Generation of Hydroxyl Radical by Chromate in Biologically Relevant Systems: Role of Cr(V) Complexes versus Tetraperoxochromate(V)

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While Cr(V) species and •OH radicals have been suggested to play significant roles in the mechanism of chromate-related carcinogenesis, controversy still exists regarding the identity of the Cr(V) species and their role in the generation of •OH radicals. Some recent studies have suggested that the primary Cr(V) species involved is the tetraperoxochromate(V) (CrO_{8}^{3-}) ion, which produces •OH radical either on decomposition or by reaction with H_{2}O_{2}. The present study utilized ESR and spin trapping techniques to probe this mechanism. The results obtained show that (i) CrO_{8}^{3-} is not formed in any significant amount in the reaction of chromate with biologically relevant reductants such as glutathione, glutathione reductase, NADPH, ascorbate, vitamin B_{6}, etc. (ii) Decomposition of CrO_{8}^{3-}, or its reaction with H_{2}O_{2} does not generate any significant amount of •OH radicals. (iii) The major Cr(V) species formed are complexes of Cr(V) with reductant moieties as ligands. (iv) These Cr(V) complexes generate •OH radicals from H_{2}O_{2} via Fenton-like reaction. The present study thus disagrees with the recently proposed “tetraperoxochromate(V) theory of carcinogenesis from chromate.” Instead, it suggests an alternative mechanism, which might be labeled as “the Cr(V)-complexation-Fenton reaction model of carcinogenesis from chromate.” —Environ Health Perspect 102(Suppl 3):231–236 (1994).

Key words: ESR, spin-trapping, Cr(V) complex, tetraperoxochromate(V), Fenton-like reaction

Introduction

Chromate and Cr(VI)-containing compounds have been found to exert serious toxic and carcinogenic effects on humans and animals and to cause mutations in bacteria and transformation of mammalian cells (1–7). Since it has been reported that such Cr(VI) compounds do not react with isolated DNA (8), the reduction of Cr(VI) by cellular reductants has been thought to be an important step in the mechanism of Cr(VI)-induced DNA damage (6,9). Using ESR spectroscopy, Jennette has shown that a long-lived Cr(V) species is formed in the course of microsomal reduction of Cr(VI) in the presence of NADPH (9). Since Cr(V) complexes are generally characterized as being labile and reactive, whereas Cr(III) complexes are substitutionally inert, the detection of Cr(V) formation led Jennette to suggest that the Cr(V) interme-

diates are the likely candidates for the “ultimate” carcinogenic forms of chromium compounds (9). While several earlier studies have shown that the Cr(VI)-induced DNA damage is strongly dependent on the Cr(V) intermediates (8,10–13), recent studies have suggested that hydroxyl (•OH) radicals may also play an important role (10–12,14–21). It has been reported, for example, that treatment of Chinese hamster V-79 cells with FAD and Cr(VI) resulted in an increase in Cr(VI)-induced DNA strand breaks over that observed upon treatment of cells with Cr(VI) alone (21). This increase in DNA strand breaks was attributed to •OH radical generation in the presence of FAD. In contrast, incubation of Chinese hamster V-79 cells with vitamin E, an •OH radical scavenger, prior to treatment with Cr(VI) led to a decrease in Cr(VI)-induced DNA strand breaks (12,16).

As to the mechanism of Cr(VI) related •OH radical generation, our studies (22–24) suggest that the reaction of a Cr(V) complex with hydrogen peroxide via a Fenton-like reaction is the important source of chromium-mediated •OH radical generation in biological systems (Equation [1]).

\[
\text{Cr(V)} + \text{H}_{2}\text{O}_{2} \rightarrow \text{Cr(VI)} + \text{•OH} + \text{OH}^{-}
\]

