Reaction of Chromium(V) with the EPR Spin Traps 5,5-Dimethylpyrroline *N*-Oxide and Phenyl-*N*-*tert*-butylnitrone Resulting in Direct Oxidation

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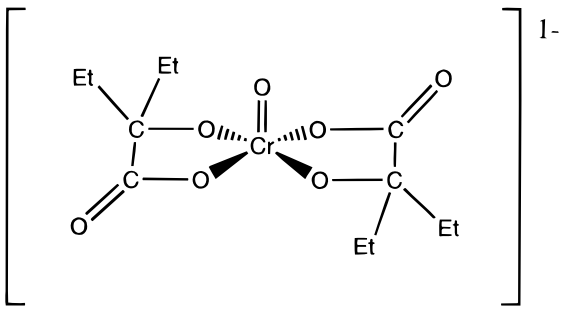
Experiments were performed to investigate potential biologically relevant oxidation reactions of a model Cr(V) complex, sodium bis(2-ethyl-2-hydroxybutyrato)oxochromate(V) (Na[Cr(ehba)$_2$O]), with the spin traps 5,5-dimethylpyrroline *N*-oxide (DMPO) and phenyl-*N*-*tert*-butylnitrone (PBN). Electron paramagnetic resonance spectroscopy (EPR) has shown that, under physiological conditions, 100 mM HEPES, pH = 7.4, the reaction of Cr(V) with DMPO resulted in the formation of the hydroxyl radical adduct of DMPO, DMPO/•OH. In the presence of 1.0 M ETOH, this reaction did not display the characteristic α-alcohol adduct of DMPO, implying that DMPO/•OH formation arises from a direct oxidative mechanism and not from a discrete diffusible radical such as the hydroxyl radical, •OH. Upon addition of hydrogen peroxide to Cr(V) and DMPO, a significant increase of the DMPO/•OH adduct was observed, which can be ascribed to the formation of a diffusible radical, •OH. Under acidic conditions, 100 mM acetic acid/sodium acetate, pH = 3.0−6.0, reaction of Cr(V) with DMPO or PBN yielded no EPR-detectable radicals, but led to decreased stability of Cr(V). Product analysis of the reaction of Cr(V) with PBN by HPLC showed cleavage of the imine moiety of the nitrone to form benzaldehyde. Experiments using ^{17}O- and ^{18}O-labeled water coupled with IR and EPR spectroscopy were used to examine exchange of the oxo group on Cr(V) and thus to postulate a mechanism for the different pH-dependent reactions. The results suggest that, at physiological pH, nitrone coordination of Cr(IV) induces nucleophilic attack of water at the β-position of DMPO resulting in homolytic bond cleavage and radical formation. At lower pH's, an even electron mechanism predominates and is best accounted for by an oxo-atom transfer from Cr(V) to the spin trap resulting in C═N oxidative cleavage but no radical formation. These same mechanisms may be responsible for the reactions observed between Cr(V) species, generated by the *in vivo* reduction of Cr(VI), and DNA under various conditions and may be important in Cr(VI) carcinogenesis.

Introduction

Chromium(V) is considered by many to play a major role in the induction of cancer by the genotoxic and carcinogenic form of chromium, chromium(VI). The Cr(V) redox state is a transient intermediate that can be formed *in vitro* via the reduction of Cr(VI) with a variety of common cellular reductants, such as ascorbate, glutathione, ribonucleotides, and NADPH.[1−4] This reduction of Cr(VI) to lower valency is considered to be essential for DNA damage and thus the potentiation of carcinogenesis.[5] The formation of the Cr(V) oxidation state intracellularly from Cr(VI) has recently been established in both cultured cells and *in vivo*.[6,7] The formation of Cr(V) has been correlated with many forms of DNA damage. Stearns *et al*. have shown that, at the ascorbate/Cr(VI) ratio that generated maximum EPR-detectable Cr(V), there was a resulting proportional increase in binding of chromium to calf thymus DNA.[8] Reduction of Cr(VI) by glutathione resulted in a Cr(V)−glutathione complex that has been implicated in the formation of GSH−Cr−DNA adducts, abasic sites, and single strand breaks on DNA.[9−11]

A fundamental question concerns the role that Cr(V) may play in the overall mechanism of carcinogenesis by Cr(VI), since the DNA damage may be attributed to other chromium valency states or free radicals known to be formed from the reduction reaction. Few complexes of Cr(V) have been isolated and characterized to date, and no Cr(V) complex with the small molecular weight reductive ligands of ascorbate, glutathione or cysteine have been unambiguously synthesized and structurally characterized. Of the Cr(V) complexes known, few show appreciable stability in aqueous systems under physiological conditions making biologically relevant studies of this oxidation state particularly difficult. Some of the best known and studied of the Cr(V) complexes are those chelated to α-hydroxy acid ligands such as bis(2-ethyl-2-hydroxybutyrato)oxochromate(V), [Cr(ehba)$_2$O]$^-$.[12]



This pentacoordinate, distorted square pyramidal Cr(V) complex has demonstrated mutagenicity, toxicity, and an ability

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to cause DNA single-strand breaks.[13] On the basis of EPR *g*-values and ^{53}Cr hyperfine values, it is our hypothesis that the pentacoordinated oxygen ligands on the inner sphere of this complex may resemble the complexes formed from reduction and complexation of Cr(VI) by ascorbate.[4] This is particularly relevant since ascorbate has been identified as the major reductant of Cr(VI) in ultrafiltrates prepared from rat tissue.[14,15]

