EPR Spectroscopic Studies of the Reactions of Cr(VI) with L-Ascorbic Acid, L-Dehydroascorbic Acid, and 5,6-*O*-Isopropylidene-L-ascorbic Acid in Water.[1] Implications for Chromium(VI) Genotoxicity

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Abstract: The characterization of the Cr(V) intermediates in the reduction of Cr(VI) by L-ascorbic acid (L-AsA) in aqueous solution is described as a function of pH and ascorbate concentration. In the reaction of Cr(VI) with AsA at least seven EPR-active Cr(V) signals are present in addition to that of the ascorbate radical. The Cr(V)/ascorbate complex at $g_{iso} = 1.9791$ ($A_{iso} = 16.4 \times 10^{-4}$ cm^{-1}) is much more stable in moderately acidic solution (pH 3−6) than in neutral and alkaline solutions. The reaction is affected by aerial O_2 producing Cr(V)/ascorbate/peroxo complexes with signals at $g_{iso} = 1.9818$ ($A_{iso} = 13.2 \times 10^{-4}$ cm^{-1}) and $g_{iso} = 1.9812$ and another at $g_{iso} = 1.9824$ ($A_{iso} = 12.9 \times 10^{-4}$ cm^{-1}). These signals are most prominent at near physiological pH values (7−8), and all were absent when the solutions were preincubated with catalase. The Cr(V)/ascorbate complex is most stabilized in a [AsA]:[Cr(VI)] ratio of 1:2, while the Cr(V)/ascorbate/peroxo complexes reach their maximum signal intensities at [AsA]:[Cr(VI)] = 1:1. None of these Cr(V) species are stable at pH > 10, and they are reduced rapidly to Cr(III) at [AsA]:[Cr(VI)] ratios above 2:1. This is the first characterization of such Cr(V)/ascorbate/peroxo complexes. They arise from the reduction of O_2 during the aerial oxidation of AsA, and their identities were confirmed by studies on the reductions of Cr(VI) with AsA in the presence of H_2O_2. Their presence explains the O_2 sensitivity of *in vitro* DNA damage, and implications for Cr(VI)-induced cancers are considered. The reductions of Cr(VI) by dehydroascorbic acid (DHAA) and 5,6-*O*-isopropylidene-L-ascorbic acid (i-p-AsA), in the presence and absence of H_2O_2 were also studied using EPR spectroscopy. From these studies, likely solution structures for the Cr(V) complexes formed in the reduction of Cr(VI) by AsA were assigned.

Introduction

Cr(V) complexes have aroused considerable interest in recent years.[2,3] Such complexes have been used as dynamically-polarized proton and deuteron targets in high-energy physics experiments,[4,5] but much more attention has focused on their presence as intermediates in Cr(VI) oxidations and Cr(VI) carcinogenesis.[6−8] Chromate causes a variety of DNA damage, such as single strand breaks, alkali-labile sites and DNA−protein cross-links *in vivo* and in cultured cells. While Cr(VI) compounds do not react with isolated DNA *in vitro*,[9,10] evidence has been gathered that Cr(V) species, formed during the reduction of Cr(VI) by intracellular reductants and enzyme systems,[11,12] are active mutagens that induce biologically adverse effects of Cr(VI). This hypothesis has been proposed by many *in vivo* and *in vitro* studies.[13,14] Among the low molecular weight cellular constituents, AsA (or ascorbate),[15,16] glutathione (GSH),[17,18] and cysteine[19,20] react with Cr(VI) at a significant rate at physiological pH values. Ascorbate is the principal reductant of Cr(VI) in rat kidney, liver, and lung ultrafiltrates and cytosols, and mediates Cr−DNA binding *in vitro*.[21,22] Although the mechanism by which AsA reduces Cr(VI)

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(1) Abbreviations: EPR, electron paramagnetic resonance; AsA, ascorbic acid; DHAA, dehydroascorbic acid; GSH, glutathione (reduced); i-p-AsA, 5,6-*O*-isopropylidene-L-ascorbic acid; AP sites, apurinic/apyrimidinic sites; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; Tris·HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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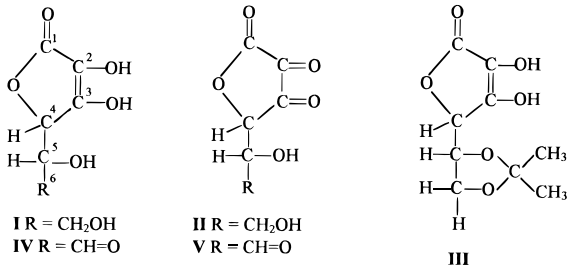
compounds is not fully understood, it is used as an "anti-chrome" agent for the treatment of chromate-poisoning[23] and the protection of inhalation of chromic acid mists.[24] Pretreatment of Chinese hamster V79 cells with AsA and chromate resulted in a decrease of DNA single strand breaks compared with those observed upon treatment of the cells with chromate alone.[25] EPR spectroscopy revealed decreased levels of Cr(V) species, but increased the levels of Cr(III) in these cells. It was deduced that the capability of altering the biological effects of carcinogenic Cr(VI) by AsA was possibly through its modification of the levels of paramagnetic Cr in cells.[26] Further evidence that AsA may be involved in the body's natural defense system against the toxicity of Cr(VI) compounds was indicated by the decrease in the levels of AsA in rat kidneys when rats were injected with $K_2Cr_2O_7$.[27] It has also been demonstrated that AsA protects against mutation and recombination induced by CrO_3 in somatic cells of *Drosophila*.[28] Because of these studies, whether the intracellular reduction of ascorbate aggravates or alleviates the toxicity and carcinogenesis of Cr(VI) remains a controversial issue.

AsA reduces Cr(VI) more rapidly than GSH when equimolar amounts of GSH and AsA are added to an excess of Cr(VI).[29] Recent studies[21] have shown that ascorbate accounted for ~80% of the Cr(VI) reductase activity of rat liver and kidney ultrafiltrates, while no more than 20% could be attributed to sulfhydryl-containing factors, including GSH. Paradoxically, until recently, the reduction of Cr(VI) by AsA or ascorbate has drawn much less attention than it deserves compared with the Cr(VI)−GSH system,[30−38] probably because of the relative instability of the Cr(V)/ascorbate species under the studied conditions[39] and the complexity of the redox processes.[20,40] As early as 1968, it was observed that AsA reduced Cr(VI) to Cr(III) with concomitant complex formation, but potential Cr(V) species produced in the reaction were not discussed.[41] The reaction of $Cr_2O_7^{2-}$ with AsA was first studied using EPR spectrometry at pH values close to those of biological relevance, and these revealed the presence of relatively stable Cr(V) species with a $t_{1/2}$ for decomposition of ~15 min.[39] More recent studies showed that, in addition to Cr(V) and ascorbate radical, Cr(IV) and other carbon-based radicals were detected by spin traps.[42]

Despite the renewed interest in the study of the reduction of Cr(VI) by AsA, there is considerable debate on the mechanism of the reaction and the identities of the species causing DNA damage. The reaction proceeds 10 times faster in deoxygenated media than in aerated media,[43] and an oxygen-activated species, capable of oxidizing formate ion, was postulated to be a Cr(V)−O_2^- complex, which was implicated as having a potential role in Cr(VI) carcinogenesis.[44,45] Since elevated levels of DNA single strand breaks occurred in an aerobic compared to an anaerobic system during the reduction of Cr(VI) with ascorbate, Cr(V)/ or Cr(IV)/peroxo/superoxo complexes were implicated as possible candidates as the DNA cleaving species.[46] Subsequently, AP sites were also shown to be caused by the same reactive species.[47] However, other researchers[42] discounted the involvement of reactive oxygen species as a major pathway in the reaction. In light of these contradictory reports, we have reinvestigated this reaction.

Here, we report our studies of the interaction of Cr(VI) with AsA (**I**) in aqueous solution over a much wider pH range, in order to better characterize the Cr(V) intermediates in the oxidation of AsA by Cr(VI) using empirical EPR methods.[38] The Cr(V) intermediates in the reactions of Cr(VI) with DHAA (**II**) and i-p-AsA (**III**) were also studied to help identify likely structures of Cr(V) intermediates.



I R = CH_2OH
IV R = CH=O

II R = CH_2OH
V R = CH=O

III

Experimental Section

Materials. L-(+)-AsA (Merck, min. 99.7%), L-DHAA (Aldrich), $Na_2Cr_2O_7 \cdot 2H_2O$ (Merck, 99.5%), $Na_2CrO_4 \cdot 4H_2O$ (Aldrich, 99%), $HClO_4$ (Merck, 70%), NaOH (Fluka, >98%), acetyl chloride (May & Baker, >99.%), H_2O_2 (Pacific, 20.4% w/w), Chelex 100 resin (Bio-Rad, Na^+ form, 100−200 mesh, AR grade), bovine liver catalase (Sigma, EC 1.11.1.6), Tris·HCl (Sigma, >99%), and Tris (Amresco, >99.8%) were used as received. The concentration of H_2O_2 was standardized using the method described in the literature.[48] ***Caution**: Cr(VI) is mutagenic and carcinogenic and the intermediates generated in the reduction of Cr(VI) by AsA are capable of cleaving DNA,[15,16] so appropriate precautions should be taken to avoid skin contact and dust inhalation while handling these chemicals.*

i-p-AsA was synthesized according to the literature method[49,50] from L-AsA (5.0 g), acetone (20 mL), and acetyl chloride (0.5 mL). ^1H and

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^{13}C NMR spectra were obtained in CD_3COCD_3. ^{1}H NMR (400 MHz): δ 0.90 (d, 3H), 0.91 (d, 3H), 3.60−3.63 (q, 1H), 3.77−3.81 (q, 1H), 3.95−3.97 (s, 1H), 4.32−4.33 (d, 1H). ^{13}C NMR (400 MHz): δ 26.16, 26.63, 66.41, 75.42, 75.87, 110.64, 120.38, 151.63, 170.48.

EPR Measurements. A Bruker ESP 300 spectrometer operating at ∼9.6 GHz was used for recording EPR spectra and for obtaining second-derivative spectra from solutions contained in a Wilmad quartz flat cell. The magnetic fields and microwave frequencies were measured with a Bruker ER 035M NMR gaussmeter and a Hewlett-Packard 5352B microwave frequency counter, respectively. Spectra were recorded 3 min after the solutions were mixed and were averaged over 20 scans with a data collection time of ∼140 s. EPR spectrometer settings: central field, 3480 G; sweep width, 100 G; microwave frequency, ∼9.6 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.968 G; receiver gain, 2×10^4; conversion time, 5.12 ms; time constant, 1.28 ms. The ratios of EPR signals remained constant when the power was changed, showing that saturation was not important.

To check for the effects of adventitious metal ions, experiments were performed where Chelex resin (5 g) was added to the Milli-Q water (100 mL) and the mixture was stirred overnight. An AsA solution (5 mM) was prepared using this water and was stirred with Chelex resin for a further 2 h. $Na_2Cr_2O_7$ solution (5 mM) was also prepared with the treated water, and both solutions (5 mL) were mixed and their EPR spectra recorded under conditions identical with those reported previously. For experiments performed in the absence of aerial O_2, stock solutions of $Na_2Cr_2O_7$ and AsA were degassed for 30 min with pure Ar. A flat quartz cell was filled with the solution under N_2 in a glovebox, and the cell was sealed before removing it and immediately recording the EPR spectrum. Except where otherwise stated, experiments were conducted at ∼20 °C in the absence of buffer solutions, which influence the reaction.[15,39]

An EPR spectrum-fitting program, e23new,[51,52] was used to estimate the spectral parameters using Lorentzian line shapes. Initial estimation of the concentrations and peak frequencies were obtained from the second-derivative spectra. The simulations did not take into account the contributions from the minor peaks that arise from the ^{53}Cr hyperfine coupling; however, the small errors that this introduces are comparable to the experimental errors involved in fitting procedures and can be ignored.