Cr(V) species has been reported to be generated in the reduction of Cr(VI) by various biological systems (6), in particular, microsomes (9,25), mitochondria (26), superoxide radical (27), certain flavoenzymes (28–30), mitochondrial electron transfer chain complexes (31), ascorbate (32,33), and thiol- and diol-containing molecules (6,23,34,35). In contrast to the mechanism outlined in Equation [1], it has been frequently suggested (10,11,15,18,36) that Cr(VI) reacts with cellular H_{2}O_{2} to form tetraperoxochromate(V) (CrO_{8}^{3-}) ions, which decompose to produce •OH radicals via Equation [2], as given below:

\[
\text{Cr(VI)} + \text{H}_{2}\text{O}_{2} \rightarrow \text{CrO}_{8}^{3-} \rightarrow \text{Cr(VI)} + \text{•OH}
\]

The Cr(VI)-mediated •OH radical generation via the CrO_{8}^{3-} intermediate was recently cited (37) as the basis of “the tetraperoxochromate(V) theory of carcinogenesis from chromate.” The first evidence for the role of CrO_{8}^{3-} in the Cr(VI)-mediated •OH radical generation was reported by Kawanishi et al. (15). They observed CrO_{8}^{3-} formation by ESR from a mixture containing 40 mM Na_{2}CrO_{4} and 400 mM H_{2}O_{2} at pH 8.0. However, the concentrations used for both Cr(VI) and H_{2}O_{2} were orders of magnitude higher than any in vivo estimate. Later Aiyar et al. (10) reaffirmed the Kawanishi model of •OH radical generation from a mixture of Cr(VI) and H_{2}O_{2}, i.e., that CrO_{8}^{3-} was the species responsible
for -OH generation. In a subsequent report (11), these authors reported the detection of CrO$_3^-$ ions from mixtures containing Cr(VI), GSH and H$_2$O$_2$, although the detected ESR signal appears to be too weak for any reliable assignment. In a separate study, Sugiyama et al. (36) reported that a mixture of Cr(VI) and vitamin B$_3$ generates CrO$_3^-$ and vitamin B$_3$ enhances the -OH radical generation from a mixture of Cr(VI) and H$_2$O$_2$. They attributed the enhancement of -OH radical generation by vitamin B$_3$ to CrO$_3^-$ formation. This conclusion disagrees with our reports (22-24) that any diol-containing molecule will generate a Cr(V)-diol complex upon reaction with Cr(VI) (23), and that this Cr(V)-diol complex will react with H$_2$O$_2$ to generate -OH radical via a Fenton-like mechanism. The present investigation was undertaken with the view of resolving this and related controversies.

Materials and Methods

Materials

Phosphate buffer (pH 7.2, 100 mM), dimethyl sulfoxide(DMSO), and ethanol were purchased from Fisher (Pittsburgh, PA). Glutathione (GSH), NADPH, ascorbate, glutathione reductase (GSSG-R), H$_2$O$_2$, K$_2$Cr$_2$O$_7$, vitamin B$_3$, ribose, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), and tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) (pH 8.0) were purchased from Sigma (St. Louis, MO). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich (Milwaukee, WI). Potassium tetraperoxodisulfate (K$_2$Cr$_2$O$_7$) was synthesized by reaction of Cr(VI) with H$_2$O$_2$ at basic pH. Chelex-100 chelating resin was purchased from Bio-Rad Laboratories (Richmond, CA). The phosphate buffer was treated with Chelex-100 to remove possible metal ion contaminants. DMPO solutions were purified using activated charcoal until free radical impurities disappeared as verified by electron spin resonance (ESR) spectroscopy.

ESR Measurements

All ESR measurements were made using a Varian E3 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separations using K$_2$Cr$_2$O$_7$ and DPPH as reference standards. Reactants were mixed in test tubes in a final volume of 250 µl. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations. All the experiments were carried out at room temperature and in ambient air.

