Free Rad. Res. Comms., Vol. 10, No. 1-2, pp. 17-26
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ESR SPIN TRAPPING DETECTION OF HYDROXYL RADICALS IN THE REACTIONS OF Cr(V) COMPLEXES WITH HYDROGEN PEROXIDE

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Electron spin resonance (ESR) measurments provide direct evidence for the involvement of Cr(V) in the reduction of Cr(VI) by NAD(P)H. Addition of hydrogen peroxide (H_2O_2) to NAD(P)H-Cr(VI) reaction mixtures suppresses the Cr(V) signal and generates hydroxyl (·OH) radicals (as detected via spin trapping), suggesting that Cr(V) reacts with H_2O_2 to generate the ·OH radicals. Reaction between H_2O_2 and a Cr(V)-glutathione complex, and between H_2O_2 and several Cr(V)-carboxylato complexes also produces ·OH radicals. These results suggest that Cr(V) complexes catalyze the generation of ·OH radicals from H_2O_2, and that ·OH radicals might play a significant role in the mechanism of Cr(VI) cytotoxicity.

KEY WORDS: ESR, spin trapping, Cr(VI) reduction, Cr(V), hydrogen peroxide, free radicals.

INTRODUCTION

This communication reports an ESR spin trapping detection of hydroxyl (·OH) radicals in reactions between some Cr(V) complexes containing biologically important ligands and hydrogen peroxide (H_2O_2). This work was undertaken because while the reduction of Cr(VI) to lower oxidation states has been suggested as a key step in the mechanism of Cr(VI) toxicity, the reaction mechanism is not yet clear.[1] Mutagencity has been well documented for Cr(VI) via various bacterial assays, while Cr(III) has been shown not to be mutagenic.[2,3] Since it has been reported that compounds containing Cr(VI) do not react with isolated DNA,[4] the reduction of Cr(VI) by other cellular constituents has been thought to be important in the mechanism of Cr(VI) induced DNA alteration.[1,4,5] For cellular systems earlier studies include those on the reduction of Cr(VI) by microsomes,[6-8] rat liver DT-diaphorase,[9] and aldehyde oxidase.[10] Regarding the reduction mechanism, Jennette used ESR spectroscopy to show that a long-lived Cr(V) complex is formed as an intermediate in the microsomal reduction of Cr(VI) in the presence of NADPH.[8] She postulated that Cr(V) is the "ultimate" carcinogenic form of chromium compounds. This conclusion has led to many further investigations of the formation of Cr(V) in the reduction of Cr(VI) under biologically relevant conditions.[11-19] More recently, however, it has been suggested that oxygen-derived radicals generated during the Cr(VI) reduction are the species responsible for the DNA alteration.[20,21] To the best of our knowledge, however, no information is available on the generation of such radicals in the reduction of Cr(VI) under biologically relevant conditions, except some preliminary results on

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the glutathionyl (GS·) radical formation in the reduction of Cr(VI) by glutathione (GSH).[17-19] We, therefore, undertook a systematic investigation of the reaction of Cr(VI) with NAD(P)H, GSH, α-hydroxycarboxylic acids, and H_2O_2. NADPH was chosen as a model reductant because of its ubiquitous existence inside the cells, its critical role in the enzymatic Cr(VI) reduction,[1,6-9] its representation of important cellular Cr(VI) reductants, and its ability to reduce other metal ions.[22] GSH was chosen as another model Cr(VI) reductant because GSH also exists inside cells, it represents another important category of cellular Cr(VI) reductants, the thiols, and it also plays an important role in the Cr(VI) toxicity.[1,14-19,23,24] For further clarification, we also studied the reaction of Cr(VI) with several α-hydroxycarboxylic acids in the presence of H_2O_2 since these acids are definitively known to reduce Cr(VI) to form stable, isolable Cr(V).[25,26] The results obtained indicate that Cr(V) complexes, produced in the reduction of Cr(VI) by cellular reductants, react with H_2O_2 to generate ·OH radicals, which might be the initiators of primary events in Cr(VI) carcinogenicity.