$[Cr(ehba)_2O]^-$ at neutral pH in aqueous solutions rapidly disproportionates through a bimolecular mechanism producing Cr(VI) and a reactive Cr(IV) intermediate (eqs 1 and 2), which ultimately leads to the final redox proportions of 2 Cr(VI) to 1 Cr(III): (eq 3). However, at more acidic pH's disproportion-

$$2Cr(V) \rightarrow Cr(IV) + Cr(VI) \quad (1)$$

$$Cr(IV) + Cr(V) \rightarrow Cr(VI) + Cr(III) \quad (2)$$

$$\text{net:} \quad 3Cr(V) \rightarrow 2Cr(VI) + Cr(III) \quad (3)$$

ation of this species is minimal and decomposition is slow and attributed to ligand oxidation.[16] Of the two oxidative species of interest, Cr(IV) has been determined to be a somewhat stronger one electron oxidant with a formal reduction potential of $E^{\circ\prime} = 1.35$ V *vs* 1.29 V for Cr(V); however, the formal reduction potential for the two-electron Cr(V)/Cr(III) redox couple has been estimated to be 1.32 V.[17] The disproportionation reaction of Cr(V) is particularly suited for monitoring the redox changes via UV−vis spectroscopy, and the d^1 Cr(V) species is readily monitored with room temperature EPR spectroscopy.

Because oxidation reactions of Cr(V) and Cr(IV) with biological molecules such as DNA are likely to be important in the mechanism of Cr(VI) carcinogenesis, we have used EPR and UV−vis spectroscopy to examine the oxidation reactions between $[Cr(ehba)_2O]^-$ and model substrates, the spin traps 5,5-dimethylpyrroline *N*-oxide (DMPO) and phenyl-*N*-*tert*-butylnitrone (PBN). The oxidation reactions appear to be pH dependent and involve a radical (odd electron) *vs* nonradical (even electron) pathway with the nonradical pathway dominating at lower pH and a radical pathway appearing at physiological pH.

Experimental Section

Chemicals. The sodium salt of the Cr(V) complex bis(2-ethyl-2-hydroxybutyrato)oxochromate(V) was prepared by the method of Krumpolc and Rocek.[12] Briefly, this involved the reaction of Cr(VI), as $Na_2Cr_2O_7 \cdot 2H_2O$, with 7 equiv of the ligand 2-ethyl-2-hydroxybutyrate (EHBA) for 24 h at room temperature in acetone followed by crystallization by the dropwise addition of hexane. Analysis by IR and UV−vis were identical with that shown previously for this complex.[12] Purity was determined to be >98% by oxidation to Cr(VI) by treatment with alkaline H_2O_2 and analysis by UV−vis at 372 nm using the empirically determined extinction coefficient, $\epsilon = 4830$ cm^{-1} M^{-1}, using $Na_2Cr_2O_7 \cdot 2H_2O$ as a standard. *Caution! Cr(VI) is a known human carcinogen and Cr(V) complexes are potentially carcinogenic. Appropriate precautions should be taken in handling these materials.* Removal of trace iron from reaction buffers was accomplished by treating the solutions with Bio-Rad Chelex-100 cation exchange resin, 5 g/100 mL, for 2 h. Further steps to insure trace iron removal was accomplished with the addition of 50 μM of diethylentriaminepentaacetic acid (DETAPAC), to the buffer solutions. Solutions of the spin traps DMPO and PBN were prepared in the appropriate buffer and then solution filtered with activated charcoal to remove impurities prior to use. At low pH, it is important to make the solutions of spin traps fresh and to use immediately. The 10% ^{17}O- and 95−98% ^{18}O-labeled water were obtained from Cambridge Stable Isotope Labs Inc., and used as received.

EPR Studies. EPR spectra were recorded using a Bruker ESP-300 spectrometer. The spectral parameters, unless otherwise specified, were 100-kHz field modulation, 1.0 G modulation amplitude, 5.12 ms time constant, 9.769−9.772 microwave frequency, 1×10^5 receiver gain, 2 mW microwave power attenuated at 20 dB, 3380−3580 G sweep width, and a 21 s scan time. All signals were averaged over nine scans. Typical reactions were carried out on 1.0 mL volumes at room temperature between 80 s and 60 min with 100 mM spin trap (DMPO or PBN) and 0.50 mM Cr(V). Measurements were carried out on *ca.* 100 μL volumes drawn into a capillary tube sealed on one end with Dow-Corning high vacuum grease and placed in a quartz EPR tube. The *g* values were determined with respect to 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), $g = 2.0036$. Concentrations of Cr(V) were calculated by peak integration from standard curves measured from $Na[Cr(ehba)_2O]$ dissolved in aqueous 100 mM EHBA ligand stock solutions. Radical concentrations were measured using peak integration from a standard curve using the stable radical 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) in 100 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), 50 μM DETAPAC, pH = 7.4.

UV−Vis Studies. Cr(VI) formed from the disproportionation reaction of Cr(V) at pH = 7.4 was determined at 372 nm ($\epsilon = 4830$ M^{-1} cm^{-1}). Typical reactions were 100 mM spin traps (DMPO or PBN) and 0.50 mM Cr(V) in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4. The reaction was allowed to proceed at room temperature for 1 h prior to determination of final Cr(VI) concentration.

