)–deferoxamine complex, if formed at all, might exhibit a very broad width, making it ESR nondetectable; or (b) the Cr(V)–deferoxamine complex might only exist as a very short-lived intermediate, rapidly converting into some Cr(III) species. No Cr(III)-related signals could be detected, however. Additional experiments are needed to distinguish between these two possibilities.

ESR spin trapping measurements were made to measure the formation of · OH radical, utilizing DMPO as a spin trap, essentially as described earlier (9–11). Figure 2a shows a typical spectrum from a reaction mixture of glutathione reductase, NADPH, Cr(VI), and DMPO. The 1:2:2:1 quartet hyperfine structure with splittings of a_H =

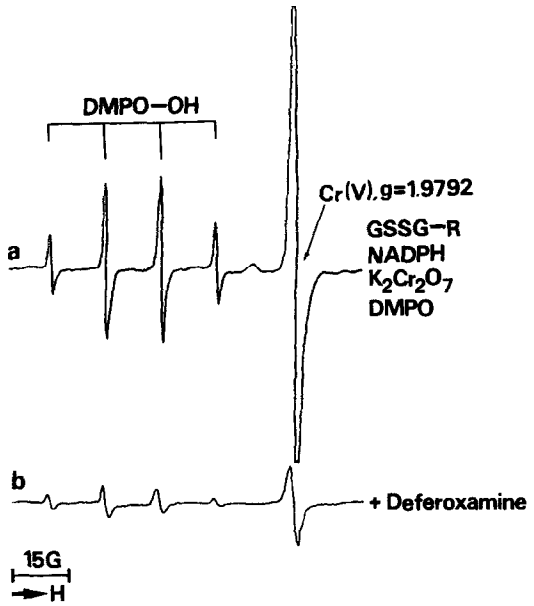


FIG. 2. (a) ESR spectrum recorded from a phosphate buffer solution (pH 7.2) of 60 mM DMPO, 2 mM NADPH, 2 mM $K_2Cr_2O_7$, and 0.2 mg/ml GSSG-R. (b) Same as (a) but with 4 mM deferoxamine added. The spectrometer settings were receiver gain, 1.0×10^5; modulation amplitude, 1.0 G; scan time, 8 min; time constant, 0.5 s.

a_N = 14.9 G was readily assigned to a DMPO–OH adduct, suggesting ·OH radical generation in this system (16, 23). This signal assignment was confirmed via standard competition experiments using ethanol or formate (24). The signal at g = 1.9792 is from the Cr(V)–NADPH complex, as discussed above. When these measurements were repeated in the presence of 4 mM deferoxamine, the intensities of both the DMPO–OH and the Cr(V)–NADPH complexes were found to be much smaller (Fig. 2b), indicating that deferoxamine does indeed inhibit ·OH radical formation by decreasing the amount of available Cr(V). Deferoxamine also inhibited the ·OH radical generation when exogenous H_2O_2 was added. A similar inhibitory effect on the ·OH radical generation was observed, though to a lesser degree, when EDTA and DETAPAC were utilized in place of deferoxamine (data not shown).

Essentially the same results were obtained using other flavoenzymes, ferredoxin–$NADP^+$ oxidoreductase, and lipoyl dehydrogenase (data not shown), showing the generality of the behavior of the flavoenzymes.

B. Inhibition of 8-OHdG Formation by Deferoxamine

As mentioned earlier, Aiyar *et al.* (8) reported that Cr(VI)-related ·OH radicals hydroxylate dG to form 8-OHdG, Eq. [2]. We examined whether the Cr(V)-mediated 8-OHdG formation could be attenuated by deferoxamine. As done by Aiyar *et al.* (8), we also measured the formation of 8-OHdG by HPLC techniques (8, 17, 21, 25). Formation of 8-OHdG either in DNA or from free nucleoside (dG) has been shown to be mediated by ·OH radicals (21, 25). Since the 8-OHdG residue in DNA is reported to induce misreading during DNA synthesis (26), this damage is considered to be mutagenic and/or carcinogenic (26), and 8-OHdG is generally used as an easily detectable marker of oxidative DNA damage (21, 25–27). Our HPLC measurements showed (Fig. 3) that a reaction mixture of Cr(VI), GSSG-R, NADPH, and dG generates 8-OHdG. The time dependence of the HPLC chromatograms (Fig. 3) shows that the amount of 8-OHdG formed increases gradually, reaching its maximum when NADPH is essentially all consumed. This observation confirms an active role for NADPH in the ·OH radical generation and hence in 8-OHdG formation. The yields of 8-OHdG were calculated to be 2.0%, utilizing synthesized 8-OHdG as a standard. This yield is essentially the same as for other biological systems (21, 28).

