Reduction of Chromium(VI) by Ascorbate Leads to Chromium−DNA Binding and DNA Strand Breaks in Vitro†

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ABSTRACT: Chromium(VI) is a known human carcinogen which requires intracellular reduction for activation. Ascorbate (vitamin C) has been reported to function as a major reductant of Cr(VI) in animals and cell culture systems. The reaction of Cr(VI) with varying concentrations of ascorbate was studied under physiological conditions in vitro in order to determine the types of reactive intermediates produced and to evaluate the reactivity of these intermediates with DNA. Reactions of 1.8 mM Cr(VI) with 0−18 mM ascorbate at pH 7.0 in *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES; 0.10 M) and tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl; 0.050 M) buffers were studied by electron paramagnetic resonance and UV/visible spectroscopy. Cr(V) and carbon-based free radical adducts of 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) were observed at 0.5 to 1 and 1 to 1 reactions of ascorbate to Cr(VI). Levels of Cr(V) were higher for reactions in HEPES buffer, and levels of carbon-based radicals were higher in Tris·HCl buffer. Levels of Cr(IV) and Cr(III) increased with increasing concentration of ascorbate in both buffers. Reaction of Cr(VI) with varying ascorbate in the presence of calf thymus DNA or pBR322 DNA resulted in Cr−DNA adducts and plasmid relaxation, respectively. Maximum binding of Cr to DNA was observed for the 1:1 reaction ratio of Cr(VI) with ascorbate in both HEPES and Tris·HCl buffers, but total Cr bound to DNA was 8-fold lower in Tris·HCl than HEPES buffer. Preincubation of Cr(VI) with ascorbate before reaction with DNA decreased Cr−DNA binding to background levels. Preincubation of Cr(III) with ascorbate resulted in only low Cr−DNA binding. Levels of Cr−DNA binding were higher with single-stranded *vs* double-stranded DNA. Reactions with ^{14}C-labeled ascorbate produced no cross-linking of ascorbate to DNA. Maximum plasmid relaxation was observed for the 1:1 ascorbate to Cr(VI) ratio in both buffers; however, single-strand breaks were 2-fold higher in Tris·HCl than HEPES buffer. Reactions with plasmid in the presence of DMPO quenched formation of single-strand breaks. Interpretation of these results in light of the spectroscopic studies suggested that Cr(V) and carbon-based radicals were responsible for Cr−DNA adducts and DNA single-strand breaks, respectively.

Chromium(VI) is a known carcinogen in humans and animals (IARC, 1987), and chromate compounds are mutagenic and genotoxic (De Flora & Wetterhahn, 1989). Although a link between Cr(VI) and lung cancer was established in the 1930s (reviewed in Léonard & Lauwerys, 1980), the mechanism of Cr(VI)-induced cancer as well as the ultimate genotoxic species are unknown. Chromium(VI) requires intracellular reduction for activation (Connett & Wetterhahn, 1983). The primary reducing agents based on kinetics and cellular concentrations are ascorbate (vitamin C) and glutathione (GSH;[1] γ-glutamylcysteinylglycine) (Connett & Wetterhahn, 1985), but other small molecules and enzyme systems are capable of reducing Cr(VI) under physiological conditions. The in vivo reduction of Cr(VI) can theoretically produce many reactive intermediates that could target DNA, namely, Cr(V), Cr(IV), free radicals, and reactive oxygen species, as well as the final end product Cr(III) (Connett & Wetterhahn, 1983). The DNA damage resulting from Cr(VI) exposure has recently been reviewed (De Flora & Wetterhahn, 1989; De Flora et al., 1990). The major lesions observed in vivo include DNA−protein cross-links, DNA interstrand cross-links, Cr−DNA adducts, and single-strand breaks. Oxidative nucleotide base modification as 8-oxo-2′-deoxyguanosine has recently been observed in Cr(VI)-treated chick embryos (Misra et al., submitted for publication). These Cr(VI)-induced DNA lesions presum-

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[1] Abbreviations: asc•⁻, ascorbate radical anion; CT, calf thymus; DABA, diaminobenzoic acid; DETAPAC, diethylenetriaminepentaacetic acid; DHA, dehydroascorbate; DKG, 2,3-diketogularic acid; DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; EHBA, 2-ethyl-2-hydroxybutyric acid; EPR, electron paramagnetic resonance; GSH, glutathione, γ-glutamylcysteinylglycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; RNase A, ribonuclease A; RT, room temperature (24−26 °C); TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy radical; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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ably interfere with normal replication and/or transcription processes, which eventually leads to cancer.

Much of the current research is concerned with the preliminary step of Cr(VI) activation, specifically the reduction of Cr(VI) to form reactive intermediates, and evaluation of these species as DNA damaging agents. The role of GSH in Cr(VI) metabolism has been the focus of previous studies (reviewed in Standeven & Wetterhahn, 1989). Glutathione levels have been correlated with Cr(VI)-induced DNA damage in vitro and in vivo. Depletion of GSH led to decreased Cr−DNA adduct formation in rat ultrafiltrates (Standeven & Wetterhahn, 1992) and 18-day chick embryo liver (Misra and Wetterhahn, unpublished results). Increased GSH levels have been correlated with an increase in Cr(VI)-induced DNA alkali-labile sites in cultured chick embryo hepatocytes (Cupo & Wetterhahn, 1985) and V-79 cells (Sugiyama et al., 1987). The ability of Cr(V)−thiol complexes to produce Cr−DNA adducts has been shown for reactions of Cr(VI) with GSH (Aiyar et al., 1990; Borges et al., 1991), and reaction of Cr(VI) with GSH in the presence of calf thymus (CT) DNA in vitro was shown to result in GSH−Cr−DNA complexes (Borges & Wetterhahn, 1989). The participation of cysteine and/or GSH in Cr−DNA cross-links was observed in Chinese hamster ovary (CHO) cells treated with Cr(VI) (Lin et al., 1992).