MATERIALS AND METHODS

ESR spectra were obtained at X-band (~9.7 GHz) using a Bruker ER 200D ESR spectrometer. For accurate measurements of the g-values and hyperfine splittings, the magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, Model ERO35M) and the microwave frequency was measured with a digital frequency counter (Hewlett-Packard, Model 5340A). An ASPECT 2000 computer was used for data acquisition and analysis. The concentrations given in the Figure legends are final concentrations. All experiments were carried out at room temperature except those specifically indicated.

Me_2SO, ethanol, phosphate buffer solution (pH = 7.2), potassium dichromate, and sodium formate were purchased from Fisher, while 1,3-dimethyl-2-thiourea (DMTU), GSH, and superoxide dismutase (SOD) from bovine blood were purchased from Sigma. All were used as received. 2-ethyl-2-hydroxybutyric acid, 2-hydroxyisobutyric acid, 2-hydroxy-2-methyl-butyric acid, and NAD(P)H were purchased from Aldrich. Spin traps 5,5,-dimethyl-1-pyrroline-N-oxide (DMPO) and α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN), were also purchased from Aldrich, and were used without further purification since very weak or no spin adduct signal was obtained from the purchased sample when used alone.

RESULTS AND DISCUSSION

Figure 1(a) shows a typical ESR spectrum from a mixture of NADPH and Cr(VI) at physiological pH (7.2). The spectrum is essentially identical with those reported earlier for Cr(V) complexes with ethylene glycol,[27] ascorbic acid,[12] glucose or lactose,[13] and galacturonic acid:[14] a strong signal centered at g = 1.9792, with four weak satellite signals due to the ^{53}Cr isotope (9.55% abundance, I = 3/2). The observed ^{53}Cr hyperfine coupling of 17.7 G (indicated in Figure 1a) is very similar to those observed for Cr(V) complexes with oxygen ligands.[27] Moreover, on scale expansion, the main (g = 1.9792) peak showed a multiplet, at least five principal components with 0.84 G spacing (Figure 1(b)). Such splittings have also been observed for other Cr(V) complexes, and are thought to be characteristic of the superhyperfine intraction of nearby hydrogens in the Cr(V) complexes of diols.[12-14,27]

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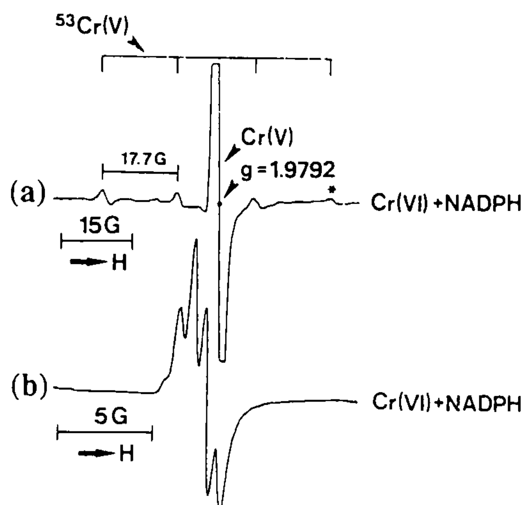

FIGURE 1 (a) A typical ESR spectrum of 25 mM $K_2Cr_2O_7$ and 25 mM NADPH in a phosphate buffer solution (pH = 7.2). The spectrum was recorded 30 minutes after the reaction was initiated. Modulation amplitude, 1.25 G; field, 3512 ± 50 G; scan time, 100 seconds; time constant, 0.5 second; receiver gain, 2×10^5. (b) same as (a) but modulation amplitude, 0.12 G; field, 2512 ± 12.5 G; receiver gain, 5×10^4. The asterisk indicates the highest field ^{53}Cr hyperfine component.