Results

**Does a Mixture of Cr(VI) and H$_2$O$_2$ Generate CrO$_3^-$ Ions under Biologically Relevant Conditions?** As mentioned in the Introduction section, Kawanishi et al. (15) observed via ESR formation of CrO$_3^-$ from a mixture containing 40 mM Na$_2$CrO$_4$ and 400 mM H$_2$O$_2$ at pH 8.0. However, the concentrations used for both Cr(VI) and H$_2$O$_2$ were orders of magnitude higher than any in vivo estimate. We tried to detect the CrO$_3^-$ formation at concentrations of K$_2$Cr$_2$O$_7$ and H$_2$O$_2$ as low as possible. As shown in Figure 1a, 25 mM K$_2$Cr$_2$O$_7$ and 25 mM H$_2$O$_2$ were required to generate detectable amount of CrO$_3^-$ ions at physiological pH (pH = 7.2). In contrast, in the presence of certain flavoenzymes such as GSSG-R, even 0.5 mM K$_2$Cr$_2$O$_7$ and 1 mM NADPH generated intense Cr(V) ESR signal (Figure 1b), which was assigned to Cr(V)-NADPH complex formation (28-30). The spectra in Figure 1 demonstrate that in an enzymatic reduction of Cr(VI), Cr(V)-complex formation is much more efficient than CrO$_3^-$ formation from direct reaction of Cr(VI) with H$_2$O$_2$.

**Does a Mixture of Cr(VI) and Vitamin B$_3$ Generate CrO$_3^-$?** As shown in Figure 2a, reaction of Cr(VI) with vitamin B$_3$ generates an ESR signal at g = 1.9795. The measured peak-to-peak linewidth is about 5 G. While in an earlier report (30) this signal had been assigned to the CrO$_3^-$ ions, this g value and the linewidth are typical of those of Cr(V)-diol complexes (23). To ascertain whether this signal was due to CrO$_3^-$, a synthesized K$_2$Cr$_2$O$_7$ crystal was added to the Cr(VI)/vitamin B$_3$ mixture. The g value of CrO$_3^-$ signal was measured to be 1.9720 (Figure 1a), in agreement with an earlier report (38). Since vitamin B$_3$ contains diol functionalities, a key group for Cr(V) formation, spectra were recorded from reaction of Cr(VI) with other diol-containing molecules (Figures 2b and 2c). We also recorded the ESR spectrum from a mixture of Cr(VI) and vitamin B$_3$ in DMSO because of the higher solubility of vitamin B$_3$ in this medium. As can be noted in Figure 2d, this spectrum was strong enough that a superhyperfine splitting of about 0.85 G could be resolved. Such superhyperfine...
splitting is considered to be characteristic of the superhyperfine interaction of the methylene-type of hydrogens in Cr(V)-dial complexes (23). At higher spectral gain and wider scan width, we observed four weak satellite signals due to the 53 Cr isotope (9.55% abundance, I = 3/2) (Figure 2e). The measured 17.7 G 53Cr hyperfine splitting (indicated in Figure 2e) is very similar to those reported earlier (23) for Cr(V) complexes, confirming that the Cr(V) species detected in the mixture of Cr(VI) and vitamin B6 is indeed a Cr(V)-dialyl type complex, and not CrO42- ions.

**Does Reaction of Cr(VI) with GSH Generate CrO42-?** We examined whether the reaction of Cr(VI) with GSH can generate CrO42-. GSH was chosen because it is considered to be one of the major Cr(VI) reductants in cellular systems (18). Figure 3a shows the ESR spectrum obtained from an aqueous solution of GSH and K2Cr2O7 in a 5:1 molecular ratio at pH = 4.0. This spectrum was recorded 2 min after solution preparation. The spectrum is dominated by signals at g = 1.995 (5.8 G peak-to-peak derivative width) and at g = 1.985. These two peaks have different decay rates and have been already assigned by earlier workers to two different Cr(V) species, coordination not defined (34,35). The spectra in Figures 3a–3g show the time dependence of the decay of the Cr(V) complexes. It may be noted that the g = 1.985 species decays at a slower rate than the g = 1.995 species; the g = 1.985 signal was still observable after at least 15 min of mixing.