Results

Cr(V) Species in the $Na_2Cr_2O_7$/AsA Reaction. $Na_2Cr_2O_7$ (10 mM, 5 mL) and AsA (10 mM, 5 mL) were mixed, the pH was measured by an Activon Model 210 pH meter fitted with an Activon BJ 321 calomel pH electrode, and the solution EPR spectrum was taken 3 min after the solutions were mixed. Subsequent spectra were taken consecutively at 15-min intervals. Figure 1a shows the EPR spectrum of an aqueous solution of $Na_2Cr_2O_7$ and AsA in a 1:1 mole ratio at pH 6.0. The spectrum is dominated by a sharp signal with a g_{iso} value of 1.9791 and $\Delta H_{p-p} = 0.73 \times 10^{-4}$ cm^{-1} with its four clearly observed ^{53}Cr hyperfine coupling bands ($A_{iso} = 16.4 \times 10^{-4}$ cm^{-1}) in agreement with literature values ($g_{iso} = 1.979$, $A_{iso} = 16.4 \times 10^{-4}$ cm^{-1}).[39] During the first hour of the reaction, its signal intensity steadily increased and then slowly decreased (Figure S1 in the Supporting Information) and the solution pH decreased from 6.0 at 3 min to 5.6 at 138 min. Two hours after the reaction began, another signal at $g_{iso} = 1.9785$ was also detected as a shoulder due to a Cr(V) species that was remarkably stable since its signal could be observed 9 d later, although its intensity was substantially reduced. Two minor short-lived signals at $g_{iso} = 1.9818$ and $g_{iso} = 1.9824$ were also observed (Figure 1). Their signal intensities decreased from the beginning of the reaction and at a much higher rate than that of the $g_{iso} = 1.9791$ signal. After *ca.* 1.5 h, they could not be detected. However, these signals were not detected in solutions preincubated with catalase (0.04 mg mL^{-1} ascorbate solution, Figure S2 in the Supporting Information). The species giving rise to the $g_{iso} = 1.9824$ signal was favored to a greater extent in alkaline solution.

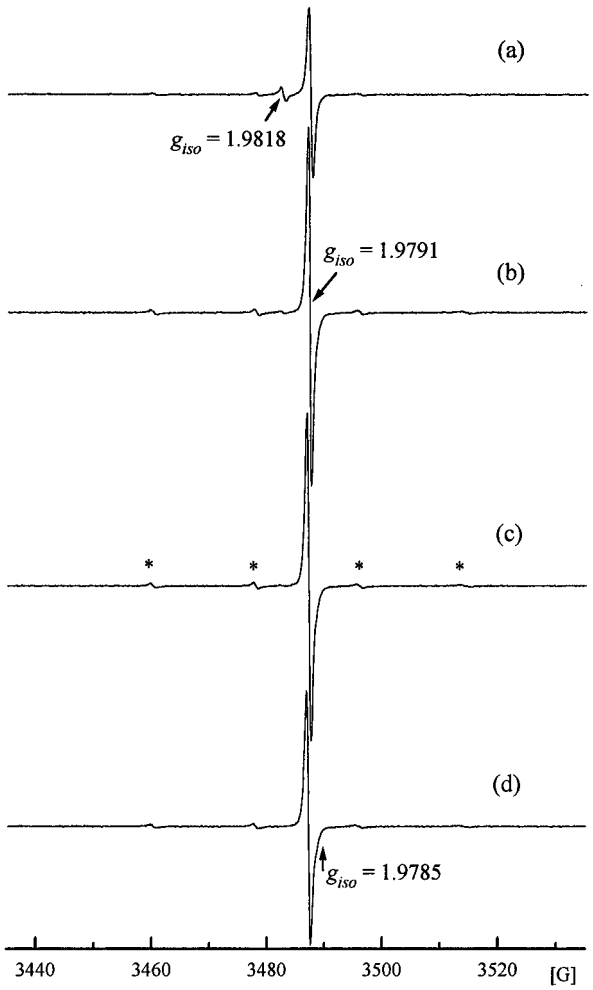

Figure 1. X-band EPR spectra of $Na_2Cr_2O_7$ (10 mM, 5 mL) and AsA (10 mM, 5 mL) ($T \approx 293$ K, operating frequency 9.6599 GHz): (a) $t = 3$ min, pH 6.0; (b) $t = 48$ min, pH 5.7; (c) $t = 93$ min, pH 5.7; (d) $t = 138$ min, pH 5.6. The ^{53}Cr hyperfine signals are shown as asterisks.

The treatment of the solutions with Chelex resin before the reaction had no effect on the EPR spectra, showing that the results were not effected by adventitious metal ions. Similar spectra were observed in Tris·HCl buffer (pH 7.4), with the exception of a further signal at $g_{iso} = 1.9765$, which increased with increased buffer strength with respect to the other signals.

Reaction of Na_2CrO_4 with AsA. In the reaction of Na_2CrO_4 (10 mM, 5 mL) with AsA (5 mM, 5 mL), seven Cr(V) EPR signals were detected at pH 8.4 (Figure 2a). The $g_{iso} = 1.9793$ signal was much less stable in alkaline solutions, as reflected in the lower signal intensity compared with that of the $g_{iso} = 1.9791$ signal formed in the reaction of $Cr_2O_7^{2-}$ with AsA. There is another signal at $g_{iso} = 1.9788$. The $g_{iso} = 1.9824$ signal was more evident, with its intensity being nearly equal to that of the $g_{iso} = 1.9820$ signal. The g_{iso} values of these species changed slightly with a change of the pH of the

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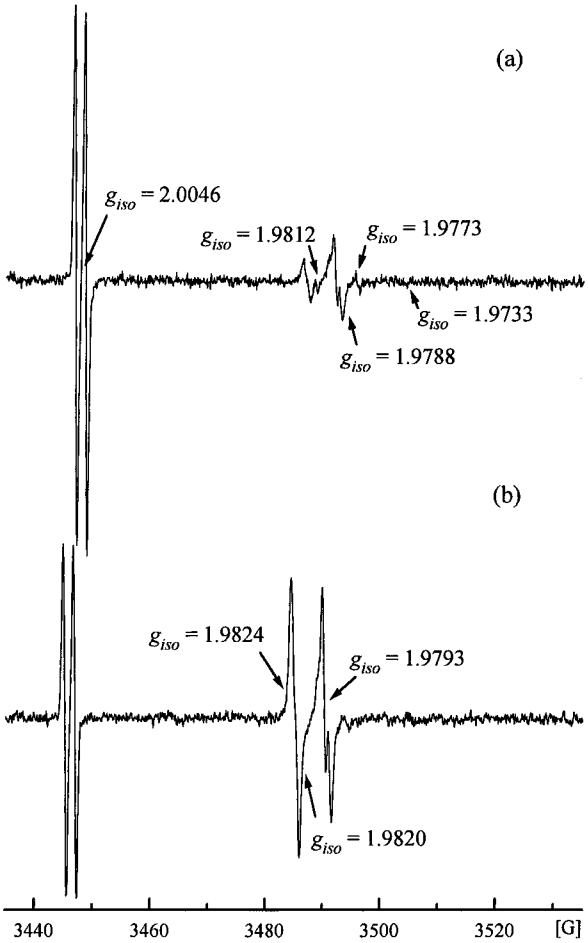

Figure 2. X-band EPR spectra of Na_2CrO_4 (10 mM, 5 mL) and AsA (5 mM, 5 mL): (a) freshly-prepared solution (pH 8.4, operating frequency 9.6742 GHz); (b) solutions allowed to stand for 3 h before the reaction was commenced (pH 8.2, operating frequency 9.6692 GHz, $T \approx 293$ K).

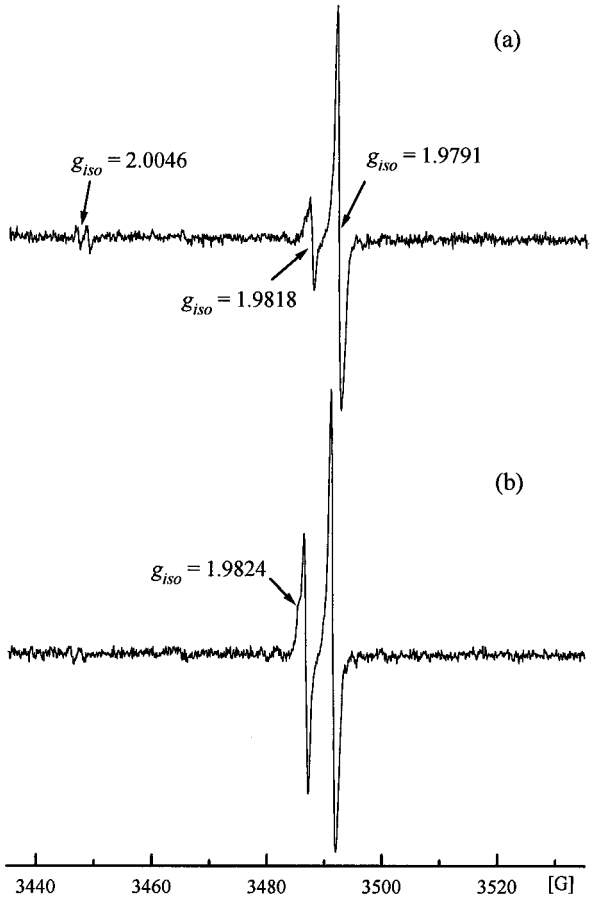

Figure 3. X-band EPR spectra of $Na_2Cr_2O_7$ (5 mM, 5 mL) and AsA (10 mM, 5 mL) (3 min after mixing, $T \approx 293$ K): (a) fresh solution (pH 6.9, $\nu = 9.6741$ GHz); (b) solution allowed to stand in the air for 3 h (pH 6.8, $\nu = 9.6719$ GHz).

solution, as will be detailed later. Three other transient signals with g_{iso} values of 1.9812, 1.9773, and 1.9733 were also observed, as well as that of the ascorbate radical ($g_{iso} = 2.0046$, $A_{iso}(^1H) = 1.61 \times 10^{-4}$ cm^{-1})[53] during the first hour of the reaction. When the solutions were exposed to air for 3 h before the reaction, the intensities of most of the Cr(V) signals increased dramatically, except that due to the ascorbate radical, which was reduced (Figure 2b).

O_2 Participation in the Reaction of Cr(VI) with AsA. Reactions of Cr(VI) with aged AsA solution gave a higher intensity for the signal at $g_{iso} = 1.9818$ than with fresh solutions. This indicated that this signal was caused by the aerial oxidation of AsA in solution. In order to clarify the identity of the species responsible for this signal, $Na_2Cr_2O_7$ and AsA solutions were exposed to the air for 0, 1, 2, and 3 h before mixing. The longer the AsA solutions were exposed to air prior to their reactions with Cr(VI), the stronger were the relative signal intensities due to these Cr(V) species. Not only did the intensity for the signal at $g_{iso} = 1.9818$ increase, but those of the $g_{iso} = 1.9791$ and $g_{iso} = 1.9824$ signals were also enhanced (Figure S3 in the Supporting Information and Figure 3).