Although these results suggest a role for GSH in mediating Cr(VI) genotoxicity, the correlation between Cr(VI) treatment and a decrease in GSH levels has been ambiguous in in vivo studies (Standeven & Wetterhahn, 1991b and references therein) and attention has shifted to the function of ascorbate in Cr(VI) genotoxicity. Ascorbate was recently shown to be the major reductant of Cr(VI) in rat kidney, liver, and lung ultrafiltrates (Suzuki & Fukuda, 1990; Standeven & Wetterhahn, 1991a, 1992). Increased ascorbate levels have been shown to correlate with decreased Cr(VI)-induced alkali-labile sites and enhanced DNA−protein cross-links in V-79 CHO cells (Sugiyama et al., 1991). The clastogenicity of particulate Cr(VI) was decreased when CHO cells were cotreated with $PbCrO_4$ and ascorbate, presumably by enhanced extracellular reduction of solublized Cr(VI) to Cr(III), which has little or no clastogenic activity on intact cells (Wise et al., 1993).

The in vitro reaction of Cr(VI) with ascorbate at pH 7.0 has been shown to produce Cr(V), Cr(IV), ascorbate radical, and carbon-based radicals as reactive intermediates (Goodgame & Joy, 1987; Stearns & Wetterhahn, 1994). The relative amounts of intermediates were dependent on buffer and stoichiometry of ascorbate to Cr(VI). This provided a model system in which different reactive species could be generated in order to evaluate their role in DNA damage in vitro. Measurement of the reactivity of these intermediates toward DNA is the subject of this paper. In the present study the extent of formation of Cr−DNA adducts and DNA single-strand breaks has been determined for reaction of Cr(VI) with a range of concentrations of ascorbate in the presence of calf thymus (CT) or pBR322 plasmid DNA. The aim of this work was to explore the relative reactivity of Cr(V), Cr(IV), and Cr(III) toward formation of Cr−DNA adducts, and to evaluate the ability of reactive species to produce DNA single-strand breaks.

MATERIALS AND METHODS

General Methods. The Cr(VI) source was $K_2Cr_2O_7$, the Cr(III) source was $Cr(NO_3)_3 \cdot 9H_2O$, and the Mn(II) source was $MnCl_2 \cdot 4H_2O$. Ascorbic acid or sodium ascorbate was used as noted. The pK_{a_1} of ascorbic acid is 4.2 (Buettner, 1988), thus at 0.9−18 mM, pH 7.0, either starting material exists in solution as the monoanion ascorbate. DMPO was obtained from Fluka (Ronkonoma, NY). DMPO solutions were purified by charcoal filtration (Buettner & Oberley, 1978). The *Escherichia coli* plasmid pBR322 DNA and 1 kb DNA ladder were obtained from GIBCO BRL (Gaithersburg, MD). Radiolabeled [^{14}C]-L-ascorbic acid was obtained from New England Nuclear (Boston, MA). Dehydroascorbate (DHA) was purchased from Aldrich (Milwaukee, WI). Chromium(VI) is a known human carcinogen and should be handled with care. All buffers and ascorbate solutions were treated with Chelex 100 resin or cation exchange resin AG50W X8 to remove trace iron. Ascorbate concentrations were determined by UV/visible spectroscopy (265 nm, ϵ = 14500 M^{-1} cm^{-1}; Buettner, 1988). All solutions were prepared immediately before use.

Spectroscopy. UV/visible spectra were recorded on a Perkin Elmer Lambda 2 spectrophotometer. EPR spectra were recorded on a Bruker ESP-300 spectrometer with previously published parameters (Stearns & Wetterhahn, 1994). EPR spectra were recorded for reaction solutions of $K_2Cr_2O_7$ (1.80−1.82 mM Cr(VI)) with varying sodium ascorbate (0.91−1.0, 1.8−1.9, 2.8−2.9, 5.5−5.8, and 18−19 mM) in either Tris·HCl buffer (0.050 M, pH 7.0, RT) or HEPES buffer (0.10 M, pH 7.0, RT). Reactions were studied in the presence and absence of 0.10 M DMPO, 1.9 ± 0.1 mM CT DNA-P, and 3.60 mM $MnCl_2 \cdot 4H_2O$. Concentrations of Cr(V) were estimated by comparison of signal intensity (as $I\Delta H^2$ where I = intensity and ΔH = width in gauss (G) from peak maximum to peak minimum) to the signal intensity of K_3CrO_8 in 0.20 M NaOH and 0.5% H_2O_2 (Dalal et al., 1981). The limit of detection of K_3CrO_8 was 1.0 μM. The Cr(V) signal was not affected by the presence of CT DNA; therefore, the presented data represents combined data for reactions ± CT DNA. Concentrations of ascorbate radical anion ($asc^{\bullet -}$) and DMPO−radical adducts were estimated by comparison of measured signal intensities (as $I\Delta H^2$) to the intensity of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) radical in HEPES buffer (0.10 M, pH 7.0, RT) (Tan et al., 1992). The limit of detection of TEMPO radical was 0.5 μM. Concentrations of Cr(IV) were estimated by comparison of Mn(II) signal intensities of standard solutions to those of reaction solutions, which showed a decrease in the Mn(II) EPR signal (Stearns & Wetterhahn, 1994). Intensities were calculated as the absolute value of signal intensity averaged for the 6 line Mn(II) signal. Plots of signal intensity *vs* concentration of Mn(II) standards were linear over 0.45−3.60 mM. Spectra of standards and control samples were acquired under identical conditions as spectra of reaction samples.