Variation of the solution pH within the range pH 3 to pH 8 yield spectra generally similar to those outlined above for pH 4.0, with an enhanced rate of decay occurring at higher pH values. This observation essentially rules out the identification of these species as CrO42- because CrO42- becomes more stable at the higher pH. Figure 4 shows the effect of changing the GSH:K2Cr2O7 ratio. A decrease in the GSH:K2Cr2O7 ratio decreases the g = 1.995 signal while increasing the g = 1.985 signal (Figures 4d–4f). However, for a GSH:K2Cr2O7 ratio of less than one, not even the g = 1.995 signal was observed (Figure 4g). We reiterate that the chemical structures of these Cr(V) species are not known at present but their dependence on the GSH:Cr(V) ratio suggests that the g = 1.985 species might be a 1:1 and the g = 1.995 species a 2:1 GSH complex of Cr(V). The results show that the major species generated in the reaction of Cr(VI) with GSH is Cr(V)–GSH complexes, with no evidence for CrO42- formation.

It was recently reported (11) that a mixture of K2Cr2O7, GSH and H2O2 in a Tris-HCl medium generates CrO42-, although the ESR peak was too weak to make any reliable assignment. We were unable to detect any CrO42- formation from a mixture of K2Cr2O7, GSH and H2O2 in Tris-HCl. To obtain further clues, we measured the stability of K2Cr2O7 in phosphate buffer as well in Tris-HCl buffer. The ESR spectrum of CrO42- in phosphate buffer is shown in Figure 5a. This spectrum can be detected even 10 minutes after solution preparation. In Tris-HCl medium, however, CrO42- rapidly decayed and converted to another Cr(V) species at g = 1.9721 (Figure 5b). This Cr(V) species is likely to be a Cr(V)-Tris-HCl complex, but the detailed structure has not yet been elucidated. The intensity of the newly formed Cr(V) species reduced to about 20% within 1.5 min after reaction initiation while that of CrO42- became

**Figure 3.** Time course of ESR spectrum recorded from a mixture of 15 mM GSH and 75 mM GSH at different time intervals.

**Figure 4.** ESR spectra recorded 2 min after mixing 15 mM K2Cr2O7 and various concentrations of GSH.

**Figure 5.** (a) ESR spectrum recorded 0.5 minute after preparation of 1 mM K2Cr2O7 in phosphate buffer (pH 7.2); (b) same as (a) but in Tris-HCl (pH 8.0) buffer; (c) same as (b) but spectrum recorded 1.5 min after sample preparation; (d) same as (a) but with 5 mM GSH added; (e) ESR spectrum recorded 30 minutes after preparation of 5 mM K2Cr2O7 and 7.5 mM NADPH in phosphate buffer (pH 7.2).
undetectable (Figure 5e). When GSH was added to a K$_2$Cr$_2$O$_7$/Tris-HCl solution, no Cr(V) species can be detected (Figure 5d), indicating that GSH also reacts with K$_2$Cr$_2$O$_7$. These results show that CrO$_3^-$ is unstable in the Tris-HCl (pH 8) medium and that CrO$_3^-$ formation in the mixture of K$_2$Cr$_2$O$_7$, GSH and H$_2$O$_2$ in Tris-HCl buffer (pH 8.0) is unlikely. We also studied the effect of NADPH on the stability of K$_2$Cr$_2$O$_7$. As shown in Figure 5e, addition of NADPH to the K$_2$Cr$_2$O$_7$ solution caused a sharp decrease in CrO$_3^-$ signal and led to the formation of the Cr(V)−NADPH complex, suggesting that CrO$_3^-$ undergoes fast ligand exchange with NADPH to form a Cr(V)−NADPH complex. Thus in biological systems in which thiols like GSH and diols such as NADPH are plentiful, it is unlikely that CrO$_3^-$ could exist in any significant amount.