In order to establish that the species giving rise to the signals at $g_{iso} = 1.9818$ and $g_{iso} = 1.9824$ were caused by products of the aerial oxidation of AsA, the reaction of $Na_2Cr_2O_7$ and AsA in the absence of O_2 was also studied. These signals were not detected in the reactions in anoxic conditions, which contrasts sharply with their presence in aerated media (Figure 4). The signal intensity due to the Cr(V)/ascorbate complex at $g_{iso} = 1.9791$ produced in the reaction of Cr(VI) with AsA in the absence of O_2, is lower than in its presence.

Reaction of Cr(VI) with AsA in the Presence of H_2O_2. H_2O_2 and O_2^- are formed in the aerial oxidation of AsA,[54,55] which prompted us to study the reaction of Cr(VI) with AsA in the presence of H_2O_2. $Na_2Cr_2O_7$ (5 mM, 5 mL) with H_2O_2 (4.8 mM, 5 mL) produced three Cr(V) EPR signals at $g_{iso} = 1.9798$, 1.9764, and 1.9724 at pH 5.1 (Figure 5a).[56] In the reaction of H_2O_2 (4.8 mM, 5 mL) with AsA (5 mM, 5 mL) only the ascorbate radical was observed (Figure 5b), but the reaction of $Na_2Cr_2O_7$ (5 mM, 5 mL) with an aqueous solution of 5 mM AsA and 2.4 mM H_2O_2 (5 mL) generated five EPR signals at $g_{iso} = 1.9818$, 1.9798, 1.9791, 1.9764, and 1.9724 (pH 5.5, Figure 5c). The intensity of the $g_{iso} = 1.9791$ signal was lower than that observed in the absence of H_2O_2. While the $g_{iso} = 1.9818$ signal quickly diminished, the $g_{iso} = 1.9791$ signal gradually increased in intensity with time. The intensity

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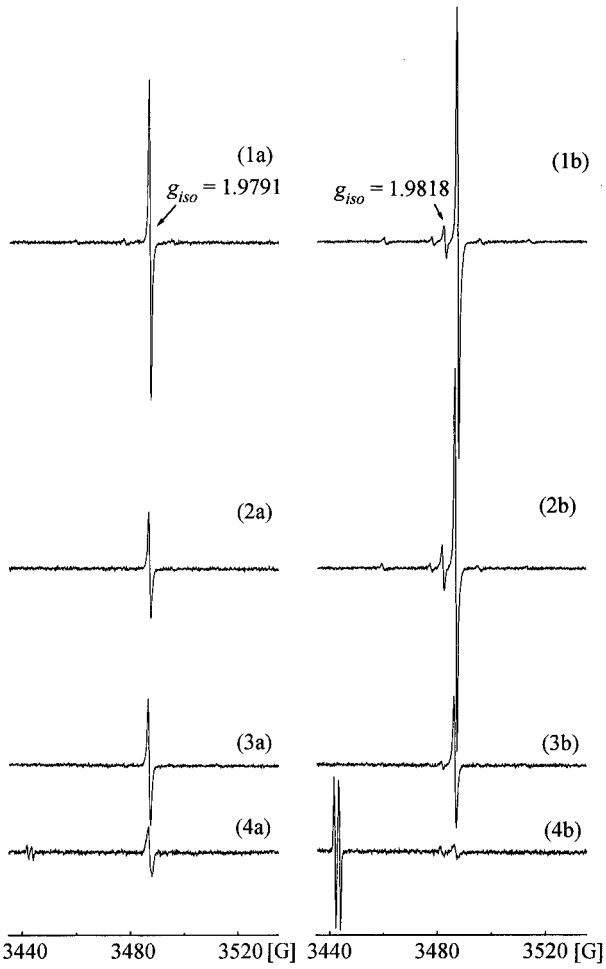

Figure 4. Comparisons among the X-band EPR spectra of $Na_2Cr_2O_7$ and AsA: (a) anoxic reactions; (b) reactions exposed to air. The spectra were collected 9 min after mixing at $T \approx 293$ K. (1) $Na_2Cr_2O_7$ (5 mM, 5 mL), AsA (5 mM, 5 mL) (pH 5.5; (1a) $\nu = 9.6596$ GHz; (1b) $\nu = 9.6607$ GHz); (2) $Na_2Cr_2O_7$ (5 mM, 5 mL), AsA (5 mM, 10 mL) (pH 5.6; (2a) $\nu = 9.6598$ GHz; (2b) $\nu = 9.6590$ GHz); (3) $Na_2Cr_2O_7$ (5 mM, 5 mL), AsA (10 mM, 5 mL) (pH 6.4; (3a) $\nu = 9.6595$ GHz; (3b) $\nu = 9.6586$ GHz); (4) Na_2CrO_4 (10 mM, 5 mL), AsA (10 mM, 5 mL) (pH 8.6; (4a) $\nu = 9.6712$ GHz; (4b) $\nu = 9.6596$ GHz).

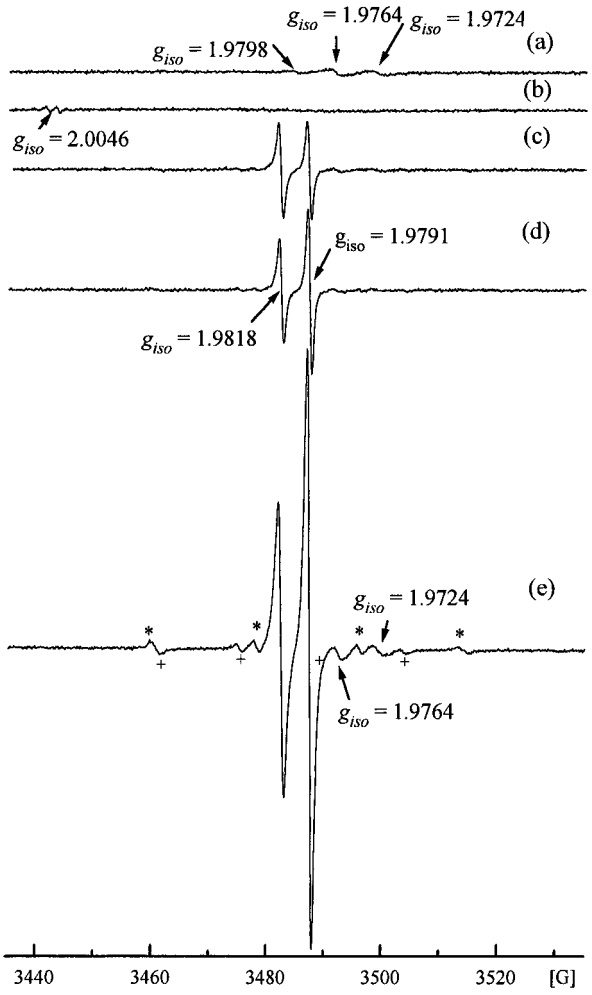

Figure 5. X-band EPR spectra of (a) $Na_2Cr_2O_7$ (5 mM, 5 mL) and H_2O_2 (4.8 mM, 5 mL) (pH 5.1, $\nu = 9.6591$ GHz); (b) AsA (5 mM, 5 mL) and H_2O_2 (4.8 mM, 5 mL) (pH 3.6, $\nu = 9.6594$ GHz); (c) $Na_2Cr_2O_7$ (5 mM, 5 mL) and 5 mL of a solution containing 5 mM AsA and 2.4 mM H_2O_2 (pH 5.5, $\nu = 9.6588$ GHz); (d) $Na_2Cr_2O_7$ (5 mM, 5 mL) and 5 mL of a solution containing 5 mM AsA and 4.8 mM H_2O_2 (pH 5.4, $\nu = 9.6600$ GHz); (e) $Na_2Cr_2O_7$ (10 mM, 5 mL) and 5 mL of a solution containing 10 mM AsA and 9.6 mM H_2O_2 (pH 5.3, $\nu = 9.6601$ GHz). All spectra were taken 3 min after mixing at $T \approx 293$ K. The hyperfine coupling signals for the $g_{iso} = 1.9791$ signal are shown as asterisks, and the plus symbols indicate those for the $g_{iso} = 1.9818$ signal.

of the $g_{iso} = 1.9818$ signal increased with increasing $[H_2O_2]$. Increasing the concentrations of the reactants (10 mM) gave an EPR spectrum of higher signal intensity from which the A_{iso} value was determined (13.2×10^{-4} cm^{-1}, Figure 5e). The species giving rise to the signal at $g_{iso} = 1.9824$ was not evident under these conditions, but became more conspicuous when the reaction took place in alkaline solution, e.g., when Na_2CrO_4 (10 mM, 5 mL) was reacted with a 5-mL solution containing 5 mM AsA and 4.8 mM H_2O_2 at pH 7.1 (Figure 6). This signal degraded faster than the signal at $g_{iso} = 1.9818$.

pH Dependence of Cr(V)/Ascorbate Species. $Na_2Cr_2O_7$ (5 mM, 5 mL) and AsA (5 mM, 5 mL) were mixed, the pH of the resultant solution was adjusted by the addition of 0.5 M NaOH or 0.5 M $HClO_4$, and the EPR spectra were recorded at 3 min after mixing. In order to minimize the effect of O_2 on the reaction, all of the solutions were prepared immediately before mixing. The nature of the Cr(V) species formed was very dependent on the pH of the solution (Figure 7). Much more intense signals and a longer lifetime for the $g_{iso} = 1.9791$ signal were observed in moderately acidic solutions than in neutral and alkaline media. Increasing the pH resulted in a marked decrease in intensity for this signal, ΔH_{p-p} increases, and the g_{iso} and A_{iso} values also changed. In alkaline solutions, the g_{iso} value increased and the A_{iso} values decreased for the signal at ~1.979 with increasing pH values (Table S2). At pH 10, the $g_{iso} \approx 1.979$ signal was only observed during the first few minutes of the reaction. At pH values above 11, no Cr(V) signal was detected. The $g_{iso} = 1.9785$ signal was best observed in pH 3−5; its g_{iso} value also changed from 1.9785 at pH 4 to 1.9788 at pH 8. Ascorbate radical was observed at pH values of 8−10.

The signal at $g_{iso} = 1.9818$ became detectable at pH ≈ 5, and its intensity increased with increasing pH. The signal at $g_{iso} = 1.9824$ began separating from that at $g_{iso} = 1.9818$ at pH ≈ 7, and its intensity also increased with increasing pH, until at pH ≈ 8, it was nearly equal to that of the $g_{iso} = 1.9818$ signal. At pH values above 8.5, the intensities of both signals decreased, and they were barely detectable at pH 10. The signal at $g_{iso} = 1.9818$ shifts to higher g values with increasing pH. The g_{iso} and A_{iso} values of the signal at $g_{iso} = 1.9824$ were less pH dependent than those of the $g_{iso} = 1.9791$ and $g_{iso} = 1.9818$ signals.