Cr−DNA Binding Studies. CT DNA solutions were demetallated by dialysis of stock solutions against either Tris·HCl buffer (0.050 M, pH 7.0, 37 °C) or HEPES buffer (0.10 M, pH 7.0, 37 °C) containing 1.0 mM diethylenediaminepentaacetic acid (DETAPAC) for >12 h at 4 °C, followed by dialysis against buffer alone for 12 h at 4 °C. Purification of CT DNA from RNA and protein with

ribonuclease A (RNase A) and proteinase K, followed by phenol/chloroform/isoamyl extraction (Standeven & Wetterhahn, 1992), did not affect levels of Cr bound to DNA. DNA concentrations were determined by UV/visible spectroscopy (260 nm, ϵ = 6600 M^{-1} cm^{-1}) (Borges & Wetterhahn, 1989). CT DNA (1.8 mM DNA-P) was incubated with $K_2Cr_2O_7$ (1.8 mM Cr(VI)) and varying amounts of ascorbic acid (0, 0.90, 1.8, 3.6, 5.4, 9.0, and 18 mM) in either Tris·HCl buffer (0.050 M, pH 7.0, 37 °C) or HEPES buffer (0.10 M, pH 7.0, 37 °C); or with $Cr(NO_3)_3 \cdot 9H_2O$ (1.8 mM Cr(III)) and varying ascorbic acid (0–18 mM) in HEPES buffer (0.10 M, pH 7.0, 37 °C). Chromium was reacted with ascorbate immediately in the presence of DNA, or solutions of Cr and ascorbate were preincubated for 30 min before incubation with DNA. Control samples lacked ascorbic acid or Cr or DNA. Incubations with DNA were carried out at 37 °C for 30 min. Noncovalently bound Cr was removed from DNA by a previously published procedure (Borges et al., 1991) modified by substitution of 40% ethanol for 20% propanol for the elution of DNA from the NENsorb 20 chromatography columns. The concentration of DNA (as DNA-P) in each sample was determined by the diaminobenzoic acid (DABA) fluorometric assay (Kissane & Robins, 1958). The detection limit was 2.0 μM DNA-P. The concentration of Cr in each sample was measured by atomic absorption spectroscopy to give Cr/DNA-P binding ratios. The detection limit was 0.024 μM Cr. The amounts of Cr detected in control reactions containing only Cr(VI), Cr(III), or ascorbic acid were subtracted from Cr present in reaction samples for the calculation of binding ratios. Spiking experiments in which 7.69 pmol of a Cr standard was added to each sample showed that Cr recovery ranged from 97% to 101%.

For experiments with single-stranded DNA, the CT DNA was denatured by heating solutions in boiling water for 10 min and then immediately placing solutions on ice to prevent reannealing. A 36% increase in the UV absorbance at 260 nm was observed for denatured DNA solutions in HEPES and Tris·HCl buffers.

Cross-Linking of [^{14}C]Ascorbate to CT DNA. The Cr–DNA binding studies decribed above with Cr(VI) and ascorbate were carried out in HEPES and Tris·HCl buffers with C-1 labeled [^{14}C]-L-ascorbic acid (4.74 μCi/μmol in HEPES, and 3.0 μCi/μmol in Tris·HCl). The 54.0 mM ascorbic acid stock solution was prepared by addition of 1.055 μmol of ^{14}[C]ascorbic acid to 4.345 μmol of ascorbic acid in 100 μL of HEPES buffer (0.10 M, pH 7.0, 37 °C). Noncovalently bound Cr was removed from DNA as described above, and levels of [^{14}C]ascorbate bound to DNA were determined by scintillation counting in Aquasol-2 using a Packard 1900CA liquid scintillation counter. The counting efficiency was 95% based on quench curves. DNA and Cr sample concentrations were determined as described above to calculate ratios of Cr and ascorbate bound to CT DNA.