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In order to find if the Cr(V)-NADPH complex would generate ·OH radicals, we employed the ESR spin trapping technique using DMPO and 4-POBN as spin traps. Figures 2 and 3 show typical spectra. An aqueous, phosphate-buffered (pH = 7.2), solution of DMPO with Cr(VI), or NADPH, or with NADPH plus H_2O_2 did not give a detectable ESR spectrum (Figure 2(a–c)). A solution containing DMPO, Cr(VI) and NADPH together, or one containing DMPO, Cr(VI) and H_2O_2 together gave only a small DMPO spin adduct signal (Figure 2(d) and (e)). The addition of H_2O_2 to a solution of DMPO, Cr(VI) and NADPH generated a strong spin adduct ESR spectrum, consisting of a 1:2:2:1 quartet with splitting of $a_N = a_H = 14.9$ G (Figure 2(f)), where a_N and a_H denote hyperfine splitting of the nitroxyl nitrogen and the α-hydrogen respectively. Based on these splitting constants and the 1:2:2:1 lineshape this spectrum was assigned to the DMPO-OH adduct.[28-30] Since the DMPO-OH adduct could, in principle, arise from many sources, we carried the standard competition experiments in which the ·OH radical abstracts a hydrogen atom from ethanol or formate, with the trapping of a new radical.[30-32] As expected, addition of ethanol decreases the intensity of the DMPO-OH adduct signal and results in the appearance of new spin adduct signal (as indicated by the astrisks in Figure 2(g) with the splitting constants, $a_N = 15.8$ G and $a_H = 22.8$ G. These splitting constants are typical of those of the DMPO–$CHOHCH_3$ adduct,[33,34] attesting to the generation of ·OH radicals. The relative ESR signal intensities of DMPO–$CHOHCH_3$ and DMPO-OH depend on the amount of ethanol added. On increasing the concentration of ethanol, the ESR signal intensity of DMPO–$CHOHCH_3$ increased and that of DMPO-OH decreased. For checking out our procedure similar competition reaction was carried out with the Fenton reaction, a known source of ·OH radical, and essentially the same results were obtained (data not shown). Similar results were obtained with sodium formate (Figure 2(h)). These results strongly suggest that the

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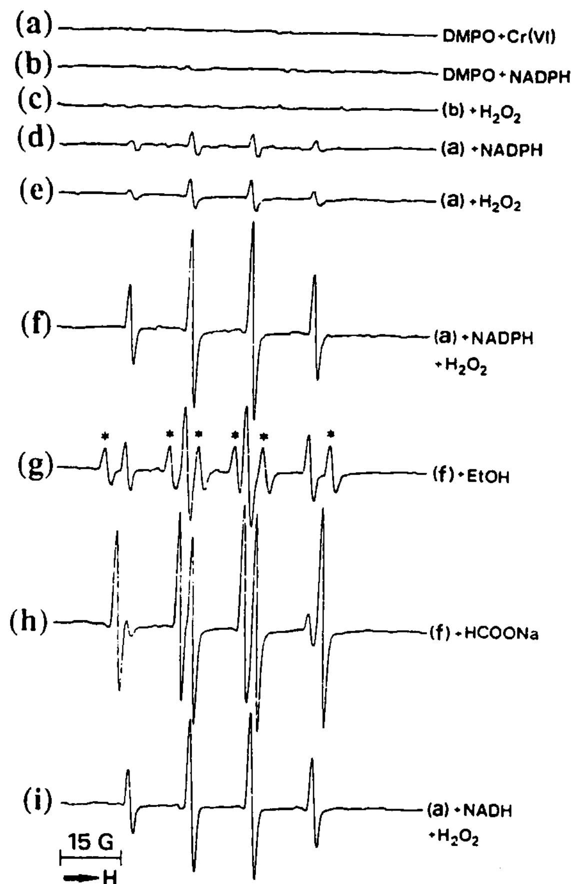