HPLC Studies. HPLC data for the product analysis of the reaction between Cr(V) and PBN were recorded using a Hewlett-Packard 1050 HPLC with diode array detection. A 4.6 mm × 25 cm, 5 μm C18 RP column with a mobile phase consisting of solvent A, 50 mM ammonium acetate, and solvent B, acetonitrile. For product analysis of the reaction of PBN with Cr(V), a gradient of 90% 50 mM ammonium acetate and 10% acetonitrile to 100% acetonitrile over 20 min was used. The wavelengths and detection limits (in parenthesis) for the starting material and possible products were as follows: benzaldehyde, 254 nm (5.0 μM); benzoic acid, 230 nm (10.0 μM); PBN, 290 nm (5.0 μM).

GC−MS Studies. Mass spectral data were recorded using a HP-5890 gas chromatograph with a HP-5971 mass selective detector. Experimental parameters were injector temperature 150 °C, detector temperature 312 °C, oven temperature 100−290 °C ramped over 22 min, and ionization potential 70 eV. Following the 30 min incubation of 0.50 mM Cr(V) and 100 mM PBN in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4 at room temperature, the samples were extracted with methylene chloride, and 0.5 μL of extract was analyzed.

IR Studies. Exchange of the oxo group of the Cr(V) complex was monitored by IR spectroscopy. 5.0 mg of the Cr(V) complex was dissolved in 50 μL of either ^{16}O- or 95−98% ^{18}O-labeled water and allowed to exchange for 15 min at room temperature. The reaction mixture was frozen on dry ice and lyophilized to dryness on a speed-vac concentrator. IR analysis was performed on a Perkin-Elmer FT-IR using a 1% KBr pellet of the complex.

Results

Reaction of Cr(V) with DMPO at Physiological pH. In order to understand the mechanism by which Cr(V) reacts with spin traps, the reaction of Cr(V) with either DMPO or PBN was monitored by EPR spectroscopy at room temperature. A typical EPR spectrum for the reaction of 0.50 mM Cr(V) and 100 mM DMPO in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4 is shown in Figure 1A. The characteristic 1:2:2:1 quartet with $A_H = A_N = 14.9$ G splitting for the DMPO/•OH spin adduct was observed. Addition of 1.0 M ETOH to the reaction mixture resulted in no change of the 1:2:2:1 quartet, which implies that

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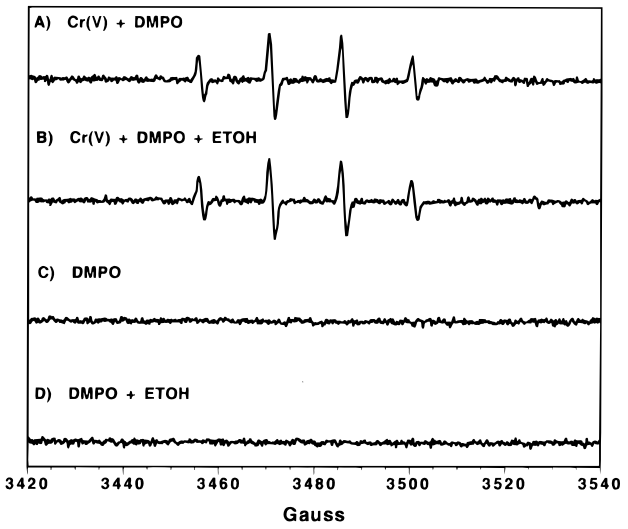


Figure 1. EPR spectra for the reaction of Cr(V) with DMPO. (A) EPR spectrum of the reaction of Cr(V) (0.50 mM) with DMPO (100 mM). (B) EPR spectrum of the reaction of Cr(V) (0.50 mM) + DMPO (100 mM) + ETOH (1.0 M). (C) EPR spectrum of DMPO (100 mM) control. (D) EPR spectrum of DMPO (100 mM) + ETOH (1.0 M) control. All spectra were acquired at room temperature in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4 after a 10 min reaction time.

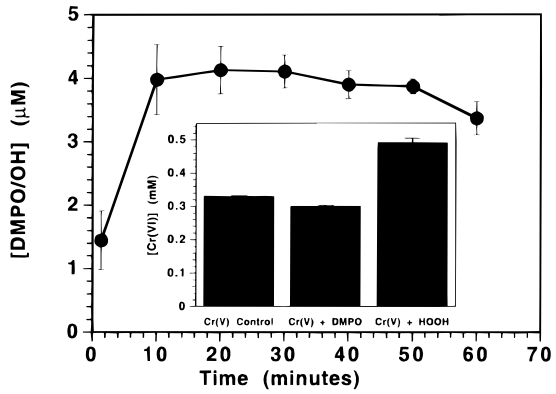


Figure 2. Time course experiment for the formation of the radical adduct of DMPO, DMPO/•OH, from the reaction of Cr(V) (0.50 mM) and DMPO (100 mM) at room temperature in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4. Concentrations of DMPO/•OH were determined from a standard curve using the stable radical species TEMPO. The inset shows the final concentration of Cr(VI) following disproportionation of Cr(V) (0.50 mM) in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4 in the presence of either DMPO (100 mM) or hydrogen peroxide (10 mM). The concentration was determined at λ = 372 nm (ϵ = 4830 M^{-1} cm^{-1}). Data represents the mean ± standard deviation (n = 3).

a discrete diffusible radical, such as •OH, is not responsible for the formation of DMPO/•OH, Figure 1B. Instead, the results suggest a direct reaction of Cr(V) with the spin trap. No dependence upon dioxygen for the formation of DMPO/•OH was observed when the same reaction was carried out under an argon atmosphere or in air. Reaction under an oxygen atmosphere did not show any oxygen dependence for the formation of DMPO/•OH, although paramagnetic signal broadening was observed (data not shown). The reaction of Cr(V) with the spin trap PBN under similar conditions did not yield significant levels of radicals.