pBR322 DNA Nicking Studies. Contaminating RNA was removed from pBR322 DNA by treatment with RNase A (20 μg/mL) at 37 °C for 30 min, and contaminating protein was removed by digestion with proteinase K (50 μg/mL) at 37 °C for 30 min, followed by phenol/chloroform/isoamyl extraction by standard methods (Standeven & Wetterhahn, 1992). Plasmid DNA was then dialyzed against 1.0 mM DETAPAC in 0.050 M Tris·HCl (pH 7.0) at 4 °C for 12 h to remove trace metals, followed by dialysis against buffer alone at 4 °C for 12 h. DNA was precipitated with ethanol and resuspended in either HEPES (0.10 M, pH 7.0, 37 °C) or Tris·HCl (0.050 M, pH 7.0, 37 °C) buffer. Plasmid DNA concentration was determined by UV/vis spectroscopy (260 nm, ϵ = 6600 M^{-1} cm^{-1}) (Borges & Wetterhahn, 1989). Stock buffer solutions contained DETAPAC at 15 μM (HEPES) or 25 μM (Tris·HCl) to slow reaction of contaminating trace metals with dioxygen (Egan et al., 1992). Addition of DETAPAC at these levels decreased background DNA nicking ~5-fold. Reaction solutions of 10 μL in either HEPES (0.10 M, pH 7.0, 37 °C) or Tris·HCl (0.050 M, pH 7.0, 37 °C) contained 1.0 μg of pBR322 DNA (0.36 mM DNA-P, 82 nM plasmid), 1.8 mM Cr(VI) (from $K_2Cr_2O_7$), and varying ascorbic acid (0, 0.90, 1.8, 3.6, or 5.4 mM). Reactions of 1.8 mM Cr(VI) and 1.8 mM ascorbic acid with DNA were also carried out in the presence of 0.10 M DMPO. All reactions were initiated by microcentrifugation of reactants, and were run at 37 °C for 30 min, followed by termination in an ice water bath. A 2 μL portion of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) was added to each reaction solution (Sambrook et al., 1989). Reaction samples (10 μL) and 0.8 μg of 1.0 kb Lambda molecular weight marker DNA were loaded onto a 0.7% agarose gel. Reactions were immediately analyzed by agarose gel electrophoresis in TBE buffer (90 mM Tris·HCl, 90 mM boric acid, 2 mM EDTA) at 120 V (4 V/cm) for 8–12 h. Gels were stained in aqueous solutions of ethidium bromide (0.5 μg/mL) and were photographed with Polaroid 55 film under short wavelength UV light. Negatives were scanned with a Helena Laboratories Quick Scan R&D densitometer connected to a Hewlett Packard HP3396A integrator to quantitate relative intensities of bands representing supercoiled (form I) and nicked circular (form II) plasmid DNA.

Statistics. Group means were compared by an unpaired, two-tailed *t* test. Multiple comparisons among group means were accomplished by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test. Differences were considered significant at $P < 0.05$.

RESULTS

Reactive Intermediates Determined by EPR Spectroscopy. The overall reaction between Cr(VI) and ascorbate is the three-electron reduction of Cr(VI) with formation of dehydroascorbate (DHA):

$$2Cr(VI) + 3AscH^- \rightarrow 2Cr(III) + 3DHA \qquad (1)$$

The reactive intermediates Cr(V), $CO_2^{\bullet -}$, $asc^{\bullet -}$, and other carbon-based radicals have been observed for this reaction by EPR spectroscopy in the presence of free-radical spin traps, and Cr(IV) has been detected by reaction with Mn(II) (Stearns & Wetterhahn, 1994).

In the current study levels of intermediates were determined at reaction concentrations identical to those in which DNA damage was measured. Reaction of $K_2Cr_2O_7$ (1.8 mM Cr(VI)) with varying sodium ascorbate (0.9–19 mM) was observed by EPR spectroscopy in the presence and absence of 0.10 M DMPO, 3.60 mM $MnCl_2 \cdot 6H_2O$, and 1.9 ± 0.1 mM CT DNA-P in either 0.10 M HEPES or 0.050 M Tris·HCl buffers (pH 7.0, RT). Representative EPR spectra have been published elsewhere (Stearns & Wetterhahn, 1994; Stearns et al., 1994).