FIGURE 2 ESR spectra recorded 2 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM DMPO with (a) 2 mM $K_2Cr_2O_7$: (b) 2 mM NADPH; (c) 3 mM H_2O_2; (d) 2 mM $K_2Cr_2O_7$ and 2 mM NADPH; (e) 2 mM $K_2Cr_2O_7$ and 3 mM H_2O_2; (f) 2 mM NADPH, 3 mM H_2O_2; (g) same as (f) but with 0.2 M ethanol added; (h) same as (f) but with 0.6 M sodium formate added; (i) 2 mM $K_2Cr_2O_7$, 2 mM NADH, 3 mM H_2O_2. Spectrometer setting were: receiver gain, 1.25×10^5; modulation amplitude, 1.25 G; scan time, 200 seconds; field, 2480 ± 50 G; time constant, 0.5 second. The asterisks indicate the DMPO-$CHOHCH_3$ spin adduct signal.

detection of the DMPO–OH spin adduct (Figure 2(f)) is the result of the trapping of ·OH radicals formed during the reaction and not an artifact. Similarly, the ·OH radicals were detected from a mixture containing DMPO, Cr(VI), H_2O_2, and NADH (Figure 2(i)), showing that both NADPH and NADH are capable of generating ·OH radicals in the presence of Cr(VI) and H_2O_2 and to nearly the same extent.

Additional support for the ·OH radical identification was obtained from ESR spectra using 4-POBN as another spin trap. The ESR spectrum obtained from a mixture of 4-POBN, Cr(VI), NADPH, and H_2O_2 was a triplet of doublets with $a_N = 14.9$ G and $a_H^\beta = 1.6$ G (Figure 3(a)). When recorded with a small modulation amplitude (0.2 G) and a smaller scan width, the spectrum shows another doublet, arising from the coupling of $a_H = 0.3$ G from the hydroxyl hydrogen (Figure 3(b)). These hyperfine splittings are typical of those of the 4-POBN-OH spin adduct.[35-37]

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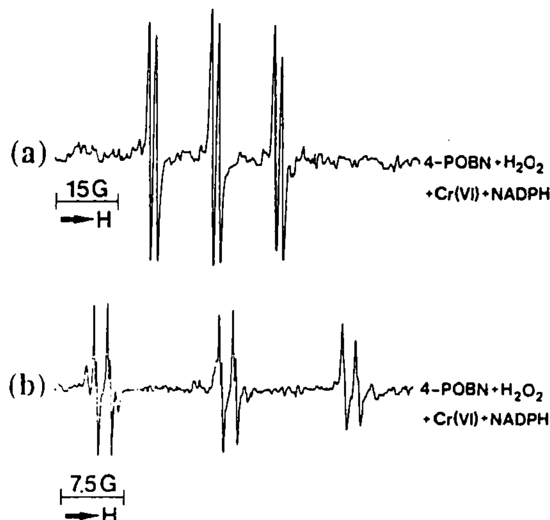

FIGURE 3 ESR spectra recorded 2 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM 4-POBN, 2 mM $K_2Cr_2O_7$, 2 mM NADPH, and 3 mM H_2O_2 at different spectrometer settings: (a) gain, 2.5×10^5; scan time, 200 seconds; modulation amplitude, 1.0 G; scan width, 3480 ± 50 G; time constant, 0.5 seconds; (b) gain, 5×10^5; scan time, 500 seconds; modulation amplitude, 0.2 G; scan width, 3480 ± 25 G; time constant, 0.5 second.

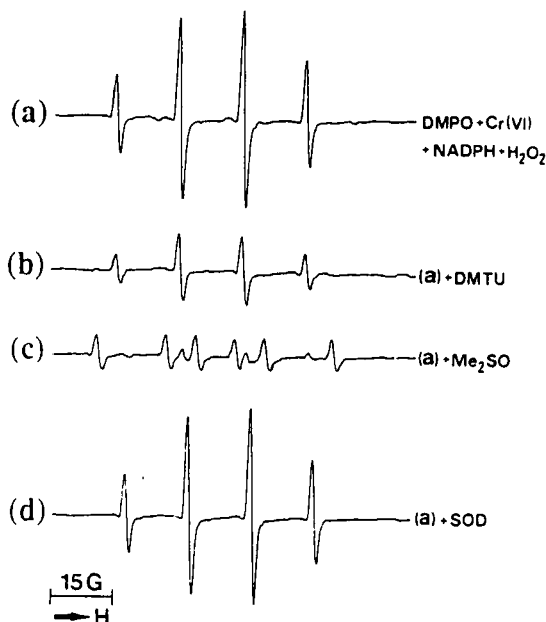

FIGURE 4 ESR spectra recorded 2 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM DMPO and (a) 2 mM $K_2Cr_2O_7$, 2 mM NADPH and 3 mM H_2O_2; (b) same as (a) but with 200 mM DMTU added; (c) same as (a) but 5% DMSO added; (d) same as (a) but with 200 units/ml SOD added. Spectrometer settings were the same as those in Figure 2.