**Does Reaction of Cr(VI) with Ascorbate Generate CrO$_3^-$?** We used ascorbate as another Cr(VI) reductant since ascorbate is also thought to be a major Cr(VI) reductant in cell systems (6,32). As shown in Figure 6a, a mixture of Cr(VI) and ascorbate in phosphate buffer (pH 7.2) generates an ESR signal at g = 1.9794, which can be assigned to a Cr(V)-ascorbate complex according to an earlier report (33). No CrO$_3^-$ ESR signal was detected. When H$_2$O$_2$ and DMPO (as a spin trap) were added, a 1:2:1:1 quartet with hyperfine splittings of $a_{g}$ = $a_{a}$ = 14.9 G was observed (Figure 6b). Based on these splittings (39), the 1:2:1:1 signal was assigned to the DMPO/OH adduct, as evidence of OH radical generation. Upon addition of H$_2$O$_2$, however, Cr(V) becomes non-detectable (Figure 6b), indicating that •OH radicals were generated in the reaction between Cr(V) and H$_2$O$_2$ via a Fenton-like mechanism.

**Does K$_2$Cr$_2$O$_7$ Generate •OH Radical?** Figures 7a and 7b present spin trapping studies of possible •OH generation from K$_2$Cr$_2$O$_7$. Decomposition of CrO$_3^-$ in phosphate buffer does not generate any detectable amount of •OH radicals (Figure 7a). Addition of H$_2$O$_2$ to the CrO$_3^-$ solution did not cause any significant enhancement in •OH generation, nor any significant decrease in the CrO$_3^-$ peak (Figure 7b). Figure 7c shows the Cr(V)-NADPH complex formation from a mixture of Cr(VI) and NADPH. Addition of H$_2$O$_2$ reduced the intensity of the Cr(V)-NADPH signal to a barely detectable level with a concomitant appearance of the DMPO/OH spin adduct signal (Figure 7d), indicating that reaction of Cr(V)-NADPH with H$_2$O$_2$ does generate •OH radicals. Similarly, reaction of Cr(VI) and GSH generated Cr(V)-GSH complexes and the DMPO/GS spin adduct (Figure 7e)(23). Addition of H$_2$O$_2$ reduced the intensity of Cr(V)-GSH signals and generated DMPO/CHOHCH$_3$ spin adduct signal (as indicated by asterisks) (Figure 7f). Since the hyperfine splitting of DMPO/GS and DMPO/OH is very similar, ethanol was added for the identification of •OH radical. It is known that •OH radical efficiently reacts with ethanol to form •CHOHCH$_3$ radical, which, in turn, is trapped by DMPO to produce DMPO/CHOHCH$_3$. These results show that •OH radicals were generated via reaction of the Cr(V)-GSH complex with H$_2$O$_2$, without any significant contribution from CrO$_3^-$. Earlier studies have shown that reaction of Cr(VI) with H$_2$O$_2$ at high concentrations generates •OH radical (15). The present study confirms that while •OH radicals can be generated in the reaction of Cr(VI) with H$_2$O$_2$, the yield is very low. As can be noted from Figure 8, a mixture of 2 mM K$_2$Cr$_2$O$_7$, 2 mM NADPH and 3 mM H$_2$O$_2$ in phosphate buffer (pH 7.2) generates •OH radicals in a yield 15 times higher than from a mixture of 5 mM K$_2$Cr$_2$O$_7$ and 10 mM H$_2$O$_2$. Addition of GSSG-R increases this yield by a factor of 90. These results demonstrate that in cellular systems, the majority of Cr(VI)-mediated •OH radicals would be expected to be generated via a Fenton-like reaction of Cr(V)-complexes rather than decomposition of CrO$_3^-$. 

![Figure 6](image-url)  
**Figure 6.** (a) ESR spectrum recorded from a mixture of 10 mM K$_2$Cr$_2$O$_7$ and 10 mM ascorbate in phosphate buffer (pH 7.2); (b) same as (a) but with 60 mM DMPO and 2.5 mM H$_2$O$_2$ added.