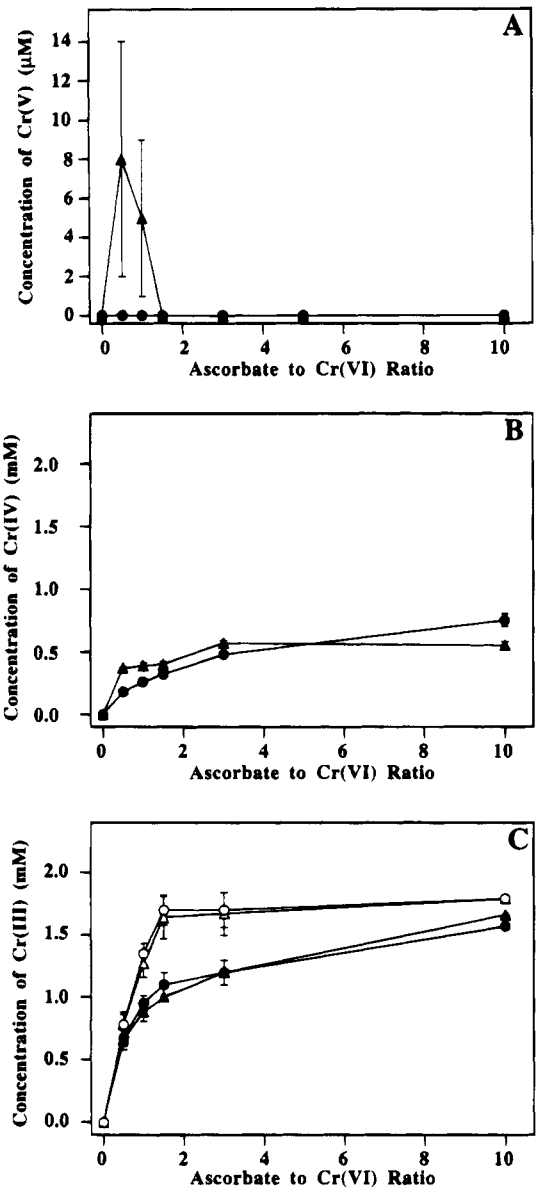

FIGURE 1: Amounts of chromium species observed in the reaction of $K_2Cr_2O_7$ (1.80 mM Cr(VI)) and ascorbate (0.90−18.0 mM) as a function of reaction ratio and buffer (pH 7.0, RT). Values plotted are mean concentrations ± SD for $n = 2-6$. (**A**) Cr(V) observed by EPR spectroscopy for reactions in 0.10 M HEPES (▲) or 0.050 M Tris·HCl (●) buffer, 1.3 min reaction time. Differences between buffers were significant for ascorbate to Cr(VI) ratios of 0.5:1 and 1:1 at $P < 0.01$ (ANOVA). (**B**) Cr(IV) measured by EPR spectroscopy for reactions in the presence of 3.60 mM $MnCl_2 \cdot 4H_2O$ in 0.10 M HEPES (▲) or 0.050 M Tris·HCl (●) buffer, 1.3 min reaction time. (**C**) Cr(III) measured by UV/visible spectroscopy for 1.3 min reactions in 0.10 M HEPES (▲) or 0.050 M Tris·HCl (●) buffer, and for 36 min reactions in 0.10 M HEPES (△) or 0.050 M Tris·HCl (○) buffer.

Chromium(V) was observed in HEPES buffer at $g = 1.980$, $\Delta H = 1.17$ G, at the 0.5:1 and 1:1 ratios of ascorbate to Cr(VI) (Figure 1A). The level of Cr(V) corresponded to 0.4−0.3% of total Cr as Cr(V). The signal intensity had peaked by the time of acquisition (1.3 min); therefore, quantitation refers to the amounts of Cr(V) observed, not necessarily the total amounts formed, but this still allows comparison of the relative effect of reactant ratios. No Cr(V) was detected above the 1:1 ratio in HEPES, or at any reaction ratio in Tris·HCl buffer (Figure 1A). The instability of Cr(V) in Tris·HCl buffer has been postulated to arise from

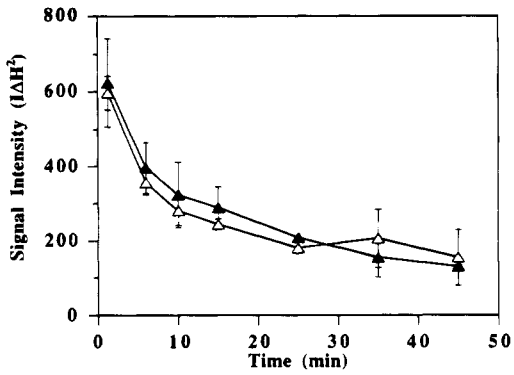

FIGURE 2: The effect of DMPO on the decay of the Cr(V) EPR signal ($g = 1.980$) formed upon reaction of Cr(VI) with ascorbate. The Cr(V) signal was generated by reaction of $K_2Cr_2O_7$ (9.0 mM Cr(VI)) with sodium ascorbate (4.5 mM) in the presence (▲) and absence (△) of 0.10 M DMPO in HEPES buffer (0.10 M, pH 7.0, RT). Reactions were run in duplicate.

Tris coordination (Goodgame & Joy, 1987; Stearns & Wetterhahn, 1994). The presence of DNA did not affect the Cr(V) signal within experimental error. The effect of DNA on the decay of the Cr(V) EPR signal could not be monitored at these concentrations; however, reactivity was evaluated through experiments described below.

The presence of DMPO did not affect the *formation* of Cr(V). However, because DMPO was used in the plasmid nicking experiments (see below) to evaluate the reactivity of Cr(V) *vs* radicals toward formation of DNA single-strand breaks, the potential reactivity of Cr(V) with DMPO was explored further. The experiments were conducted at 10 mM Cr(VI) in order to generate a Cr(V) signal that could be monitored accurately. Chromium(VI) (10.0 mM) was reacted with ascorbate (10.2 mM) in the presence and absence of 0.10 M DMPO in HEPES buffer (0.10 M, pH 7.0, RT). The Cr(V) EPR signal at $g = 1.980$ was monitored from 1.3 to 45 min. The presence of DMPO had no effect on the rate of decay of the Cr(V) signal (Figure 2). Therefore, under these conditions DMPO does not react with Cr(V).

Chromium(IV) was estimated by reaction with Mn(II) and observed as a decrease in the Mn(II) EPR signal (Stearns & Wetterhahn, 1994). Chromium(VI) (1.8 mM) was reacted with ascorbate (0.9−18 mM) in the presence of 3.60 mM $MnCl_2 \cdot 6H_2O$ ± 0.10 M DMPO in 0.10 M HEPES or 0.050 M Tris·HCl buffer (pH 7.0, RT), and the concentrations of Cr(IV) were measured. The amount of Cr(IV) in the reaction of Cr(VI) with ascorbate increased with increasing concentration of ascorbate (Figure 1B). The Cr(IV) levels ranged from 21% to 31% of total Cr as Cr(IV) for the 0.5:1 to 10:1 reaction ratios in HEPES buffer and 10−42% Cr(IV) in Tris·HCl buffer. These levels correspond to total Cr(IV) trapped by Mn(II) during the 1.3 min reaction time.