Table I provides a comparison of ·OH and 8-OHdG formation from various components. It can be noted that the whole enzymatic Cr(VI) reduction system, but without any chelator, causes 8-OHdG formation. Omission of any one component reduces the 8-OHdG formation, as well as ·OH generation. Replacement of NADPH by oxidized NADPH (i.e., $NADP^+$) does not cause 8-OHdG formation. Addition of the GSSG-R inhibitor, *N*-ethylmaleimide, of catalase (to remove the hydrogen peroxide intermediate), or of DMSO (to scavenge the ·OH radicals) causes a substantial reduction in the 8-OHdG formation. HPLC measurements also showed that addition of H_2O_2, to enhance the Cr(V)-mediated ·OH radicals, causes enhanced 8-OHdG formation and that deferoxamine reduces the 8-OHdG formation.

Similar results were obtained using other flavoenzymes, ferredoxin–$NADP^+$ oxidoreductase, and lipoyl dehydrogenase and using NADH in place of NADPH (data not shown).

C. Cr(V)-Induced Radical Formation from Organic Hydroperoxides and Its Inhibition by Deferoxamine

In order to explore the possibility of inhibiting other types of radical reactions involving Cr(V), we examined Cr(VI)-induced decomposition of simple organic hydroperoxides, since it is known that metal ions such as Fe^{2+} can catalyze the decomposition of organic hydroperoxides to generate organic radicals (29). Figure 4a shows the Cr(V)–NADH formation from a reaction mixture of Cr(VI), NADH, catalase, and DMPO. Addition of cumene–OOH decreases the intensity of the Cr(V)–NADH signal and leads to appearance of the spin adduct signal (Fig. 4b). Omission of either Cr(VI) or NADH results in an essentially nondetectable ESR signal (Figs. 4c and 4d). Spectral analysis showed that the major signals (Fig. 4b) exhibit hyperfine splittings of a_N = 16.5 G and a_H = 23.5 G, which can be assigned to the DMPO adduct of carbon-

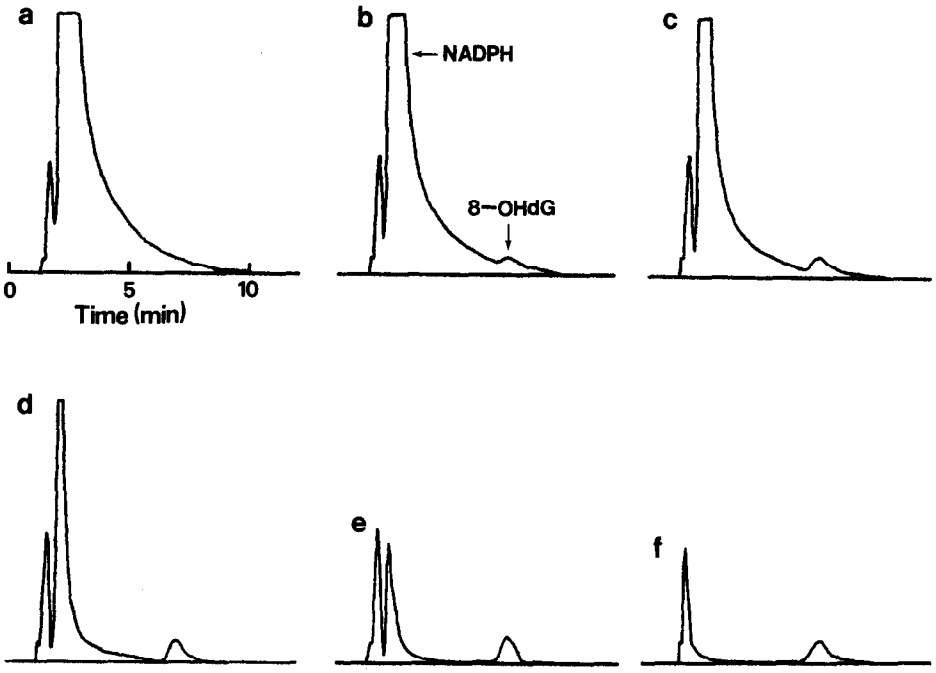


FIG. 3. HPLC chromatograms from a phosphate buffer (pH 7.2) containing 2 mM $K_2Cr_2O_7$, 2 mM NADPH, 0.3 mM dG, and 0.5 mg/ml GSSG-R. The HPLC analyses were performed at different time intervals after reaction initiation: (a) 1 min; (b) 13 min; (c) 35 min; (d) 50 min; (e) 65 min; (f) 75 min. Note the gradual increase in the 8-OHdG peak and reduction in the NADPH peak.

centered ($R\cdot$) radicals, in analogy with earlier studies of other biological systems (24, 30). Figure 4e shows that addition of 15 mM deferoxamine inhibits the radical formation. Similarly Fig. 4f shows Cr(V)-induced organic radical generation from *t*-butyl hydroperoxide, while Fig. 4g shows the inhibitory effect of deferoxamine.