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Additionally, the ·OH radical adduct signals decreased when ·OH radical scavengers were added. For example, the spectrum in Figure 4(a) shows the result without scavengers, while the weaker signal in Figure 4(b) and (c) were obtained in the presence of the scavengers DMTU and Me_2SO. All these measurments lead to the conclusion that ·OH radicals are generated significantly in the reaction mixture of Cr(VI), NADPH, and H_2O_2.

To examine the involvement of the O_2^- radicals in the mechanism of the ·OH radical generation, superoxide dismutase (SOD), the well known O_2^- scavenger, was added to the reaction mixture containing DMPO, Cr(VI), NADPH, and H_2O_2. The spectrum obtained with 200 units/ml of SOD (Figure 4(d)) had essentially the same spectral intensity and lineshape as that obtained without SOD (Figure 4(a)), which indicated that the involvement of O_2^- in the mechanism of the ·OH generation was not significant.

To find additional clues to the ·OH radical generation, we examined the dependence of the ·OH radical formation on the concentration of H_2O_2 (Figure 5). Figure

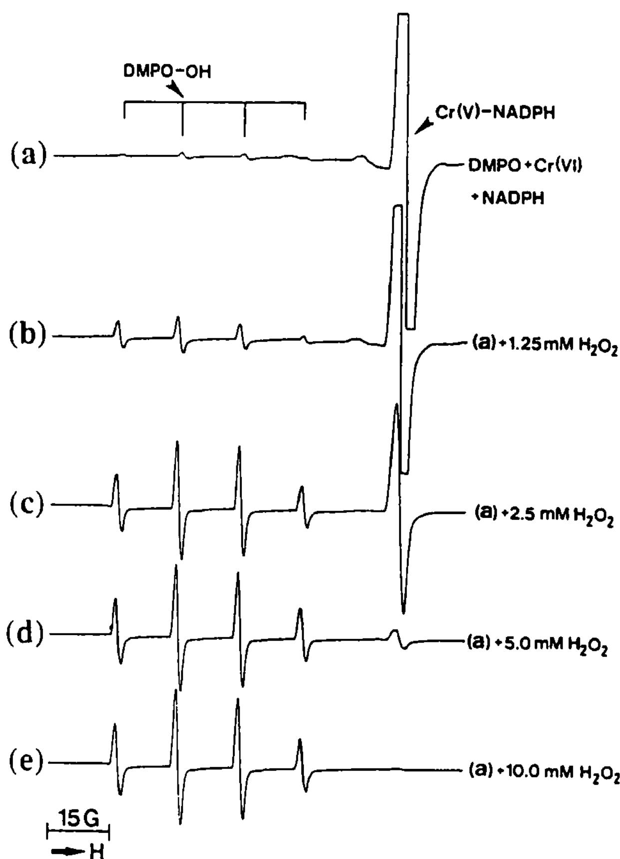

FIGURE 5 ESR spectra recorded 2 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM DMPO with (a) 25 mM $K_2Cr_2O_7$ and 25 mM NADPH; (b) same as (a) but with 1.25 mM H_2O_2 added; (c) same as (a) but with 2.5 mM H_2O_2 added; (d) same as (a) but with 5 mM H_2O_2 added; (e) same as (a) but with 10 mM H_2O_2 added. Spectrometer settings were: receiver gain, 2.5×10^4; modulation amplitude, 1.25 G; scan time, 200 seconds; field, 3490 $\pm$ 50 G; time constant, 0.5 second.