![Figure 7](image-url)  
**Figure 7.** (a) ESR spectrum of 100 mM DMPO and 10 mM K$_2$Cr$_2$O$_7$ in phosphate buffer (pH 7.2); (b) same as (a) but with 15 mM H$_2$O$_2$ added; (c) ESR spectrum of 100 mM DMPO, 25 mM K$_2$Cr$_2$O$_7$ and 25 mM NADPH in phosphate buffer (pH 7.2); (d) same as (c) but with 5 mM H$_2$O$_2$ added; (e) ESR spectrum of 100 mM DMPO, 2 mM K$_2$Cr$_2$O$_7$, 50 mM GSH, 5% ethanol; (f) same as (e) but with 5 mM H$_2$O$_2$ added. The asterisks indicate the DMPO/CHOHCH$_3$ adduct.
Disscussion

The results obtained in the present study demonstrate that under biologically relevant conditions, Cr(VI) does not generate CrO$_8^{3-}$ in significant amounts. Moreover, CrO$_8^{3-}$ decomposition or its reaction with H$_2$O$_2$ does not generate any significant amount of -OH radicals. Instead, reduction of Cr(VI) by NADPH, GSH, NADPH/GSSG-R, and ascorbate generates significant amount of Cr(V) complexes. No CrO$_8^{3-}$ species could be detected in the reaction of Cr(VI) with these materials. The Cr(V) complexes thus produced readily react with H$_2$O$_2$ to generate -OH radicals via a Fenton-like mechanism [Cr(V) + H$_2$O$_2$ $\rightarrow$ Cr(VI) + -OH + OH$^-$]. This mechanism is the likely major pathway for Cr(VI)-mediated -OH radical generation because Cr(V) complexes can be generated in various biological systems, including cytochrome P-450 (6), cytochrome b$_5$ (40), the electron transport complexes of the inner mitochondrial membrane (31), microsomal systems (9,25), certain flavoenzymes such as glutathione reductase (28-30), superoxide radicals (27), and a number of thiol and diol-containing molecules (6,23,34,35). In particular, GSH is considered by far the most important among the possible thiol reductants of Cr(VI), mainly because of its ubiquitous occurrence in mammalian cells in millimolar concentrations (18,41), and because GSH levels can be easily modulated with various agents (42). In addition to GSH, ascorbate is also considered the likely Cr(VI) non-enzymatic reductant in vivo on the basis of its high reactivity with Cr(VI) and its abundance within the cell (6,32,33).

As for the molecular mechanism of -OH radical generation by Cr(V) complexes with H$_2$O$_2$, it may be noted that CrO$_8^{3-}$ has a tetrahedral structure with all four covalent bonds fully occupied by O$_2$- moieties as shown below.

Thus the CrO$_8^{3-}$/H$_2$O$_2$ complex would not easily split H$_2$O$_2$ to -OH radical. On the other hand, a Cr(V) complex such as Cr(V)-MED, is expected to be octahedral, with one vacant site as shown below:

Thus the H$_2$O$_2$ can attach to the vacant coordination site (indicated by the arrow) and form a long-lived complex to generate -OH radical. This mechanism is similar to the oxidation of Fe(II) with H$_2$O$_2$ in the Fenton reaction as the production of -OH radical from Fe(II) via Fenton reaction is facilitated greatly by the formation of Fe(II) complexes that have vacant sites for H$_2$O$_2$ coordination (42).

In conclusion, the present study demonstrates that the reduction of Cr(VI) by cellular reductants does not generate any significant amount of CrO$_8^{3-}$ ions. ESR spin trapping studies using laboratory synthesized K$_2$CrO$_7$ showed that CrO$_8^{3-}$ decomposition by itself, or the reaction of CrO$_8^{3-}$ with H$_2$O$_2$ does not generate any significant amount of -OH radicals. Reactions of Cr(VI) with several major Cr(VI) reductants generate Cr(V) complexes, which produce -OH radical via a Fenton-like mechanism. The present investigation does not support the "tetraperoxochromate(V)" theory of carcinogenesis from chrome." Instead, we propose that the mechanism involves two species, Cr(V) complexes and -OH radicals generated via a Fenton-like mechanism. Hence, this mechanism might be labeled as "Cr(V)-complexation-Fenton reaction model of carcinogenesis from chrome."

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