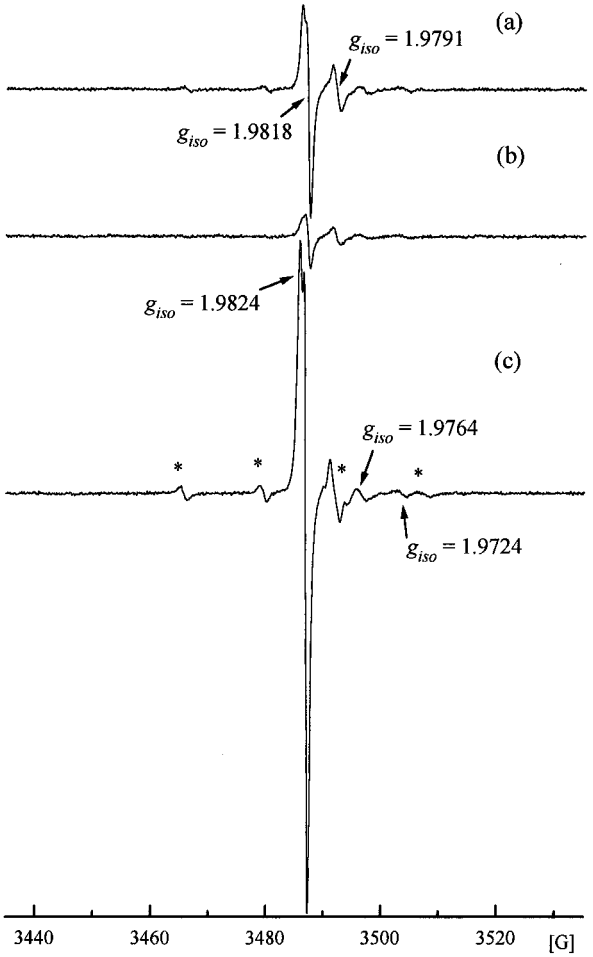

Figure 6. X-band EPR spectra of Na_2CrO_4 (10 mM, 5 mL) and AsA (5 mM, 5 mL) in the presence of H_2O_2 ($T \approx 293$ K): (a) $[H_2O_2] = 2.4$ mM, pH 7.1, $\nu = 9.6732$ GHz, 3 min after the reaction was commenced; (b) $[H_2O_2] = 2.4$ mM, pH 6.9, $\nu = 9.6732$ GHz, 10 min after the reaction was commenced; (c) $[H_2O_2] = 4.8$ mM, pH 7.1, $\nu = 9.6723$ GHz, 3 min after the reaction was commenced. The hyperfine coupling signals for the $g_{iso} = 1.9818$ signal are shown by asterisks.

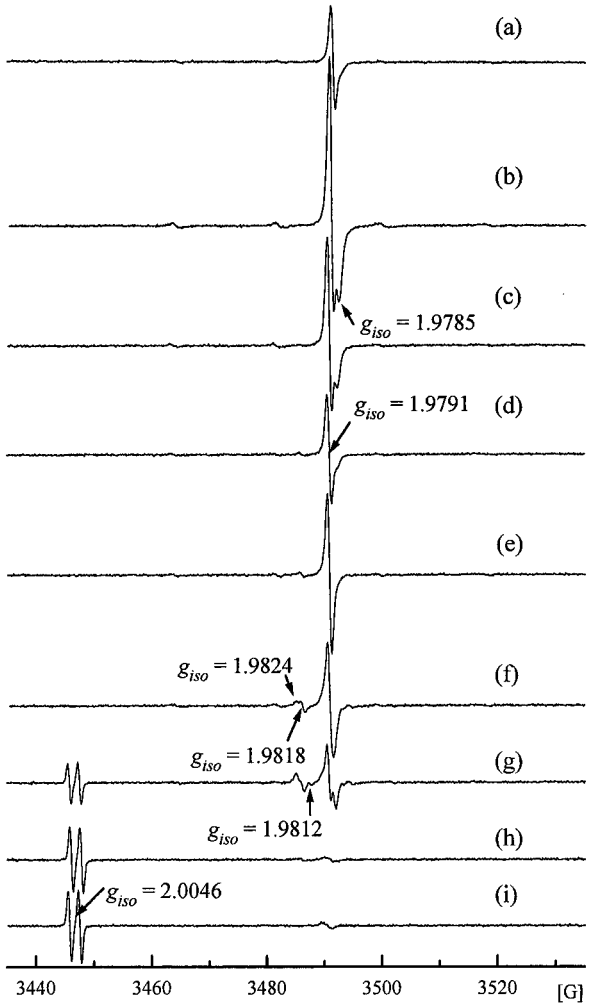

Figure 7. pH dependence of the X-band EPR spectra of $Na_2Cr_2O_7$ (5 mM, 5 mL) and AsA (5 mM, 5 mL) (3 min after mixing, $T \approx 293$ K): (a) pH 2, $\nu = 9.6726$ GHz; (b) pH 3, $\nu = 9.6695$ GHz; (c) pH 4, $\nu = 9.6696$ GHz; (d) pH 5, $\nu = 9.6713$ GHz; (e) pH 6, $\nu = 9.6702$ GHz; (f) pH 7, $\nu = 9.6697$ GHz; (g) pH 8, $\nu = 9.6719$ GHz; (h) pH 9, $\nu = 9.6728$ GHz; (i) pH 10, $\nu = 9.6708$ GHz.

Reactant Ratio Dependence of the Cr(V)/Ascorbate Complexes. When freshly-prepared $Na_2Cr_2O_7$ (5 mM, 5 mL) and AsA (5 mL) solutions were mixed, the distribution of Cr(V) complexes after 3 min was dependent on the relative mole ratio of reactants, AsA:Cr(VI) (Figure 8). Increasing the [AsA]:[Cr(VI)] ratio from 1:4 to 1:2 resulted in an increase in intensity of the $g_{iso} = 1.9791$ signal relative to the $g_{iso} = 1.9818$ signal. The former signal was at its highest intensity when the mole ratio was 1:2. On further increasing the mole ratio to 3:4, the signal intensity at $g_{iso} = 1.9791$ was reduced, while that of the signal at $g_{iso} = 1.9818$ was increased, and this signal was at its highest intensity when the [AsA]:[Cr(VI)] ratio was 1:1. When the [AsA]:[Cr(VI)] ratio was increased to 3:2, the signal at $g_{iso} = 1.9791$ was observed only in the very early stages of the reaction, and the signal at $g_{iso} = 1.9818$ was scarcely observed. Only the ascorbate radical was observed in the EPR spectrum of the reaction of $Na_2Cr_2O_7$ and AsA in a [AsA]:[Cr(VI)] ratio of 2:1.

Reaction of Cr(IV) with DHAA. The reaction of $Na_2Cr_2O_7$ (5 mM, 5 mL) with DHAA (10 mM, 5 mL) at pH 3.5 resulted in four EPR signals (Figure 9). The signal intensity at $g_{iso} = 1.9791$ ($A_{iso} = 16.4 \times 10^{-4}$ cm^{-1}) increased during the first 2 h of the reaction, while the signal at $g_{iso} = 1.9785$ ($A_{iso}(^{53}Cr) = 16.6 \times 10^{-4}$ cm^{-1}) had a ^{1}H superhyperfine structure due to three inequivalent protons ($A_{iso} = 0.96 \times 10^{-4}$, 0.85×10^{-4}, and 0.30×10^{-4} cm^{-1}), and its intensity decreased with time. Unlike the Cr(V) species in AsA solution, the Cr(V) intermediates generated in the reaction of Cr(VI) and DHAA were stable at higher concentrations of DHAA and their signal intensities increased proportionally with increasing [DHAA] (Figure 10). Three minor signals were observed at $g_{iso} = 1.9772$, 1.9727, and 1.9718, which became more evident at higher [DHAA]. The signal with $g_{iso} = 1.9772$ overlapped with that at $g_{iso} = 1.9785$. The $g_{iso} = 1.9727$ signal overlapped with a ^{53}Cr hyperfine signal of the main signal. A comparison of the observed and simulated spectra obtained from the reaction of $Na_2Cr_2O_7$ (5 mM, 5 mL) and DHAA (20 mM, 5 mL) is shown in Figure 11. In the reaction of Na_2CrO_4 (10 mM, 5 mL) with DHAA (5 mM, 5 mL) at pH 6.6, only a singlet was observed at $g_{iso} = 1.9791$ (Figure 12).

Cr(VI)/DHAA/H_2O_2 Reaction. $Na_2Cr_2O_7$ (5 mM, 5 mL) was reacted with DHAA (20 mM, 2.5 mL), and H_2O_2 (9.6 mM, 2.5 mL) was added. Besides the signals observed in the Cr(VI)/DHAA reaction at $g_{iso} = 1.9791$ and 1.9785, new signals at $g_{iso} = 1.9818$ and 1.9777 were present at pH 3.6 (Figure 13). The signal intensity at $g_{iso} = 1.9818$ quickly diminished, and that at $g_{iso} = 1.9791$ increased with time.

Cr(VI)/i-p-AsA Reaction. $Na_2Cr_2O_7$ (5 mM, 5 mL) was reacted with i-p-AsA (5 mM, 5 mL) at pH 6.2 to produce three EPR signals (Figure S4 in the Supporting Information). One

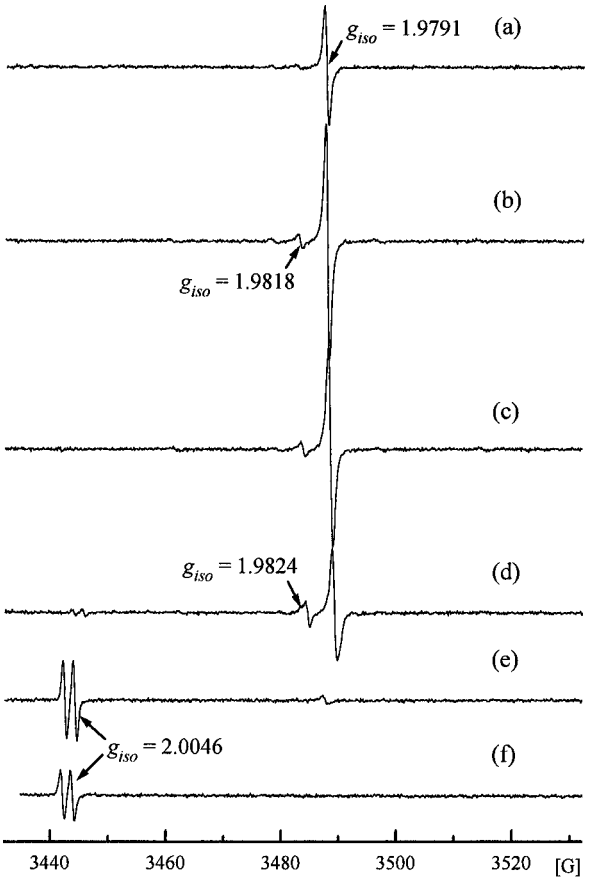

Figure 8. X-band EPR spectra of $Na_2Cr_2O_7$ (5 mM, 5 mL) and AsA (5 mL) at different [AsA] ($T \approx 293$ K): (a) [AsA] = 2.5 mM, pH 5.5, ν = 9.6689 GHz; (b) [AsA] = 5 mM, pH 5.7, ν = 9.6698 GHz; (c) [AsA] = 7.5 mM, pH 5.9, ν = 9.6717 GHz; (d) [AsA] = 10 mM, pH 6.0, ν = 9.6741 GHz; (e) [AsA] = 15 mM, pH 6.0, ν = 9.6698 GHz; (f) [AsA] = 20 mM, pH 6.1, ν = 9.6601 GHz.

signal had the same g_{iso} and A_{iso} values as that of Cr(V) species formed in the reaction of $Na_2Cr_2O_7$ with AsA (g_{iso} = 1.9791, $A_{iso} = 16.4 \times 10^{-4}$ cm^{-1}). The intensities of the other two signals at g_{iso} = 1.9818 and g_{iso} = 1.9824 increased with increasing [i-p-AsA]:[Cr(VI)] ratio from 1:2 to 1:1, while the g_{iso} = 1.9791 signal intensity was reduced. At the ratio [i-p-AsA]:[Cr(VI)] of 2:1, the signal intensities of all of the Cr(V) complexes were weak (Figure 14). The i-p-AsA radical was also observed when i-p-AsA was reacted with Cr(VI) in a ratio of 1:1 and 2:1. Its g_{iso} and A_{iso} values were the same as those of the ascorbate radical. Two other signals at g_{iso} = 1.9773 and g_{iso} = 1.9733 were observed in the reaction of Na_2CrO_4 (10 mM, 5 mL) with i-p-AsA (5 mM, 5 mL) at pH 8.0 (Figure 15).