The level of DMPO/•OH radical plateaued between 10 and 50 min after mixing Cr(V) and DMPO and then slightly decreased, Figure 2. This response correlates with the lifetime of the Cr(V) EPR signal which decayed completely by 10 min at pH = 7.4, Figure 3A. Aged solutions of Cr(V) that had

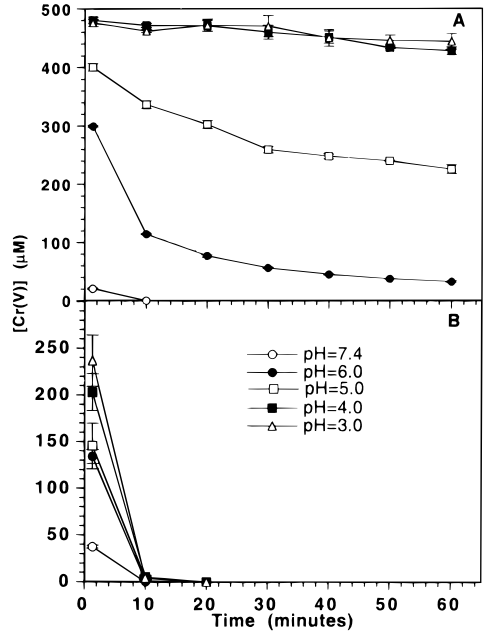


Figure 3. Time course experiment for the determination by EPR of the stability of (A) Cr(V) (0.50 mM) at room temperature in either 100 mM HEPES, 50 μM DETAPAC, pH = 7.4, or 100 mM acetic acid/sodium acetate, 50 μM DETAPAC, pH = 6.0−3.0 or (B) Cr(V) (0.50 mM) + PBN (100 mM) at room temperature in either 100 mM HEPES, 50 μM DETAPAC, pH = 7.4 or 100 mM acetic acid/sodium acetate, 50 μM DETAPAC, pH = 6.0−3.0. Cr(V) concentrations were determined from a standard curve of Cr(V) in 100 mM aqueous solution of EHBA. Data represents the mean ± standard deviation (n = 3).

completely disproportionated to Cr(VI) and Cr(III) did not show any measurable DMPO/•OH generation (data not shown). This suggests that DMPO/•OH formation arises from either Cr(V) or the Cr(IV) intermediate formed during disproportionation. The DMPO/•OH concentration following reaction of 0.50 mM Cr(V) with 100 mM DMPO was *ca.* 4.0 μM or 0.8% of the total Cr(V) in the reaction mixture. Thus, this radical pathway accounts for only a small amount of the total Cr(V) present.

If the disproportionation reaction of Cr(V) is monitored in the presence of DMPO, any reaction which results in reduction of the chromium, and thus oxidation of the substrate should be detectable by a change in the proportions of the final Cr(VI) and Cr(III) redox states. The theoretical yield for Cr(VI) from pure disproportionation of (0.50 mM) Cr(V) is 67% (0.33 mM), with 33% (0.17 mM) being Cr(III). Results of the reaction of Cr(V) with DMPO at pH = 7.4 showed a 7.8 ± 0.1% loss of Cr(VI) over controls, presumably resulting in more reduced Cr(III) species (Figure 2 inset). While this change was greater than the 0.8% reaction product of DMPO/•OH from EPR experiments, it must be noted that the DMPO/•OH concentration in the EPR is the steady-state level and not necessarily the total amount generated. It is possible that all of the loss of Cr(VI) in the disproportionation reaction is accounted for by DMPO/•OH production, or alternatively, a separate nonradical pathway involving an even electron substrate oxidation may account for this discrepancy.

Reaction of Cr(V) and DMPO with Hydrogen Peroxide at Physiological pH. In the presence of 10 mM hydrogen peroxide, the DMPO/•OH, adduct was generated at nearly 5-fold higher levels (18 μM) in the reaction with 0.50 mM Cr(V) and 100 mM DMPO in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4. With the addition of 1.0 M ETOH to the same reaction, the characteristic hyperfine splitting for the α-alcohol DMPO/ radical adduct of ETOH having hyperfine splittings of A_H = 22.8 G and A_N = 15.8 G, was overlaid on the 1:2:2:1 quartet of

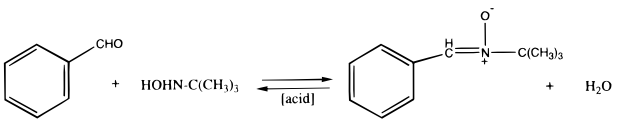
DMPO/•OH. A small amount of Cr(V) with $g = 1.980$ was seen as well, indicating some unreacted Cr(V)−EHBA complex remained due to stabilization in the slightly more nonpolar solvent. These results imply that only in the presence of hydrogen peroxide was there significant production of a diffusible radical, in this case the hydroxyl radical. UV−vis monitoring of the disproportionation reaction revealed that as expected, a shift in the redox proportions toward Cr(VI) was observed (Figure 2 inset). This result is consistent with pseudo-Fenton chemistry in the generation of •OH as shown in eq 4.

$$Cr(V) + H_2O_2 + H^+ \rightarrow Cr(VI) + {}^{\bullet}OH + H_2O \qquad (4)$$

Once again, the stoichiometric yield of radicals cannot be determined since EPR measurements give the steady-state concentrations of DMPO/•OH, which in this case would also depend on the radical trapping efficiency of the spin trap.