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5(a) shows the spectrum from a mixture containing DMPO, Cr(VI) and NADPH (without H_2O_2). It may be noted that this mixture generates only a small concentration of the DMPO–OH spin adduct but high concentration of Cr(V). When 1.25 mM H_2O_2 was added, a relatively strong DMPO–OH spin adduct signal appeared (Figure 5(b)). On increasing the H_2O_2 to 2.5 mM, the intensity of the DMPO–OH spin adduct signal increased significantly, with a concommitant decrease in the intensity of the Cr(V) signal (Figure 5(c)). 5 mM H_2O_2 caused an additional increase in the intensity of the DMPO–OH spin adduct signal with further decrease in the intensity of Cr(V) signal (Figure 5(d)). With 10 mM H_2O_2 the intensity of the DMPO–OH spin adduct signal leveled off and the intensity of the Cr(V) signal decreased to a barely observable level (Figure 5(e)). These results indicate that the reduction of Cr(VI) by NADPH generates some Cr(V) species which can react with H_2O_2 to form the ·OH radicals.

To ascertain the generality of the ·OH radical generation by H_2O_2 and Cr(V) complexes, we examined the reaction systems using α-hydroxycarboxylic acids which are known to form stable Cr(V) complexes as shown by Rocek and coworkers.[25,26] The systems investigated were mixtures containing Cr(VI), H_2O_2 and 2-ethyl-2-hydroxybutyric acid, or 2-hyroxy-isobutyric acid, or 2-hydroxy-2-methyl-butyric acid. All of these reactions generated DMPO–OH spin adduct signals (Figure 6), showing that reactions of Cr(V)-carboxylato complexes with H_2O_2 also have an ability to generate ·OH radicals. We also investigated the reaction between H_2O_2 and a Cr(V) complex with GSH, a thiol-containing cellular entity. Figure 7(a) is the ESR spectrum obtained from H_2O_2 and GSH in the presence of DMPO. It is seen that in the absence of Cr(VI) this reaction generates little or no ·OH radical. Figure 7(b) is the spectrum obtained from the reaction between Cr(VI) and GSH in the presence of DMPO. The spectrum was assigned to be a composite of the DMPO-GS spin adduct signal (as indicated) and the Cr(V)-GSH complex (the broad peak at g = 1.9951, as also marked), according to earlier reports.[17-19] Addition of either ethanol or sodium formate to the reaction mixture containing Cr(VI), GSH, and, DMPO yielded essentially the same ESR spectra (Figure 7(c) and (d)) as that in Figure 7(b). These results show that

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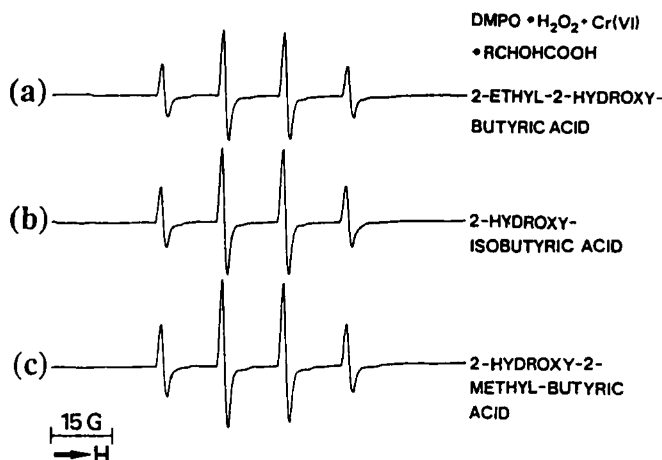

FIGURE 6 ESR spectra recorded 10 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM DMPO, 3 mM H_2O_2, 2 mM $K_2Cr_2O_7$ with (a) 2-ethyl-2-hydroxy-butyric acid; (b) 2-hydroxy-isobutyric acid; (c) 2-hydroxy-2- methyl-butyric acid. Spectrometer setting were the same as those in Figure 5.