The different Cr(V) EPR-active signals observed in the reactions of Cr(VI) with AsA, DHAA, and i-p-AsA are summarized in Table 1.

Discussion

Cr(VI)/AsA Reaction. Generally, it is accepted that the reaction of Cr(VI) with AsA involves a 3-electron reduction of Cr(VI) to form Cr(III) and DHAA:[42,57−59]

$$2\text{Cr(VI)} + 3\mathbf{I} \rightarrow 2\text{Cr(III)} + 3\mathbf{II} \quad (1)$$

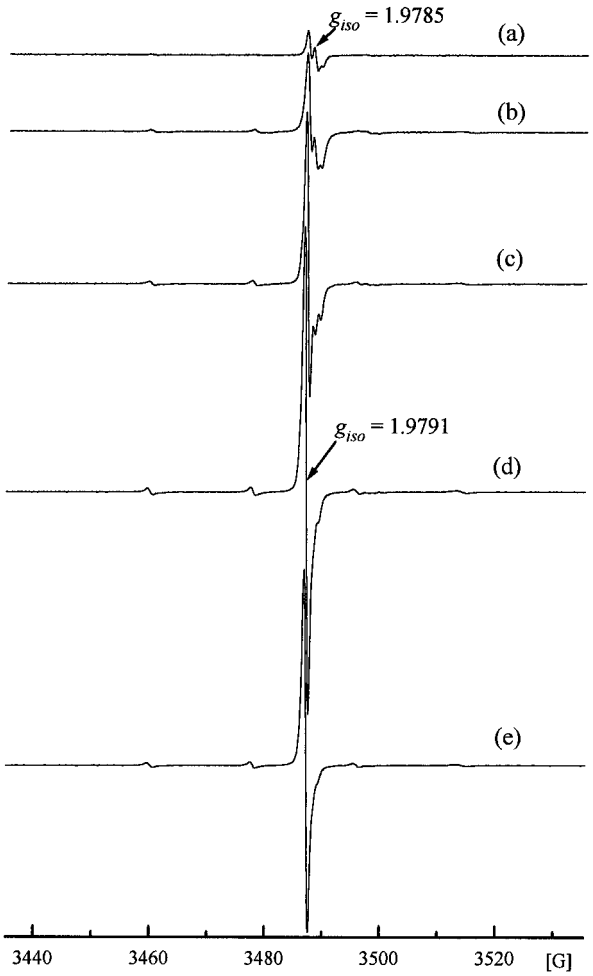

Figure 9. X-band EPR spectra of $Na_2Cr_2O_7$ (5 mM, 5 mL) and DHAA (10 mM, 5 mL) ($T \approx 293$ K): (a) t = 3 min, pH 3.2, ν = 9.6595 GHz; (b) t = 6 min, pH 3.1, ν = 9.6594 GHz; (c) t = 15 min, pH 3.1, ν = 9.6595 GHz; (d) t = 60 min, pH 3.1, ν = 9.6596 GHz; (e) t = 120 min, pH 3.4, ν = 9.6597 GHz.

The first step forms Cr(IV) and **II** via a Cr(VI)−ester intermediate (eq 2).[20] Cr(IV) reacts with another Cr(VI) to form Cr(V) (eq 3).

$$\mathbf{I} + \text{Cr(VI)} \rightarrow \mathbf{II} + \text{Cr(IV)} + 2\text{H}^+ \quad (2)$$

$$\text{Cr(IV)} + \text{Cr(VI)} \rightarrow 2\text{Cr(V)} \quad (3)$$

The EPR study shows the presence of many Cr(V) intermediates involving binding of hydroxylate groups on the lactone ring and the side chain of the ligand. Cr(VI) can attack the hydroxyl group on the C-6 to form Cr(IV) and 6-aldehydoascorbic acid (**IV**) and subsequently Cr(V) (eqs 3 and 4). The C-6 oxidation of AsA was shown by labeling studies during the metabolic process of AsA in *Pelargonium crispum*.[60]

$$\mathbf{I} + \text{Cr(VI)} \rightarrow \mathbf{IV} + \text{Cr(IV)} + 2\text{H}^+ \quad (4)$$

When Cr(VI) is in excess, it is also probable that two Cr(VI) species react with one AsA molecule; one reacts with the lactone ring and the other reacts with the side chain, ultimately forming

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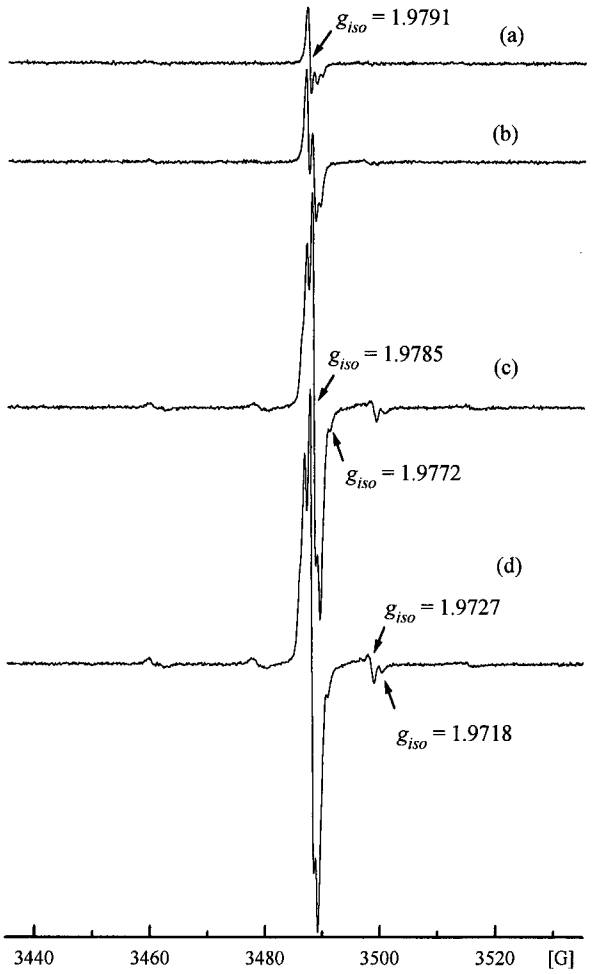


Figure 10. X-band EPR spectra of $Na_2Cr_2O_7$ (5 mM, 5 mL) and DHAA (5 mL) at different [DHAA] ($T \approx 293$ K, 3 min after mixing): (a) [DHAA] = 5 mM, pH 3.5, $\nu = 9.6599$ GHz; (b) [DHAA] = 10 mM, pH 3.3, $\nu = 9.6595$ GHz; (c) [DHAA] = 20 mM, pH 2.7, $\nu = 9.6597$ GHz; (d) [DHAA] = 30 mM, pH 2.6, $\nu = 9.6595$ GHz.

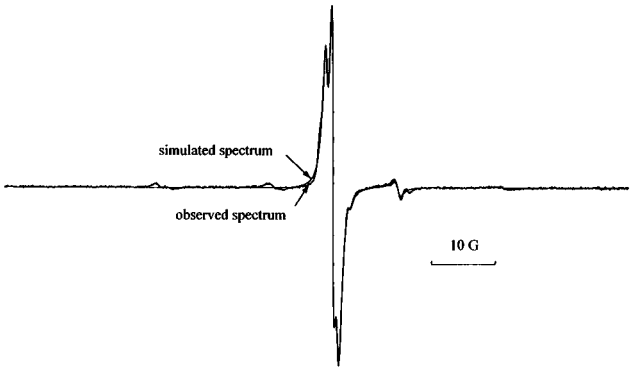


Figure 11. Simulated and observed X-band EPR spectra for the Cr(V) species produced in the reaction of $Na_2Cr_2O_7$ (5 mM, 5 mL) and DHAA (20 mM, 5 mL) at pH 2.7 ($T \approx 293$ K, 3 min after mixing). The spectrum was simulated with four signals at g_{iso} values of 1.9791, 1.9785, 1.9772, and 1.9727 and relative intensities of 840.2, 1939.0, 22.15, and 104.8, respectively. The signal at $g_{iso} = 1.9785$ exhibited ^{1}H superhyperfine coupling to three different protons ($A_{iso} = 1.038$, 0.916, and 0.326 G). As the time or ratio of reactants changed, the relative intensities changed, but the parameters remained approximately constant.

Cr(V) intermediates and 6-aldehydodehydroascorbic acid (**V**).

$$\mathbf{I} + 2\mathrm{Cr(VI)} \rightarrow \mathbf{V} + 2\mathrm{Cr(IV)} + 2\mathrm{H}^+ \qquad (5)$$

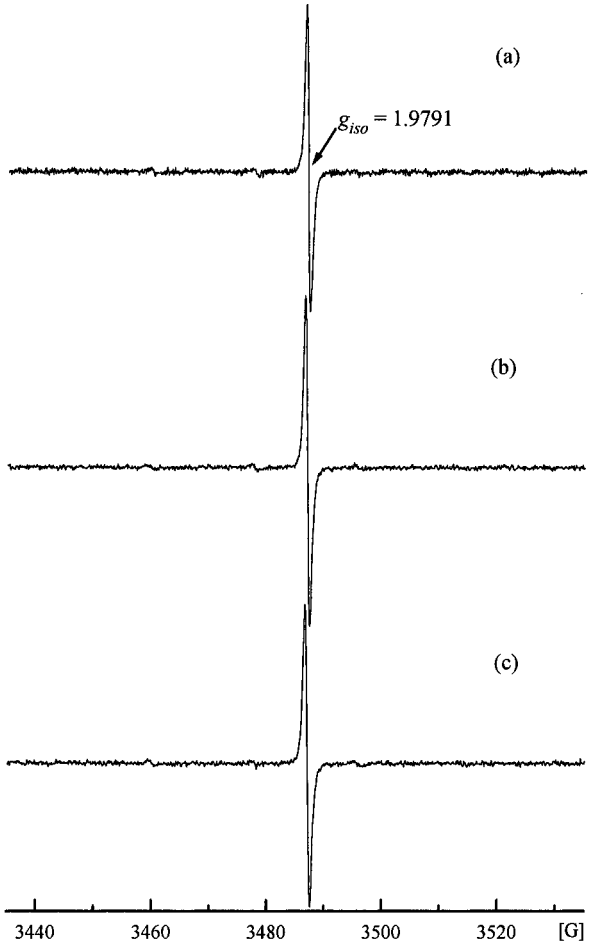


Figure 12. X-band EPR spectra of Na_2CrO_4 (10 mM, 5 mL) and DHAA (5 mM, 5 mL) ($T \approx 293$ K): (a) $t = 3$ min, pH 6.6, $\nu = 9.6597$ GHz; (b) $t = 15$ min, pH 6.6, $\nu = 9.6597$ GHz; (c) $t = 30$ min, pH 6.5, $\nu = 9.6597$ GHz.

Cr(V) generated in these reactions will coordinate AsA, or oxidation products, to form transient Cr(V) complexes that give rise to the range of Cr(V) EPR signals observed prior to undergoing intramolecular 2-electron redox reactions to form Cr(III) and oxidized ligands. When AsA is in excess, Cr(IV) reacts directly with more AsA to form ascorbate radical and Cr(III). This explains why the major EPR signal is due to this radical with excess ascorbate, whereas its intensity is weak under other conditions.