Reaction of Cr(V) with DMPO and PBN at Acidic pH's. The fact that Cr(V) has been shown to have significant reactivity toward DNA at pH's lower than the normal physiological range,[13] led us to investigate these same reactions of Cr(V) with spin traps at pH = 3.0−6.0. Cr(V) stability in aqueous solution is highly dependent on pH. Figure 3A shows that, in aqueous 100 mM HEPES, 50 μM DETAPAC, pH = 7.4, the stability of Cr(V) was quite low, with complete decay of the EPR signal observed at 10 min. In contrast, at pH = 3.0−4.0, Cr(V) was quite stable with pH = 5.0 and 6.0 showing intermediate stability. Reaction of Cr(V) with spin traps showed little or no EPR-detectable spin adducts at lower pH's in the reaction of 0.50 mM Cr(V) with 100 mM spin trap. However, a significant loss in the stability of the Cr(V) signal was observed in the presence of both spin traps. The most dramatic loss in Cr(V) stability is shown in Figure 3B, where the reaction with 100 mM PBN resulted in nearly complete Cr(V) signal decay in 10 min for all pH's tested. These results suggest that a nonradical process involving the reaction of Cr(V) and spin traps predominates at lower pH. At lower pH's, disproportionation is no longer stoichiometric, and Cr(V) was stable enough that UV−vis could not be used to accurately measure changes in final redox proportions. However, it should be noted that reduction predominated in these reactions and the major product was a Cr(III) species with an absorbance maximum at 552 nm (ϵ = 49 M^{-1} cm^{-1}) and a less distinct maximum at 405−415 nm (ϵ = ∼60 M^{-1} cm^{-1}). This product is considered to be a chelated monocarboxylato−Cr(III) complex by analogy to that demonstrated by Franchiang *et al.*[18]

Product Analysis of the Reaction between Cr(V) and PBN at Acidic pH. To determine the end product of the reaction of Cr(V) with spin traps, product analysis for the reaction of Cr(V) with the spin trap PBN was performed. PBN was chosen in this study for both its high degree of reactivity with Cr(V) and the presence of an easily detected chromophore. An initial concern was the stability of the spin trap itself at lower pH's. Synthesis of the spin trap PBN involves a condensation reaction between benzaldehyde and *tert*-butylhydroxylamine with a corresponding dehydration reaction.[19] Under acidic conditions, hydrolysis of the spin trap may occur resulting in regeneration of the original reactants.



Under the conditions used in this assay, 30 min at room temperature, hydrolysis of PBN to benzaldehyde was only detected for pH ≤ 4.0. Reaction of Cr(V) with PBN in 100 mM acetic acid/sodium acetate, 50 μM DETAPAC, at pH = 5.0 resulted in oxidation of PBN to benzaldehyde as measured by HPLC, Figure 4. Formation of benzaldehyde was confirmed with HPLC using a benzaldehyde standard as well as by GC−MS which yielded a molecular ion with *m*/*z* 106. Increasing concentrations of Cr(V) resulted in an increase in the formation of benzaldehyde, but not in a linear fashion. However, only at the lowest Cr(V) concentration (0.50 mM) was the decomposition of the Cr(V) EPR signal observed to have gone to completion by 30 min accounting for this lack of linearity. Figure 4 (inset) shows that formation of benzaldehyde from 100 mM PBN and 0.50 mM Cr(V) was similar for the pH range of 4.0−6.0. No benzaldehyde was observed for the reaction of Cr(V) with PBN in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4. The reaction was not stoichiometric since the formation of benzaldehyde was only ∼10−12% of the expected yield for a two-electron oxidation of the substrate. The lack of a readily detectable chromophore for DMPO and the propensity for this compound to decompose at higher temperatures did not allow us to investigate Cr(V) oxidation products of DMPO. However, a similar pH dependent instability of the Cr(V) EPR signal in the presence of DMPO suggests that an identical mechanism is occurring, albeit more slowly (results not shown).

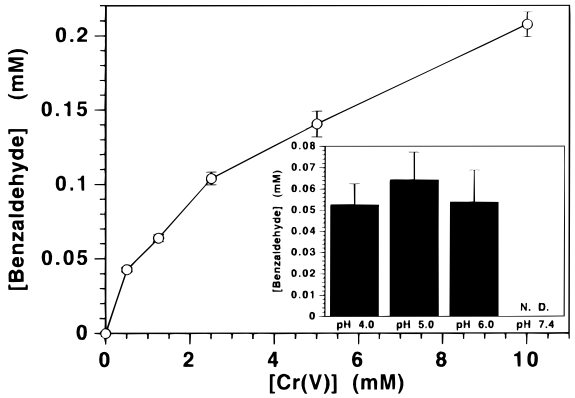


Figure 4. Dependence of the formation of benzaldehyde on Cr(V) concentration in reactions of Cr(V) (0.5−10 mM) with 100 mM PBN at room temperature after a 30 min reaction time in 100 mM acetic acid/sodium acetate, 50 μM DETAPAC, pH = 5.0. Inset shows the concentration of benzaldehyde formed from the reaction of 0.50 mM Cr(V) with 100 mM PBN for 30 min at room temperature at different pH's in 100 mM acetic acid/sodium acetate, 50 μM DETAPAC, pH = 4.0−6.0, or in 100 mM HEPES, 50 μM DETAPAC, pH = 7.4.