The presence of DMPO did not affect the formation of Cr(IV). Control samples of Mn(II) with ascorbate alone showed a decrease in Mn(II) only with 18 mM ascorbate, corresponding to a signal decrease of 11% and 13% for samples in HEPES and Tris·HCl, respectively. The Cr(IV) amounts calculated for the 10:1 ascorbate to Cr(VI) reaction were corrected for this small decrease by ascorbate alone. At these concentrations there was no reaction between Mn(II) and 2,3-diketogularic acid (DKG), the open-ring form of DHA which is predominant at pH 7.0 (Tolbert & Ward, 1982). The concentrations of Cr(IV) detected may be

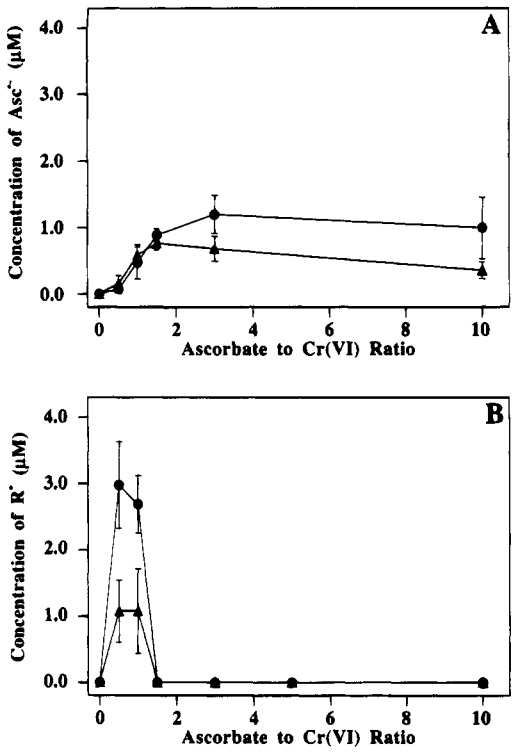

FIGURE 3: Amounts of free-radical species observed for the reaction of $K_2Cr_2O_7$ (1.80 mM Cr(VI)) and ascorbate (0.90−18.0 mM) as a function of reaction ratio and buffer (pH 7.0, RT). Reactions were observed by EPR spectoscopy in the presence of 0.10 M DMPO after a 1.3 min reaction time. Values plotted are mean concentrations ± SD for $n = 4-6$. (**A**) $Asc^{\bullet-}$ observed for reactions in 0.10 M HEPES (▲) or 0.050 M Tris·HCl (●) buffer. (**B**) Carbon-based DMPO radical adducts observed for reactions in 0.10 M HEPES (▲) or 0.050 M Tris·HCl (●) buffer. DMPO adduct levels were significant between buffers for ascorbate to Cr(VI) ratios of 0.5:1 and 1:1 at $P < 0.01$ (ANOVA).

underestimated if there are competing side reactions; thus quantitation shows only the relative trend of Cr(IV) *vs* ascorbate concentration. Control solutions showed that Mn(II) reacted with CT DNA, resulting in a decrease in the Mn(II) EPR signal intensity for any reaction or control solution containing DNA. Chromium(IV) levels were therefore calculated from data collected in the absence of CT DNA. Thus, this method does not allow for detection of a reaction of Cr(IV) with DNA; however, reactivity could be evaluated by comparison of the Cr−DNA binding and plasmid nicking results at the various reaction ratios (discussed below).

The $asc^{\bullet-}$ ($g = 2.006$, $A_H = 1.82(8)$ G) was observed at all reaction ratios of ascorbate to Cr(VI) in both buffers, reaching a maximum of 1 μM for reactions with excess ascorbate in Tris·HCl buffer (Figure 3A). The $asc^{\bullet-}$ was not trapped by DMPO, and DMPO did not affect the signal intensity; nor was the signal intensity affected by DNA.

Carbon-based free radicals presumed to result from oxidative fragmentation of ascorbate by Cr(VI) were trapped with DMPO as previously described (Stearns & Wetterhahn, 1994). The DMPO−radical adducts were only observed at the 0.5:1 and 1:1 ascorbate to Cr(VI) reaction ratios (Figure 3B). Radicals were observed in both buffers, but DMPO adduct levels were 2-fold higher in Tris·HCl buffer. In HEPES buffer DMPO−radical adducts were measured at 1.1 ± 0.6 μM for the 0.5:1 and 1:1 ratios, whereas in Tris·HCl buffer DMPO adducts were detected at 2.8 ± 0.6 μM for the 0.5:1 and 1:1 reaction ratios (Figure 3B). Within the limits of detection there was no difference in the signal intensities of the DMPO adducts in the presence and absence of CT DNA. Spectroscopic evaluation of the reactivity of the carbon-based radicals with DNA was obstructed by concentration limits, but reactivity was assessed through the plasmid nicking experiments described below.