***g*$_{iso}$ = 1.9791 and *g*$_{iso}$ = 1.9785 Signals.** The coordination modes were ascertained by a combination of the presence or absence of ^{1}H superhyperfine coupling and reactions with DHAA and i-p-AsA, and the g_{iso} values that are typical of five-coordinate Cr(V) complexes.[38] Cr(V) can react with another AsA molecule at either the lactone ring or the side chain, forming **VI** and **VII**, respectively. It is also likely that two Cr(V) species can bind with one AsA molecule, one coordinating with the oxygen atoms on C-2 and C-3 and the other with the oxygen atoms on C-5 and C-6, resulting in the formation of a Cr(V) dimer, **VIII**. Cr(V) may also coordinate with the oxidative products **II** and **IV**, forming **IX** and **X**. The other ligands bound to Cr(V) have been assigned as hydroxo groups on the basis of an EXAFS structure of a Cr(V)/ascorbate complex isolated from methanolic solution. This species has EPR characteristics similar to those of species **VI** reported here

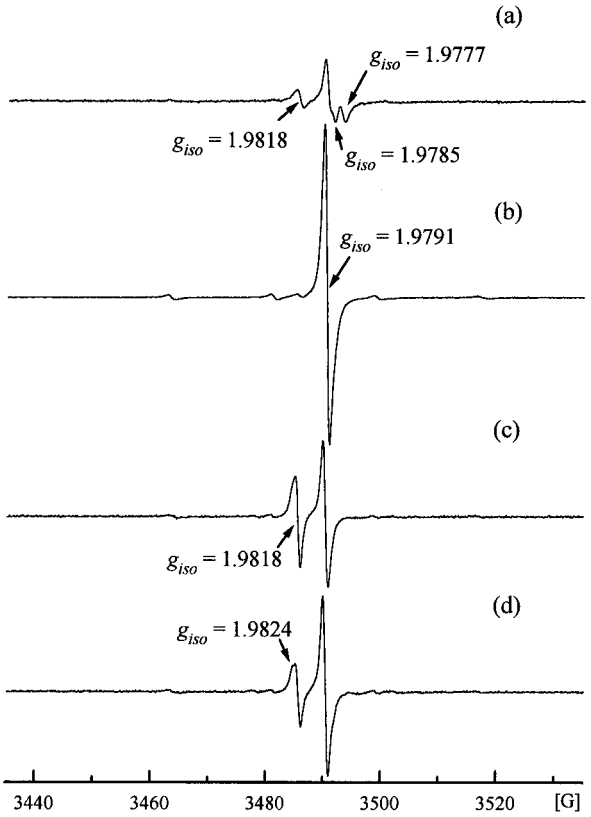
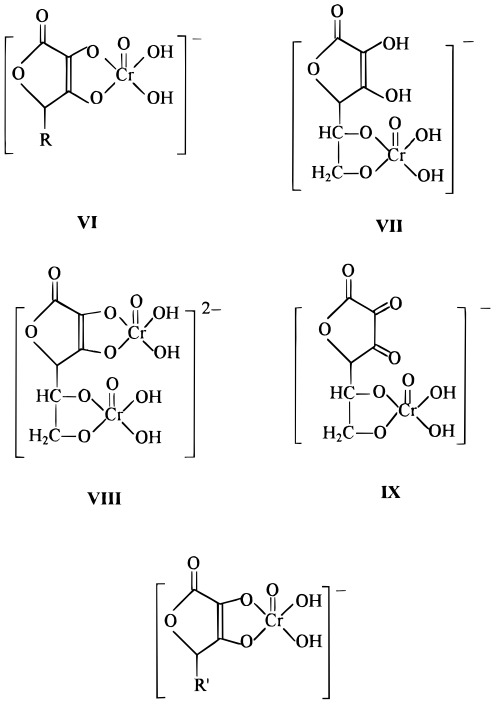


Figure 13. X-band EPR spectra of Cr(VI) and DHAA in the presence of H_2O_2 ($T \approx 293$ K): (a) $Na_2Cr_2O_7$ (5 mM, 5 mL) with DHAA (10 mM, 2.5 mL) and H_2O_2 (9.6 mM, 2.5 mL) ($t = 3$ min, pH 3.6, $\nu =$ 9.6691 GHz); (b) same as (a) ($t = 15$ min, pH 3.7, $\nu = 9.6692$ GHz); (c) Na_2CrO_4 (10 mM, 5 mL) with DHAA (10 mM, 2.5 mL) and H_2O_2 (9.6 mM, 2.5 mL) ($t = 3$ min, pH 6.7, $\nu = 9.6689$ GHz); (d) same as (c) ($t = 15$ min, pH 6.8, $\nu = 9.6697$ GHz).

when dissolved in aqueous solution.[61]



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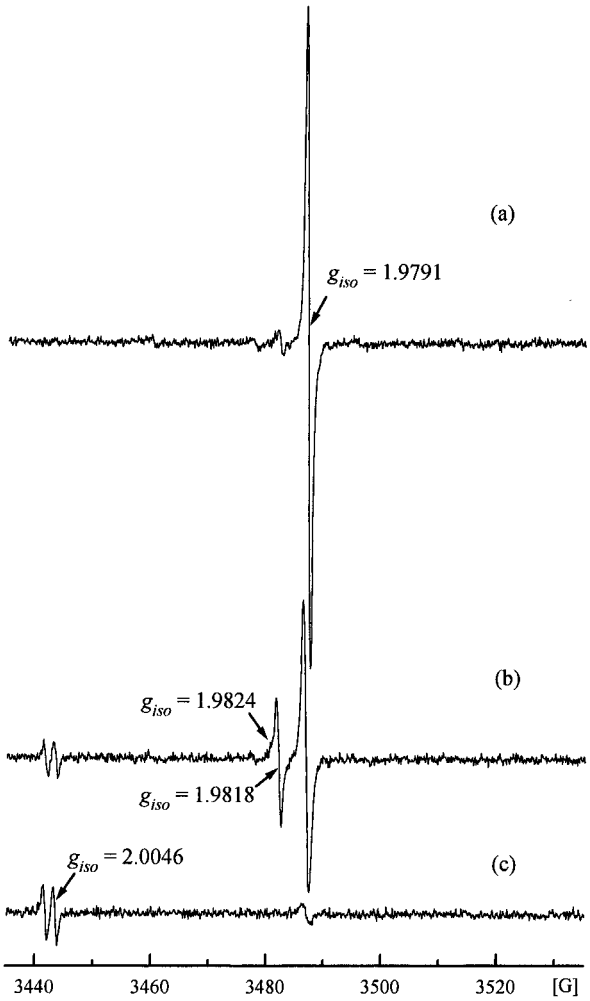


Figure 14. X-band EPR spectra of $Na_2Cr_2O_7$ (5 mM, 5 mL) and i-p-AsA (5 mL) at different [i-p-AsA ($T \approx 293$ K): (a) [i-p-AsA] = 5 mM, pH 6.2, $\nu = 9.6596$ GHz; (b) [i-p-AsA] = 10 mM, pH 6.8, $\nu =$ 9.6590 GHz; (c) [i-p-AsA] = 20 mM, pH 5.8, $\nu = 9.6591$ GHz.

All of these Cr(V) complexes have similar coordination environments, except that **VI** and **X** have enediolate and **VII** and **XIX** have aliphatic diolate coordination, giving g_{iso} values of 1.9791 and 1.9785, respectively. The latter signal was not observed in the reaction of Cr(VI) with i-p-AsA in which the aliphatic diolate group is protected. The difference in g_{iso} values reflects the change of the coordination environment around the Cr(V) center. Cr(V) complexes give higher g_{iso} values when the enediolate binds than when the aliphatic diolate binds. The enediolate binds to Cr(V) in preference to the aliphatic diolate, since there is a correlation between the g_{iso} value of the donor strength for oxygen donors.[38] The higher g_{iso} values observed when the enediolate coordinates compared with aliphatic diolates are consistent with the enediolate moiety being a stronger donor to Cr(V). This explains the preference for binding to this functional group, as evidenced by EPR spectroscopic results. The g_{iso} values of both signals showed slight increases with increasing pH values, which were attributed to the deprotonation of one of their hydroxo groups (eq 6).

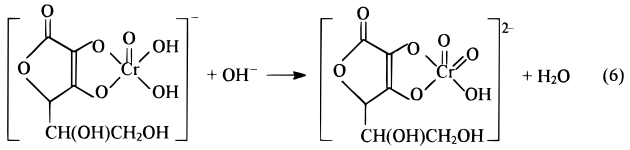


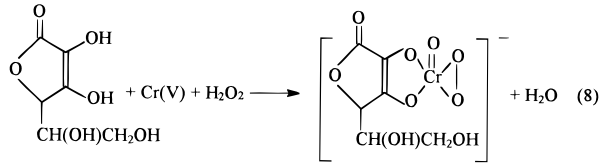
Table 1. Summary of the Signals Present in the Reactions of Cr(VI) with AsA, DHAA, and i-p-AsA

			g_{iso}[a]						
Cr(VI) (10 mM)[b]	ligand (5 mM)	pH	1.9824	1.9818	1.9812	1.9791	1.9785	1.9773	1.9733
$Na_2Cr_2O_7$	AsA	6.0	+	+	−	+	+	−	−
Na_2CrO_4	AsA	8.4	+	+	+	+	+	+	+
$Na_2Cr_2O_7$	DHAA	3.5	−	−	−	+	+	+	+
Na_2CrO_4	DHAA	6.6	−	−	−	+	−	−	−
$Na_2Cr_2O_7$	i-p-AsA	6.2	+	+	−	+	−	−	−
Na_2CrO_4	i-p-AsA	8.0	+	+	−	+	−	+	+

[a] Key: +, EPR active; − not observed. [b] [Cr(VI)].

***g*iso = 1.9818 and *g*iso = 1.9824 Signals.** These were observed in the reduction of Cr(VI) with AsA in aerated media, but not under anaerobic conditions, and were more prominent when aged AsA solutions reacted with Cr(VI) compared with freshly-prepared solutions. These factors taken together unambiguously demonstrate the involvement of O_2 in the reaction. It is well established that H_2O_2 is formed during the aerial oxidation of AsA in aqueous solution,[54] and addition of H_2O_2 into the reaction mixture of Cr(VI) with AsA markedly increased the intensity of the $g_{iso} = 1.9818$ signal. This, together with its disappearance in the presence of catalase, demonstrates that the species responsible for this signal is likely to be a Cr(V)/peroxo complex, which has been verified by the observation that increasing the $[H_2O_2]$ augments the intensity of this signal correspondingly. The reaction of Cr(VI) with H_2O_2 produces three minor EPR signals at $g_{iso} = 1.9798$, 1.9764, and 1.9724 due to Cr(V)/peroxo complexes without ascorbate as a coligand.[56] Only the signal due to the ascorbate radical was observed in the reaction of AsA with H_2O_2. Therefore, the species giving rise to the signal at $g_{iso} = 1.9818$ is a Cr(V)/ascorbate/peroxo complex, which may have a structure like **XI**.

$$\mathbf{I} + O_2 \rightarrow \mathbf{II} + H_2O_2 \qquad (7)$$

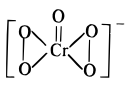


XI

The Cr(V)/ascorbate/peroxo complex is very reactive, since its signal decays very quickly. The signals due to the Cr(V)/ascorbate complexes increase as those due to the peroxo complex decrease, but it is not clear whether this occurs through a ligand exchange reaction (eq 9) or the reaction of Cr(VI) with more ascorbate to generate more of the $g_{iso} = 1.9791$ species after the peroxo complex has decomposed.