Isotope Studies of Cr(V) and DMPO/•OH at Physiological pH. Further determination of a mechanism for the reaction of Cr(V) with the spin traps at physiological pH was attempted using isotopic labeling experiments. At physiological pH, Cr(V)/DMPO reactions were carried out in 10% ^{17}O-enriched water to determine the source of the hydroxyl moiety of DMPO/•OH. The ^{17}O stable isotope has a nuclear spin $I = {}^5/_2$ *vs* the spin $I = 0$ for ^{16}O. Assuming that exchange of the oxo group of Cr(V) with solvent is slower than the accompanying disproportionation reaction, then reaction of Cr(V) with DMPO should not give ^{17}O hyperfine splittings of the DMPO/•^{17}OH adduct when carried out in ^{17}O-water if the reaction involves transfer of the oxo-group to DMPO. However, if the hydroxyl moiety is derived from the solvent, then splitting from the ^{17}O should be observed in the DMPO/•^{17}OH EPR spectra at approximately 10% concentration. Figure 5A shows the actual spectra of the reaction between Cr(V) and DMPO in 10% [^{17}O]-water, 100 mM HEPES, 50 μM DETAPAC, pH = 7.4. Figure 5B shows the computer simulation of what would be expected if the hydroxyl group is derived from the solvent and not the

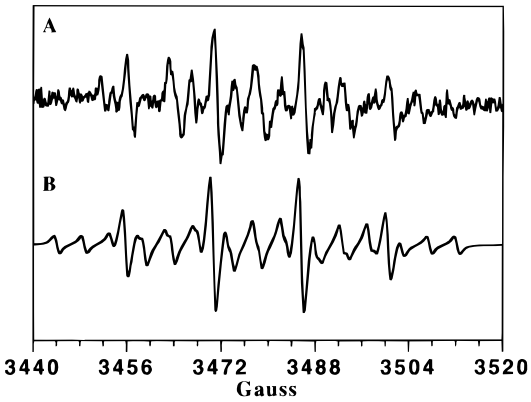


Figure 5. EPR spectra for the reaction of Cr(V) (0.50 mM) with DMPO (100 mM) in 10% ^{17}O-labeled water, 100 mM HEPES, 50 μM DETAPAC, pH = 7.4 after 10 min reaction time at room temperature: (A) actual EPR data; (B) simulated spectrum using the superhyperfine splitting value of 4.66 G[20] for the $I = {}^5/_2$ nucleus of ^{17}O.

oxo group of Cr(V) using the $A_{^{17}O} = 4.66$ G hyperfine splitting shown previously for this adduct.[20] The splitting of the 1:2:2:1 quartet of DMPO/•OH arising from the $I = {}^5/_2$ ^{17}O nucleus shows that the hydroxyl group is most likely derived from the solvent and not from the oxo group of Cr(V) at neutral pH. Control reactions of Cr(V) in 100 mM HEPES, 50 μM DETAPAC, pH =7.4, in [^{17}O]water displayed no $Cr^V(=^{17}O)$ splitting prior to EPR signal loss suggesting that oxo-atom exchange with water is slower than the corresponding disproportionation reaction. However, this result cannot rule out a Cr(IV)-based oxo-atom exchange with solvent prior to reaction with the spin trap, which would not be detectable by EPR. The fact that oxo-atom exchange is a two-electron oxidation would also seem to rule out this mechanism since formation of the DMPO/•OH adduct requires an odd electron mechanism.

Isotope Labeling Studies of Cr(V) at Acidic pH's. The reaction of Cr(V) with the spin trap PBN at acidic pH's demonstrated a different mechanism than that observed at physiological pH. The lack of radical generation at lower pH coupled with the reaction observed by EPR leading to decreased stability of Cr(V) leads us to postulate an even electron mechanism for the reaction. Oxo-atom transfer is the most likely candidate for this mechanism, since the two-electron reduction of Cr(V) would lead to the generation of the stable Cr(III) species, and no radical species with the spin traps have been observed. However, hydride abstraction could also account for these results.[21] Isotopic labeling studies were undertaken to ascertain a possible mechanism for this reaction. Control reactions of Cr(V) at pH = 4.0 demonstrated that the oxo atom of Cr(V) is in a dynamic exchange equilibrium with the solvent. This is in contrast to the lack of oxo-atom exchange at pH = 7.4. Computer simulations of a 10% ^{17}O Cr(V)−oxo complex with $A_{^{17}O} = 3.5$ G were virtually identical with actual EPR spectra of 0.50 mM Cr(V) in acetic acid/sodium acetate, 50 μM DETAPAC, pH = 4.0, taken at 80 s. The EPR result cannot be unambiguously assigned as oxo-atom exchange, EHBA ligand exchange, or formation of a six-coordinate species with water. IR studies with [^{18}O]water were also carried out on this complex to determine the lability of the oxo group of Cr(V) in aqueous systems. Hooke's law predicts a 43 cm^{-1} IR shift for a $Cr^V(=^{16}O)$ to $Cr^V(=^{18}O)$ exchange of the oxo ligand. Results of the exchange reaction using 95−98% [^{18}O]water, obtained in a difference spectra between the Cr(V) complex in ^{16}O- and ^{18}O-labeled water, showed that within 15 min, complete exchange of the oxo group had occurred resulting in a 44 cm^{-1} shift in $\nu_{Cr(=O)}$ from 998 to 954 cm^{-1}. The EPR data, coupled with the IR results, show that at low pH under aqueous conditions, oxo-group exchange with water occurs rapidly. The rapid oxo-atom exchange that we observe with the Cr(V)−EHBA complex precluded labeling studies of oxidized products under acidic conditions.