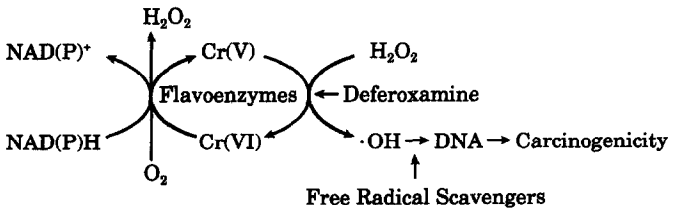
The peaks with hyperfine splittings of a_N = 15.2 G and a_H = 16.1 G, indicated by arrows in Figs. 4a and 4c, appear to be due to a DMPO adduct of alkoxy ($RO\cdot$) radical (24, 30). Since the hyperfine coupling constants of DMPO–O*R* and DMPO–OH are similar (24) and the addition of ethanol reduced the intensity of these signals, a portion of the adduct may be DMPO–OH. However, due to their weak spectral intensity, it has not been possible to establish whether the ·OH radical is indeed generated.

DISCUSSION

The data presented in Fig. 2 show that the metal ion chelator, deferoxamine, suppresses the free radical generation in the reactions of Cr(V) with H_2O_2. Similarly, the results shown in Fig. 4 establish for the first time that Cr(V) can induce the decomposition of simple organic hydroperoxide to generate alkyl ($R\cdot$) and alkoxy ($RO\cdot$) radicals and that deferoxamine can be used as an inhibitor.

The current study shows also that ·OH radicals generated by the Cr(V)-mediated Fenton type of reaction can react with deoxyguanine to form 8-OHdG. This result is significant because ·OH radicals generated in the reaction of H_2O_2 with some other metal ions, such as nickel (31), copper, or zinc containing superoxide dismutase (32), exhibit very limited reactivity. For example the ·OH radical generated by these systems cannot be scavenged by either ethanol or formate. The reason for this nonreactivity is thought to be that the ·OH radicals are generated within the domain of certain macromolecules (31, 32) and hence are not "free" to exhibit significant reactivity (27, 28). The present study establishes that the ·OH radicals generated in the enzymatic reduction of Cr(VI) are free and do have the potential to react with deoxyguanine. This observation corroborates earlier suggestion by Aiyar *et al.* (8) that Cr(VI)-related ·OH radicals cause DNA damage via hydroxylation reactions.

The current results together with some earlier studies (9–11) lead us to suggest the following schematic diagram to outline the enzymatic generation of ·OH radicals, the sites of ·OH-induced damage, and the radical's possible attenuation:



In this model, Cr(VI) serves as a catalyst, such that only a trace amount of Cr(VI) can generate significant amounts of ·OH radicals, in a continuous manner. Moreover, since

TABLE I

Comparison of 8-OHdG Formation and ·OH Radical Generation in Enzymatic Reduction of Cr(VI)

Reaction mixture	8-OHdG (pmol)[a]	Relative ·OH concentration[b,c]
GSSG-R, 0.2 mg/ml Cr(VI), 4 mM NADPH, 2 mM	5.3	15
GSSG-R, 0.2 mg/ml Cr(VI), 4 mM	Nondetectable	1
GSSG-R, 0.2 mg/ml NADPH, 2 mM	0.1	2
NADPH, 2 mM Cr(VI), 4 mM	0.3	4
GSSG-R, 0.2 mg/ml Cr(VI), 4 mM $NADP^+$, 2 mM	0.1	2
GSSG-R, 0.2 mg/ml NADPH, 2 mM Cr(VI), 4 mM *N*-Ethylmaleimide, 40 mM	0.2	3
GSSG-R, 0.2 mg/ml NADPH, 2 mM Cr(VI), 4 mM Catalase, 5000 units/ml	0.1	1
GSSG-R, 0.2 mg/ml NADPH, 2 mM DMSO, 1 M Cr(VI), 4 mM	0.2	2
GSSG-R, 0.2 mg/ml NADPH, 2 mM Deferoxamine, 4 mM Cr(VI), 4 mM	0.7	4
GSSG-R, 0.2 mg/ml Cr(VI), 4 mM NADPH, 2 mM H_2O_2, 0.5 mM	11	42

Note. All reactions were carried out in a medium containing 0.3 mM dG at pH 7.2 (phosphate buffer).

[a] The error is estimated to be ±0.1.

[b] The error is estimated to be ±0.5.