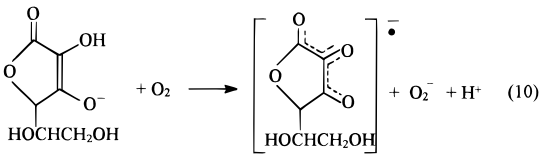
$$\mathbf{XI} + 2H_2O \rightarrow \mathbf{VI} + H_2O_2 \qquad (9)$$

The species at $g_{iso} = 1.9824$ is possibly a Cr(V)/peroxo species that may have a structure like **XII**. It is most stable at pH ≈ 7−8. Addition of H_2O_2 into the reaction mixture of Na_2CrO_4 and AsA augmented its signal intensity (Figure 6).



XII

Superoxide is formed as a transient during the aerial oxidation of AsA in aqueous solution with the generation of ascorbate radical (**XIII**).[54,55] The changed product distribution of adducts with the spin trap DMPO when superoxide dismutase (SOD) was added to a Cr(VI)/ascorbate reaction[44] indicated the presence of $O_2^{\bullet-}$.



XIII

The aerial oxidation of **I** ultimately leads to the formation of **II**, which reacts more slowly with Cr(VI) than **I**, as confirmed in the following section dealing with the EPR study on the Cr(V) species produced in the reaction of Cr(VI) with **II**. Thus, the involvement of O_2 in the reaction significantly extends the lifetime of the Cr(V) intermediates and efficiently retards the whole reduction process. This is likely to be the reason why the rate constants of Cr(VI) reductions with **I** obtained in the presence of O_2 were about 10 times smaller than those obtained in anaerobic conditions.[43] The sensitivity of the reduction of Cr(VI) by **I** to the presence of O_2 is illustrated by the observation that a freshly-prepared solution of **I**, which is exposed to the air for just 3 min, results in the formation of detectable Cr(V)/ascorbate/peroxo complexes.

The Cr(V)/ascorbate/peroxo complexes may be at least partially responsible for the oxygen-activated intermediates that oxidize formate to carboxylate radicals. The latter were detected by spin-trap techniques[44,45] during the reduction of Cr(VI) by ascorbate in aerated phosphate buffer. Because the formation of this species is dependent on the presence of O_2 and ultimately H_2O_2, addition of catalase into the reaction media caused a depletion of the DMPO−$COO^{\bullet-}$ spin-trap product and addition of SOD induced an increased intensity for this signal.

Varying the relative mole ratio of the reactants resulted in a change of the relative intensities of the Cr(V) signals. The $g_{iso} = 1.9791$ signal was at its highest intensity at an AsA:Cr(VI) ratio of 1:2 according to eq 11.

$$2Cr(VI) + \mathbf{I} \rightarrow 2Cr(V) + \mathbf{II} \qquad (11)$$

Increasing the [AsA] resulted in an increase in the $[H_2O_2]$, such that the $g_{iso} = 1.9818$ signal was better observed at the ratio of ascorbate to Cr(VI) of 1:1 than at the ratio of 1:2. The observed decreases in signal intensities of Cr(V) intermediates with further increases in the [AsA] were due to the ability of AsA to function as an antioxidant, which reduced Cr(V) rapidly to Cr(III).

***g*iso = 1.9812 Signal.** This was observed in the reaction of Cr(VI) with **I** over a narrow pH range around 8. It was not detected in the reaction in the absence of O_2, but was observed in the reaction of Cr(VI) with **I** in the presence of H_2O_2 in dimethyl sulfoxide.[62] On the basis of these facts, it may be

(62) Zhang, L.; Lay, P. A. Unpublished results.

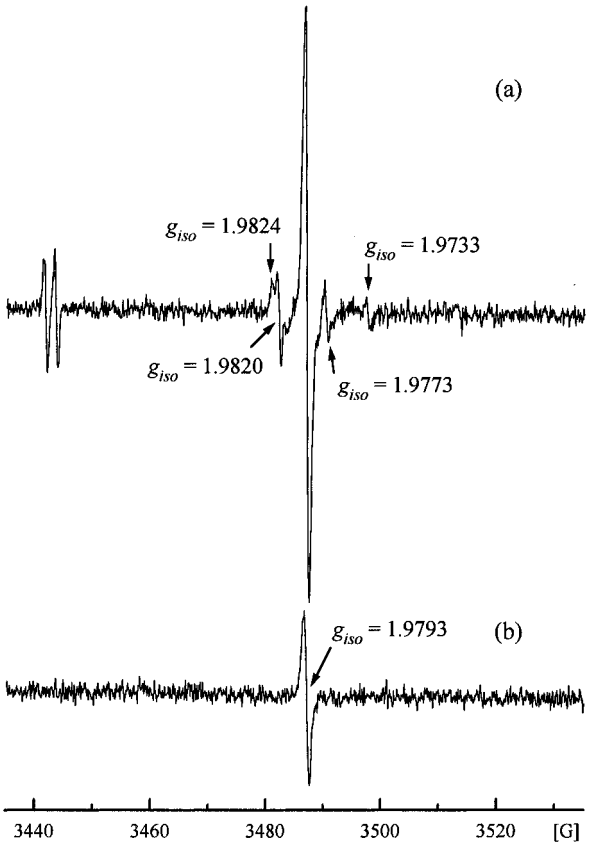
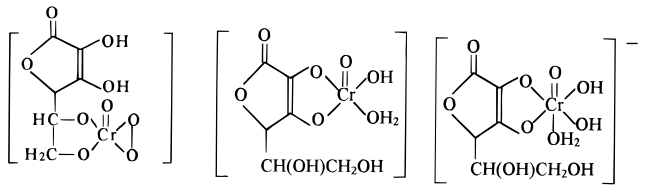


Figure 15. X-band EPR spectra of Na_2CrO_4 (10 mM, 5 mL) and i-p-AsA (5 mM, 5 mL) ($T \approx 293$ K); (a) $t = 3$ min, pH 8.0, $\nu = 9.6594$ GHz; (b) $t = 12$ min, pH 8.4, $\nu = 9.6594$ GHz.

another Cr(V)/peroxo complex coordinating with the side chain as shown in **XIV**.



XIV **XV** **XVI**

***g*iso = 1.9773 and *g*iso = 1.9733 Signals.** These low-intensity signals were detected at pH values around 7−8.5. The $g_{iso} = 1.9773$ species may have one hydroxo ligand being replaced by one water molecule, **XV**. The $g_{iso} = 1.9733$ signal is typical for six-coordinate Cr(V) complexes,[38] and is assigned as having a water molecule occupying the sixth coordination site, e.g., **XVI**. The Cr(V) species produced in the reaction of Cr(VI) and **I** are shown in Figure 16 with their corresponding structures indicated as Roman numbers in the second-derivative spectrum (Figure 16b).

Cr(V) Complexes with DHAA. DHAA is the first stable oxidative product of AsA. ^{1}H NMR and ^{13}C NMR spectroscopy showed that DHAA mainly exists as a dimer in the solid state, but it is converted to the monomer when it is dissolved in water.[63] In aqueous solutions, the open side-chain 2,3-diketo compound is very unstable due to the high positive charge associated with the three carbonyl groups and it is hydrolyzed to the DHAA dihydrate (**XVII**). This undergoes ring closure to give the semiketal form **XVIII**.[63] ^{13}C NMR spectroscopy[64]

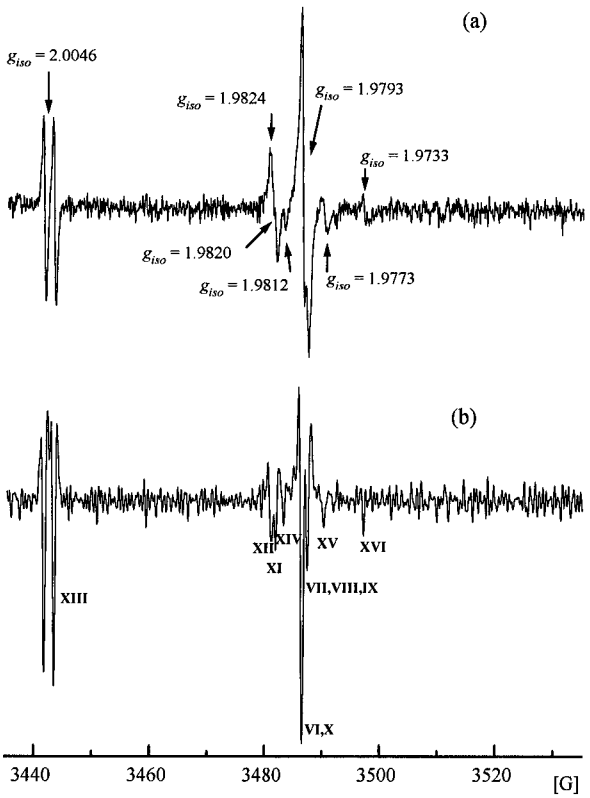
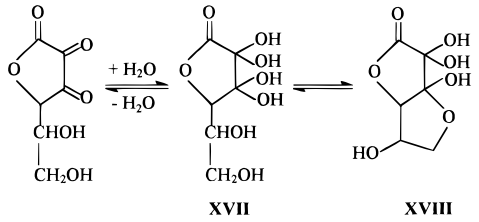


Figure 16. (a) X-band EPR spectra of Na_2CrO_4 (20 mM, 5 mL) and AsA (5 mM, 5 mL) ($T \approx 293$ K): (a) $t = 3$ min, pH 8.4, $\nu = 9.6594$ GHz; (b) second derivative of (a).

and potentiometric measurements[65] established that this form predominates in the equilibrium (eq 12).



XVII **XVIII** (12)

The aliphatic alcohols of DHAA reduce Cr(VI) and Cr(V) much less efficiently than the enediol group of AsA, so that the Cr(V) species produced from the reaction of Cr(VI) with DHAA are stable even in high concentrations of the ligand. The slow dissolution of the DHAA dimer into monomer in water and the predominance of **XVIII** in solution also cause the reduction of Cr(VI) by DHAA to proceed slowly compared with the Cr(VI) reduction by AsA.

$$\mathbf{II} + \text{Cr(VI)} \rightarrow \mathbf{V} + \text{Cr(IV)} + 2\text{H}^+ \qquad (13)$$

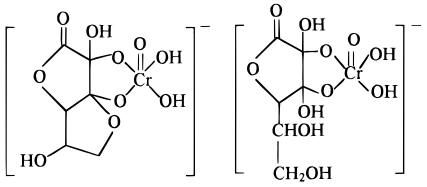
Potentially, Cr(V) could react with any form of DHAA. It could either coordinate to the O atoms on the hydrated lactone ring, giving a singlet, or coordinate to the O atoms on the side chain, giving a species with ^{1}H superhyperfine coupling. Its signal intensity decreased with time because of the ring closure of the side chain. The signal intensity of the singlet assigned to **XIX** increased with time, until it became the major signal that was observed in the spectrum after 1 h of reaction (Figure 9). This is expected because the semiketal form predominates after equilibrium is obtained among the different forms of

(63) Davies, B; Austin, J.; Partridge, D. A. *Vitamin C: Its Chemistry and Biochemistry*; Royal Society of Chemistry: Cambridge, 1991; pp 35, 66−71.

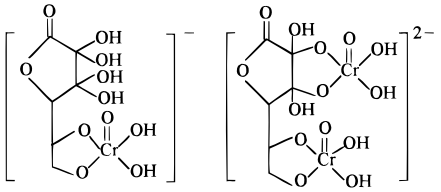
(64) Rudolf, M. *Org. Chem.* **1977**, *32B*, 562−568.