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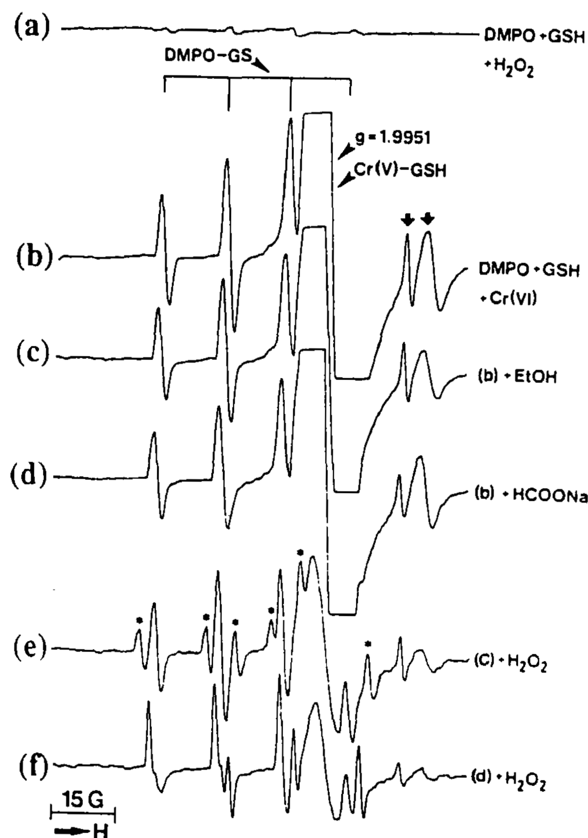

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FIGURE 7 ESR spectra recorded 5 minutes after mixing, in a phosphate buffer solution (pH = 7.2), of 60 mM DMPO with (a) 5 mM H_2O_2 and 50 mM glutathione; (b) 2 mM $K_2Cr_2O_7$ and 50 mM glutathione; (c) same as (b) but with 20% ethanol added; (d) same as (b) but with 0.6 M sodium formate added; (e) 5 mM H_2O_2, 50 mM glutathione, 2 mM $K_2Cr_2O_7$, and 5% ethanol; (f) 5 mM H_2O_2, 50 mM glutathione, 2 mM $K_2Cr_2O_7$, and 0.6 M soidum formate added. The spectrometer settings were: receiver gain, 1.25×10^5; modulation amplitude, 1.25 G; scan time, 200 seconds; field, 3480 ± 50 G; time constant, 0.5 second. The asterisks indicate the DMPO-$CHOHCH_3$ spin adduct signal while the arrows indicate minor Cr(V) species.

ethanol or sodium formate do not react with GS· radicals to generate new spin adduct signals. Addition of H_2O_2 to the reaction mixture containing Cr(VI), GSH, DMPO, and ethanol causes the appearance of the DMPO–$CHOHCH_3$ spin adduct signal (Figure 7(e)) (as indicated by the asterisks), which is indicative of the ·OH radical involvement in the reaction as discussed above. We note that the possibility of ·OH generation in the reaction of GS· with H_2O_2 was excluded by earlier investigators.[38–40] The sharp decrease in the Cr(V) signal accompanying the appearance of DMPO–$CHOHCH_3$ indicates that both the Cr(V)-GSH complex and H_2O_2 are involved in the ·OH radical formation, while GS· is not. The same conclusion was obtained by the scavenging effect of sodium formate (Figure 7(f)).

In conclusion, Cr(V) complexes are generated in the reduction of Cr(VI) by NAD(P)H and related dial of thiol molecules. It is a general property of the Cr(V)

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complexes of cellular reductants to react with H_2O_2 to generate $\cdot$OH radicals. Since H_2O_2 is a normal biological metabolite, reactions of Cr(V) complexes with H_2O_2 might be an important source of $\cdot$OH radicals, which might be the "ultimate" carcinogenic species in the mechanism of Cr(VI) carcinogenicity.

Acknowledgements

This research has been supported by the Department of the Interior's Mineral program administered by the Bureau of Mines through the Generic Mineral Technology Center for Respirable Dust under grant G1135142.

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Accepted by Prof. E.G. Janzen

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