[c] Solutions contained 60 mM DMPO as the ·OH radical trap.

it is known that Cr(V) can bind directly to DNA (5, 8, 33), the reaction between H_2O_2 and a DNA-bonded Cr(V) species may cause "site-specific" ·OH radical generation which in turn may cause DNA breakage or base modification. From this viewpoint, at least two strategies may be developed to block Cr(VI)-related genotoxicity. First, one could utilize a metal ion chelator, such as deferoxamine, to block the ·OH radical generation. Second, one may investigate the use of an antioxidant to scavenge the oxygen radicals. The former appears to be a viable approach based on the following reasoning: (a) deferoxamine is widely used for prevention and treatment of iron overload (18, 34) as well as for combating toxic effects of vanadium (18, 19); (b) deferoxamine seems to bind with Cr(V) directly and therefore it may prevent Cr(V) from interacting with such targets; and (c) since Cr(VI) serves as a catalyst in the ·OH radical generation, the blockage of Cr(V) will efficiently prevent Cr(V)-mediated ·OH generation via the Fenton reaction. Regarding the second approach, one could investigate the possible use of antioxidants such as vitamin E. Vitamin E is an ·OH radical scavenger and it has been reported that treatment with vitamin E prior to exposure to Cr(VI) results in a decrease in Cr(VI)-induced single-strand breaks of DNA from Chinese hamster V-79 cells (5).

In conclusion, the present study adds the following results to the mechanism of Cr(VI) toxicity: (a) deferoxamine can decrease the concentration of Cr(V) intermediates in reactions involving one-electron reduction of

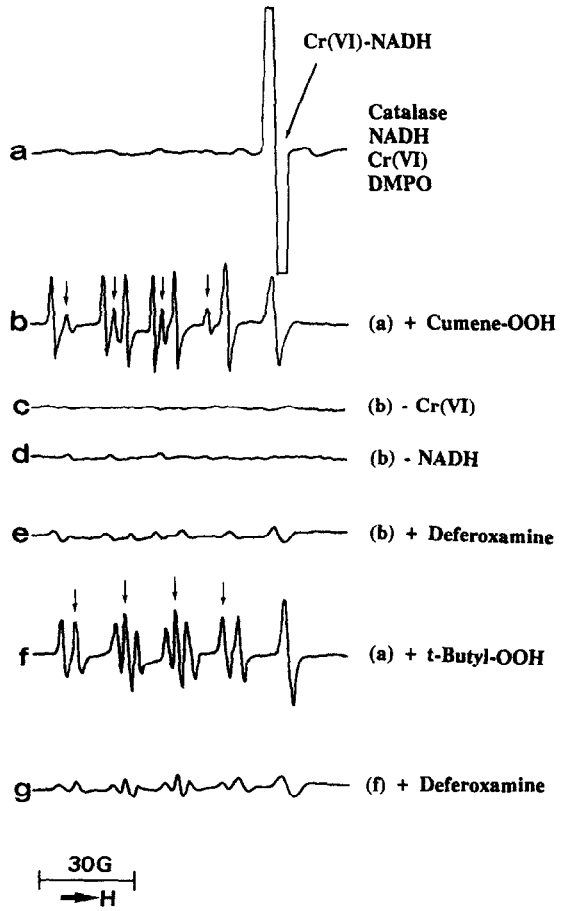


FIG. 4. Cr(V)-induced radical generation from organic hydroperoxides and its inhibition by deferoxamine. (a) Spectrum recorded 5 min after mixing, in a phosphate buffer solution (pH 7.2) of 60 mM DMPO, 25 mM NADPH, 20 mM $K_2Cr_2O_7$, and 5000 units/ml catalase. (b) Same as (a) but with 5 mM cumene hydroperoxide added. (c) Same as (b) but without $K_2Cr_2O_7$. (d) Same as (b) but without NADH. (e) Same as (b) but with 15 mM deferoxamine added. (f) Same as (a) but with 5 mM *t*-butyl hydroperoxide added. (g) Same as (f) but with 5 mM deferoxamine added. The spectrometer settings were receiver gain, 2.5×10^5; modulation amplitude, 1.0 G; scan time, 8 min; time constant, 0.5 s.

Cr(VI) by cellular constituents; (b) deferoxamine inhibits Cr(V)-mediated ·OH generation from H_2O_2; (c) ·OH radicals generated by an enzymatic Cr(V)–H_2O_2 reaction have the ability to hydroxylate deoxyguanine and deferoxamine inhibits this hydroxylation; (d) Cr(V) can induce the decomposition of organic hydroperoxides to generate alkyl and alkoxy radicals, and deferoxamine can be used to block the generation of these radicals as well. Thus deferoxamine appears to have the potential for being a therapeutic agent against Cr(VI) genotoxicity.

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