(65) Tur'yan, Y. I.; Kohen, R. *J. Electroanal. Chem.* **1995**, *380*, 273−277.

DHAA.[63,66] In neutral or alkaline solutions, the hydrated bicyclic semiketal form **XVIII** is probably the most stable form of DHAA,[63,66] resulting in the formation of **XIX** in the reaction of Na_2CrO_4 and DHAA, so only the singlet was observed (Figure 12). In the reaction of Cr(VI) with AsA in close to equimolar ratios, the Cr(V)/dehydroascorbate complexes will eventually replace the Cr(V)/ascorbate complexes as the predominant species as it proceeds. This is consistent with the experimental observations on aged Cr(VI)/AsA reaction mixtures.



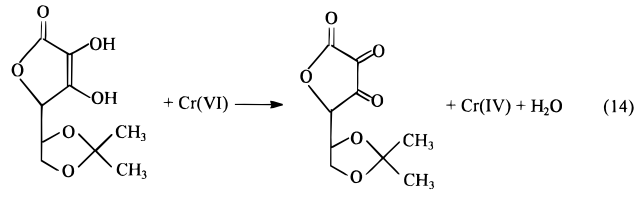
XIX **XX**



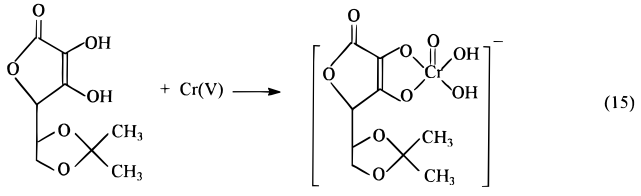
XXI **XXII**

Cr(V) Complexes with DHAA in the Presence of H_2O_2. In the aerial reaction of Cr(VI) with DHAA, the signals at $g_{iso} = 1.9818$ and $g_{iso} = 1.9824$ were not detected, because DHAA is a much weaker reductant than AsA and it is not susceptible to the attack by O_2. Unlike the reaction with AsA, O_2 will not participate in the reaction to produce H_2O_2, so that the Cr(V)/peroxo species will not form under any conditions. However, by adding H_2O_2 into the Cr(VI)/DHAA reaction mixture, Cr(V)/dehydroascorbate/peroxo complexes were detected. They are also unstable and quickly decompose in aqueous solution.

Cr(V) Complexes with i-p-AsA. The reduction of Cr(VI) by i-p-AsA (**III**) produces Cr(V) and i-p-DHAA (**XXIII**), eqs 14 and 3. The Cr(V) species **XXIV** formed in the reaction is unstable in higher concentrations of i-p-AsA because it still possesses the easily-oxidized enediol group. Like the ascorbate radical, the i-p-AsA radical is also stable due to the delocalization of the electrons over a highly conjugated tricarbonyl system.[67]



III **XXIII** (14)



XXIV (15)

As an additional check on the identities of the Cr(V) complexes, experimental and calculated[38] g_{iso} values for the Cr(V) complexes produced in the reactions of Cr(VI) with AsA, DHAA, and i-p-AsA are compared (Table 2). The excellent agreement between the observed and calculated values adds confidence to the assignments.

Implications of the Presence of Cr(V)/Ascorbate/Peroxo Complexes for Cr(VI)-Induced Genotoxicity. Several studies have demonstrated that the intermediates generated in the reaction of Cr(VI) with AsA are able to cause DNA damage, including Cr−DNA adducts, DNA strand breaks, and AP sites.[46,47,68,69] Since there are many different types of Cr(V) species formed in the reaction, different kinds of Cr(V) species may be responsible for different kinds of DNA damage because their reactivities are different. Much speculation has been offered as to which intermediates are responsible for inducing DNA single strand breaks. It has been proposed that a Cr(V)−O_2^- complex may play an important part in the initiation and promotion steps in chromate carcinogenesis.[44] Others have imputed the DNA strand breaks to carbon-based radicals,[15,68] or ascorbate-derived free radicals and hydroxyl OH• radicals.[70] Other results[46,68] seem to preclude the possibility that a Cr(V)/ascorbate complex is the DNA cleaving species because no DNA single strand breaks occurred in HEPES buffer (pH 7.0) in which a Cr(V)/ascorbate complex was formed preferentially.[42] Furthermore, from the study reported here and elsewhere,[42] the Cr(V)/ascorbate complexes were best observed in a Cr(VI):AsA ratio of 2:1, while the DNA single strand breaks were most evident in a Cr(VI):AsA ratio of 1:1.[46,68] Since higher levels of Cr−DNA binding were observed in HEPES buffer, it was assumed that Cr(V)/ascorbate complexes reacted with DNA to generate Cr−DNA adducts. H_2O_2 can be ruled out as the DNA cleaving agent because it does not react with DNA in metal ion free systems.[71] Moreover, H_2O_2 in combination with AsA in the absence of Cr(VI) did not induce single strand breaks in demetaled buffers.[46] The correlation of our EPR study with the DNA cleavage experiments of other workers suggests that the Cr(V)/ascorbate/peroxo complexes are the major species responsible for DNA single strand breaks and AP-sites. These species were best observed in a Cr(VI):AsA ratio of 1:1, which is coincident with the observation that DNA single strand breaks[46,68] and AP sites[47] occurred to the greatest extent in the same reactant ratio. These species were most evident at pH values of 7−8, which are close to physiological values. The significant decrease in the number of DNA strand breaks in the absence of O_2 is consistent with the notion that the Cr(V)/ascorbate/peroxo complexes identified in the current study are the major DNA cleaving agents. The removal of O_2 did not completely prevent DNA strand breaks from occurring. This may be due to H_2O_2 formation in the AsA solution before it was degassed, which could subsequently produce Cr(V)/peroxo complexes in the absence of air, and/or because the Cr(V)/ascorbate complexes are themselves capable of causing cleavage, albeit to a lesser extent. This is the case with 2-hydroxyacid/Cr(V) complexes that cleave DNA,[72] but are even more reactive when mixed-ligand Cr(V)/peroxo complexes are formed in the

(66) Seib, P. A.; Tolbert, B. M. *Ascorbic Acid: Chemistry, Metabolism, and Uses*; American Chemical Society: Washington, DC, 1982; pp 101−123.

(67) Kirino, Y. *Chem. Lett.* **1974**, 153−158.

(68) Stearns, D. M.; Kennedy, L. J.; Courtney, K. D.; Giangrande, P. H.; Phieffer, L. S.; Wetterhahn, K. E. *Biochemistry* **1995**, *34*, 910−919.

(69) Kortenkamp, A.; Casadevall, M.; da Cruz Fresco, P. *Ann. Clin. Lab. Sci.* **1996**, *26*, 160−175.

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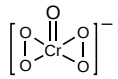
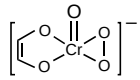
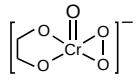
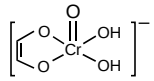
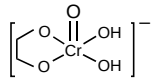
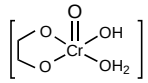
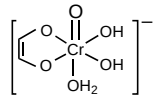
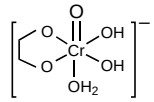
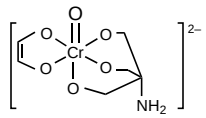
Table 2. Assignments of Cr(V) Intermediates in Cr(VI)/**I** and /**II** Reactions

complex	g_{iso}(obsd)	g_{iso}(calcd)	A_{iso}(obsd) ($\times 10^{-4}$ cm^{-1})	A_{iso}(calcd) ($\times 10^{-4}$ cm^{-1})
	1.9824	1.9823	12.9	
	1.9818	1.9818	13.2	
	1.9812	1.9812		
	1.9791	1.9791	16.4	16.4
	1.9785	1.9785	16.7	16.7
	1.9773	1.9777		
	1.9733	1.9732		
	1.9727	1.9726		
	1.9765[a]	1.9764		

[a] This species was observed in the reaction of Cr(VI) with AsA in Tris·HCl buffer.

presence of H_2O_2.[73] Since we have shown that the Cr(V)/ascorbate/peroxo complexes disappear in the presence of catalase, this adds further support to the hypothesis that peroxo species formed in the course of the reaction caused the DNA cleavage, because such damage is inhibited by catalase.[46,47] That the addition of SOD, which rapidly catalyzes the conversion of O_2^- to H_2O_2 and O_2, to the reaction mixture did not alter the level of single strand breaks indicates that superoxo species are not involved in the DNA strand breaks. In a recent study,[70] it was found that incubation of Cr(VI) with AsA in the presence of H_2O_2 caused much higher levels of double strand breaks in λ *Hin*dIII-digested DNA compared to incubation of Cr(VI) with AsA alone, without adding H_2O_2, which is also consistent with our EPR results and postulate on the nature of the active species. Finally, the variation in the intensity of the signals in different buffers (and the presence of new signals) may explain the variability in the results of the DNA assays.

There is a considerable amount of evidence that points to the role of reactive oxygen species in carcinogenesis.[74−76] Kortenkamp *et al.*[77] have shown that the DNA cleavage is also induced by oxygen-activated species during the reduction of Cr(VI) by GSH. Recently, Casadevall and Kortenkamp[69,78] showed that the formation of both AP sites and single strand breaks induced in the reaction of Cr(VI) with GSH were dependent on O_2. Since addition of catalase almost completely inhibited the generation of AP sites and single strand breaks, Cr(V)/peroxo or Cr(IV)/peroxo species are considered as possible candidates for causing the DNA damage. H_2O_2 also participates in DNA damage induced by many carcinogenic metals,[76,79] including Cr(VI),[37,80] Co(II),[81] Ni(II),[82] and Fe(III)[83] *via* the formation of active oxygen species. On the basis of these findings, it is possible that these metal/peroxo species may be the mutagenic intermediates that eventually lead to metal-ion-induced carcinogenesis. However, the low concentrations of H_2O_2 ($\sim 10^{-7}-10^{-9}$ M) in cells[75] due to enzymatic scavenging would mean that such complexes are in extremely low concentrations, and hence, the *in vitro* DNA work in the absence of O_2 may be more relevant than that in its presence. In this case, the low level of DNA damage caused in the absence of O_2 and hence the absence of H_2O_2 as a result of Cr(V)/ascorbate complexes may be more relevant to the biological situation. Other Cr(V) complexes must also be considered because of the rapid ligand exchange reactions.[2,38]

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(74) O'Brien, P.; Kortenkamp, A. *Environ. Health Perspect.* **1994**, *102* (Suppl 3), 3−10.
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(81) Yamamoto, K.; Inoue, S.; Yamazaki, A.; Yoshinaga, T.; Kawanishi, S. *Chem. Res. Toxicol.* **1989**, *2*, 234−239.
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It is clear, however, that whichever of the classes of Cr(V) species may have the greatest relevance to Cr genotoxicity, excess ascorbate rapidly diminishes the concentration of such species and hence the carcinogenic potential. This appears to add weight to the suggestion[23,24] that high vitamin C intake by workers in the chromate industry may be beneficial.

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Supporting Information Available: Tables of the time and pH dependences of the X-band EPR spectra of the $Na_2Cr_2O_7$/AsA reactions and Figures S1−S4 showing X-band EPR spectra of the time dependence, the effect of catalase and the effect of preincubation of the AsA solution in air on the $Na_2Cr_2O_7$/AsA reaction, and the time dependence of the $Na_2Cr_2O_7$/i-p-AsA reaction (6 pages). See any current masthead page for ordering and Internet access instructions.

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