Discussion

Reaction of Cr(V) with the spin trap DMPO at physiological pH resulted in the formation of DMPO/•OH radical adduct without going through a discrete diffusible radical intermediate. O'Brien and Wang failed to observe the DMPO/•OH adduct with a similar Cr(V) species, bis(2-hydroxy-2-methylpropionato)oxochromate(V); however, their work was carried out in an unbuffered aqueous system where the resultant pH would be expected to be around 4.0, which our work has demonstrated does not show these radical adducts.[22] The authors did show DMPO/•OH formation with a different Cr(V) species, tetrachlorooxochromate(V) which they believed was due to hydroxyl radical formation from decomposition of an aquated intermediate.[22] However, no competition studies with ETOH were presented, and therefore a direct oxidation of DMPO cannot be ruled out.

Previous work employing spin traps to characterize the reaction of Cr(VI) with reductants and hydrogen peroxide have implicated a Fenton-type mechanism for reaction of the Cr(V) formed during Cr(VI) reduction with hydrogen peroxide.[23−27] Our work with a distinct Cr(V) complex, in contrast to *in situ* generated Cr(V) complexes, supports this mechanism. However, due to the very low levels of endogenous hydrogen peroxide in cellular systems (10^{-7}−10^{-9} M),[28] it is likely that other mechanisms are required to explain the reactivity of high valent chromium in biological systems. It is possible that the direct oxidative mechanism of Cr(V) with biological molecules may be a more significant pathway under physiological conditions than previously considered.

Reactions of Cr(V) with DMPO in ^{17}O-labeled water show that the hydroxyl group in DMPO/•OH is likely to originate from water through nucleophilic addition to DMPO at physiological pH. In aqueous nonbuffered solutions, Makino *et al.*[29] and Hanna *et al.*[30] have shown that Fe(III) can form the DMPO/•OH adduct from DMPO. The mechanism proposed by Hanna *et al.*[30] involves Fe(III)-induced nucleophilic addition of water to DMPO resulting in formation of a hydroxylamine intermediate at the nitrone. The addition of buffers or Fe chelators resulted in a complete loss of the DMPO/•OH EPR signal, suggesting that coordination was a necessary step prior to

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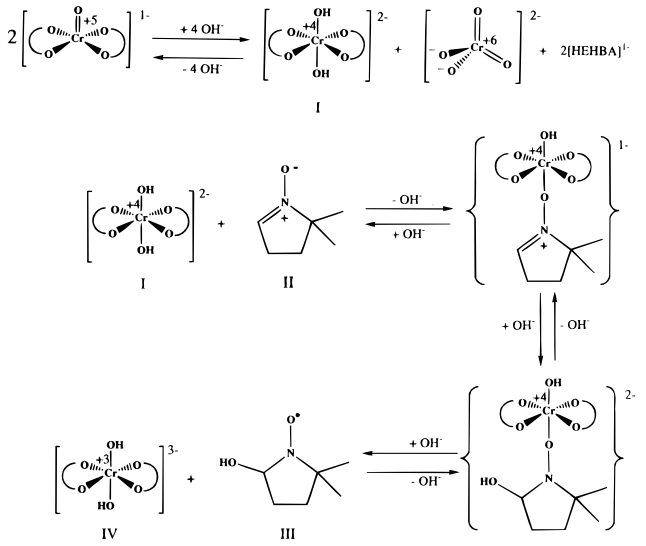
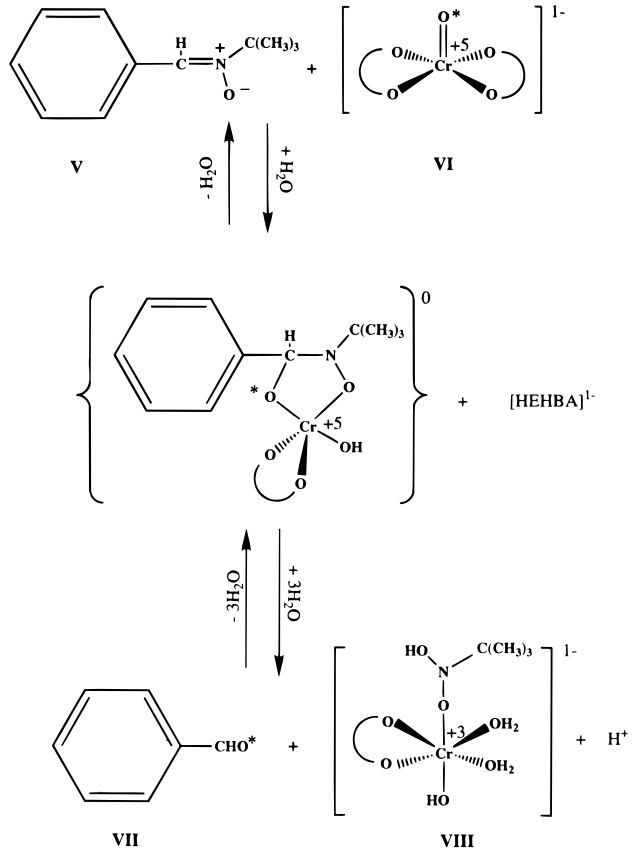
Scheme 1