Chromium(III) Is the Final Reaction Product. Formation of the Cr(III) end product(s) was monitored by UV/vis spectroscopy. Reaction on $K_2Cr_2O_7$ (1.8 mM Cr(VI)) with sodium ascorbate (0.90−18 mM) in 0.10 M HEPES or 0.050 M Tris·HCl buffer (pH 7.0, RT) was followed over time at room temperature. Reactions had gone to completion by 30 min. For all ratios of ascorbate to Cr(VI) the spectra showed a decrease in the Cr(VI) charge transfer band at 370 nm over time and a concomitant increase in visible bands at ~400 and ~570−580 nm, consistent with the previously published spectra (Dixon et al., 1993). The final λ_{max} for Cr(III) varied between buffers and reaction ratios. For a 36 min reaction in HEPES buffer the 0.5:1 reaction showed a visible absorbance at 568 nm ($\epsilon = 54\ M^{-1}\ cm^{-1}$) with the higher energy visible absorbance masked by unreacted Cr(VI). The 10:1 reaction gave a final spectrum with an absorbance at 584 nm ($\epsilon = 48\ M^{-1}\ cm^{-1}$) and a shoulder (obstructed by unreacted ascorbate) at 400 nm. Reactions in Tris·HCl buffer showed the same shift to higher λ_{max} for increasing concentrations of reactant ascorbate, with reaction spectra showing a final λ_{max} of 558 nm ($\epsilon = 56\ M^{-1}\ cm^{-1}$) and 580 nm ($\epsilon = 49\ M^{-1}\ cm^{-1}$) for 0.5:1 and 10:1 reactions, respectively. In the presence of excess ascorbate the Cr(III) would presumably be coordinated by ascorbate, whereas in reactions with excess Cr(VI) the only potential ligands for Cr(III) would be DHA or, more accurately, its ring open form DKG (Tolbert & Ward, 1982).

The amounts of Cr(III) product were estimated at a 1.3 min reaction time to correspond with the EPR experiments, and at 36 min to measure total Cr(III) formed over the time of DNA incubations (Figure 1C). The calculated extinction coefficient for Cr(III) in a particular sample was most likely an average of coefficients for Cr(III) products, which differed by coordinating ligand and/or oligomerization, and thus could not necessarily be applied to different reaction ratios. The extinction coefficients were therefore estimated in two different ways. First, the total yield of Cr(III) was calculated on the basis of stoichiometry for reactions assumed to be complete at 36 min. For reactions with stoichiometric or excess Cr(VI) the total yield of Cr(III) was also calculated from the loss of Cr(VI). Concentrations of Cr(III) ranged from ~37% to 90% of total Cr as Cr(III) for the 1.3 min reaction in both buffers, and 43−100% for the completed reaction at 36 min. The error bars for Cr(III) concentrations in Figure 1C reflect the range of possible extinction coefficients.

To summarize, the above spectroscopic experiments showed that the levels of intermediates and Cr(III) were dependent on reaction ratio and buffer. Levels of Cr(III), Cr(IV), and $asc^{\bullet-}$ increased with increasing ascorbate concentration. Levels of Cr(V) and DMPO/$R^{\bullet}$ were observed at 0.5 and 1 equiv of ascorbate to Cr(VI), or less than stoichiometric ascorbate (eq 1). Reactions of Cr(VI) with ascorbate in HEPES buffer showed lower levels of DMPO/$R^{\bullet}$ and higher levels of Cr(V) than reactions in Tris·HCl buffer. Thus, these reaction conditions could be used to

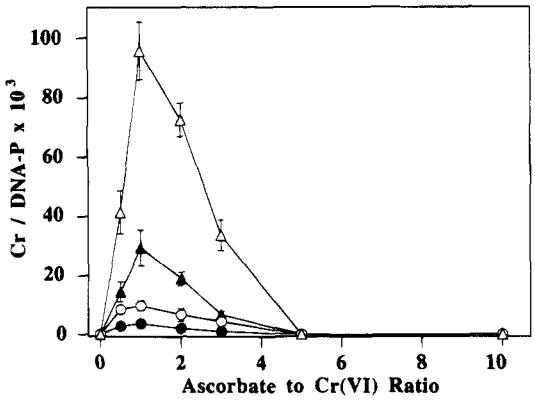

FIGURE 4: Binding of chromium to double-stranded and single-stranded CT DNA as a function of the ascorbate to Cr(VI) reaction ratio and buffer. Double-stranded (closed symbols) or denatured (open symbols) CT DNA (1.8 mM DNA-P) was incubated with $K_2Cr_2O_7$ (1.8 mM Cr(VI)) and ascorbic acid (0−18.0 mM) in either 0.10 M HEPES (▲, △) or 0.050 M Tris·HCl (●, ○) buffer at pH 7.0, 37 °C, for 30 min. Values plotted are mean binding ratios ± SD for n = 10−16. Within experiments the differences between buffers were significant for ascorbate to Cr(VI) ratios of 0.5:1 to 3:1 at $P \leq 0.01$ (ANOVA).

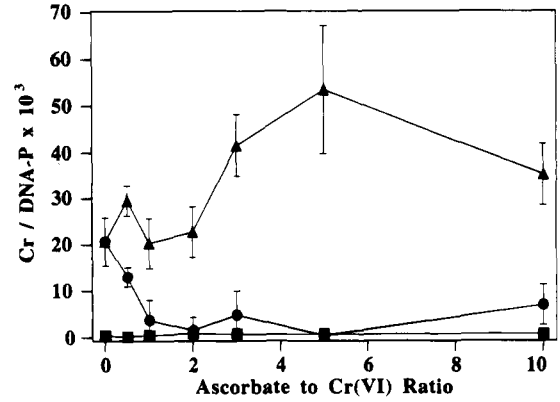

FIGURE 5: Effect of preincubations of Cr(VI) and Cr(III) with varying concentrations of ascorbate on the binding of Cr to native CT DNA. Final reaction concentrations were 1.8 mM Cr, 0−18 mM ascorbic acid, and 1.8 mM DNA-P in 0.10 M HEPES (pH 7.0, 37 °C). (■) Preincubation of Cr(VI) ($K_2Cr_2O_7$) and ascorbic acid for 30 min at 37 °C before addition to DNA; (●) preincubation of Cr(III) ($Cr(NO_3)_3 \cdot 9H_2O$) and ascorbic acid for 30 min at 37 °C before addition to DNA; (▲) immediate reaction of CT DNA with Cr(III) in the presence of ascorbic acid. Values plotted are mean binding ratios ± SD for n = 2−7.

evaluate reactivity of Cr and radical intermediates with DNA.