nucleophilic addition of water. They postulated that the hydroxylamine could autooxidize to the DMPO/•OH adduct and this autooxidation was enhanced in the presence of Fe(III). The mechanism proposed by Makino *et al.*[29] is much the same, with the exception of the final step which involves a direct oxidation of DMPO by the Fe(III) to form Fe(II). With Cr(V) at physiological pH, the reaction mechanism is considered to be much the same as that described by Makino *et al*.[29] Because of the formation of the further reduced product, Cr(III), and the lack of a buffer effect, it seems likely that the mechanism involves coordination of chromium at the nitrone, nucleophilic attack of water at the β-position of DMPO and oxidation of the nitrone to the nitroxyl radical by homolytic bond cleavage as detailed in Scheme 1. The exact oxidation state of chromium that coordinates DMPO is unknown, although it is most certainly either Cr(V) or Cr(IV) formed during the disproportionation reaction since an aged solution containing both Cr(VI) and Cr(III) did not show any radical adducts. An argument for either monodentate or bidentate coordination of chromium to DMPO could be made, but on the basis of the work of Bramley *et al.*,[31,32] shifts in Cr(V) g values and A_{iso} values would be expected upon change in coordination type or number. Since no change in the g values, A_{iso} hyperfine, or super hyperfine splitting of the Cr(V) EPR signal was observed during these reactions, it is attractive to assign the coordinating species to the EPR silent Cr(IV) species **I** formed during disproportionation of Cr(V). A homolytic cleavage of the Cr(IV) bound DMPO/OH intermediate would then give both the stable Cr(III)−EHBA species, **IV**, and the radical DMPO/•OH, **III**. Assignment of the 6-coordinate Cr(IV) transition intermediate has precedence in the work of Ghosh and Gould.[33] Their work has shown that the bis(EHBA) complex of Cr(IV) both is 6-coordinate and has a high oxidation potential for the Cr(IV)/Cr(III) redox couple, 1.35 V.[17]

At lower pH's, no appreciable radical signals were observed via EPR under these conditions. However, the stability of the Cr(V) EPR signal was significantly reduced in the presence of spin traps. EPR ^{17}O-labeling studies under these conditions demonstrated rapid exchange equilibria for the oxo group of the Cr(V) species under these conditions. The result was similar

Scheme 2



to that of Groves and Haushalter for the Cr(V)-oxoporphinato species with hyperfine splittings of $A_{^{17}O} = 5.4$ G.[34] The rapid exchange was confirmed to be at the oxo-group of Cr(V) using IR spectroscopy and ^{18}O-labeled water. A 44 cm^{-1} shift in the Cr^{V}(=O) stretch was observed, which is identical with that shown for the [^{18}O]oxo group of the Cr^{V}−oxoporphinato species described by Groves and Kruper.[35] At low pH a nonradical oxidative pathway in the reaction of Cr(V) with spin traps appears to occur through Scheme 2. The mechanism involves first the exchange of an EHBA ligand from Cr(V) which would then allow coordination to PBN, **V**. Coordination through the nitrone of PBN would polarize the imine bond allowing formation of a five-membered chelate with the nucleophilic α-carbon of PBN and the oxo atom of Cr(V). The fact that no changes in A_{iso} hyperfine splittings or g values were observed between either the Cr(V) control or Cr(V) + PBN suggests that if an intermediate is formed it is unstable or below the limits of detection of the EPR. Oxo-atom transfer would then result in oxidative bond cleavage to form benzaldehyde, **VII**, and reduction of the Cr(V) to a Cr(III) species, **VIII**. Similar oxo-atom transfers with this Cr(V) species have been suggested by Rajavelu and Srinivasan for reactions of Cr(V)−EHBA with triphenylphospine in acetone and nitrite in acetic acid/acetate buffer to give the oxidation products triphenylphosphine oxide and nitrate, respectively.[36,37] It is possible that coordination with PBN may also enhance the normal Cr(V) decomposition pathway at low pH, *i.e.*, EHBA−ligand oxidation; this would account for the lack of stoichiometric benzaldehyde formation.

The versatility of the carboxylato-bound Cr(V) and Cr(IV) oxidation states to induce a wide variety of substrate oxidation

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reactions dependent on the type of substrate or conditions has been chronicled in a set of excellent reviews by Gould.[17,38]

In conclusion, the use of EPR spin traps in reactions with Cr(V) has allowed identification of two different pH-dependent oxidation mechanisms. Neither of these two mechanisms shows the involvement of a diffusible oxygen radical species such as the hydroxyl radical. It seems likely that direct oxidation mechanisms may predominate to generate the oxidative damage that is known to be associated with Cr(VI) genotoxicity. Further investigations are underway to determine if these mechanisms may account for oxidative damage with more biologically relevant substrates.

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Supporting Information Available: EPR spectra for the reaction of Cr(V) with DMPO and hydrogen peroxide at pH = 7.4 and EPR spectra showing the oxo-atom exchange of Cr(V) with 10% [^{17}O]water at pH = 4.0 (3 pages). Ordering information is given on any current masthead page.

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