Formation of Cr−DNA Adducts. Preliminary results have shown that reaction of 1.80 mM Cr(VI) with 1.80 mM ascorbate in the presence of CT DNA or pBR322 plasmid DNA resulted in formation of Cr−DNA adducts and DNA single-strand breaks, respectively (Stearns et al., 1994). The final oxidation state of Cr bound to DNA is Cr(III); however, our working hypothesis is that the more labile intermediates, Cr(V) and/or Cr(IV), will be more likely to react with DNA than the relatively inert Cr(III). For example, reaction of Cr(V) with DNA followed by oxidation of an associated ligand, excess reductant, or DNA itself would result in the formation of a final Cr(III) product bound to DNA. The objectives of the current study were to evaluate the reactivity of radicals, Cr(V), Cr(IV), and Cr(III) toward DNA, and to shed light on the possible mechanism of Cr−DNA adduct formation.

CT DNA (1.8 mM DNA-P) was incubated with $K_2Cr_2O_7$ (1.8 mM Cr(VI)) and varying ascorbate (0−18 mM) in either 0.10 M HEPES buffer or 0.050 M Tris·HCl buffer (pH 7.0) for 30 min at 37 °C. Chromium and ascorbate were either reacted immediately with DNA or were preincubated at 37 °C for 30 min before incubation with DNA. For immediate reaction with native CT DNA higher Cr binding to DNA was observed in HEPES *vs* Tris·HCl buffer (Figure 4). Maximum binding of Cr to DNA was observed in HEPES buffer at the 1:1 ascorbate to Cr(VI) ratio, at levels of (29.4 ± 6.1) × 10^{-3} Cr/DNA-P. The 1:1 reaction in Tris·HCl buffer resulted in a binding ratio of (3.7 ± 1.1) × 10^{-3} Cr/DNA-P, which was ~8-fold lower than that in HEPES buffer. At the 5:1 and 10:1 ascorbate to Cr(VI) reaction ratios, binding levels dropped to zero in both HEPES and Tris·HCl buffers (Figure 4). The higher binding of Cr to DNA in HEPES *vs* Tris·HCl buffer and at low reaction ratios correlated with the presence of Cr(V) as detected by EPR spectroscopy. On the basis of spectroscopic estimation of the relative concentrations of reactive intermediates the levels of Cr(IV) and Cr(III) increased with increasing reactant ascorbate concentrations. If Cr(IV) or Cr(III) were mediating the binding of Cr to DNA, then Cr/DNA binding would be expected to follow the same trend; however, this was not observed.

Another possible interpretation of these results that could potentially be supported by the spectroscopic analysis was that binding of Cr to DNA was dependent on Cr(III) with "open" coordination sites (for example, H_2O) (Hneihan et al., 1993) and that the coordination of Cr(III) was a function of ascorbate concentration. Several control experiments were run with Cr(III) to explore the possible effect of ligand coordination on the ability of Cr(III) to bind DNA. Reaction of Cr(III) (2.7 mM) with ascorbic acid (1.35−27.0 mM) for 30 min at 37 °C prior to reaction with CT DNA (final concentrations 1.8 mM Cr(III), 1.8 mM DNA-P, 0−18 mM ascorbate) resulted in only low binding to DNA (Figure 5). Immediate reaction of Cr(III) with ascorbate in the presence of DNA resulted in higher binding of Cr to DNA (Figure 5). This was consistent with Cr(III) reacting with DNA before full coordination with ascorbate. The increase in Cr/DNA-P with increasing ascorbate may also reflect the ability of ascorbate coordination to decrease hydrolysis and precipitation of Cr(III) at pH 7. These experiments showed that the ability of Cr(III) to bind DNA was dependent on the Cr(III) coordination, in support of previous work (Hneihan et al., 1993); however, since the Cr(III) complexes resulting from reaction of Cr(III) with ascorbate were not necessarily the same species formed from reduction of Cr(VI) by ascorbate, a third control reaction was carried out. This consisted of preincubation of Cr(VI) with ascorbate for 30 min at 37 °C before reaction with DNA. The UV/vis and EPR studies reported above had shown that by 30 min the reaction of Cr(VI) with ascorbate had gone to completion and that the Cr(V), $asc^{\bullet-}$, and carbon-based radicals had decayed; therefore, DNA was actually incubated with product Cr(III) species, DHA (presumably in the form of open-ring DKG), organic products of ascorbate fragmentation, and depending on the reaction ratio unreacted Cr(VI) or ascorbate. Preincubation of Cr(VI) with ascorbate resulted in complete quenching of Cr bound to DNA (Figure 5) even at the low ratios of ascorbate to Cr(VI). Chromium(III) species with open coordination sites would be expected to bind DNA;

therefore, for the reaction of Cr(VI) with ascorbate the Cr(III) products were concluded to be less reactive than the other chromium intermediates.

The coordination environment of the final Cr(III)−DNA adduct is unclear. Chromium(III) has been proposed to react at the phosphate backbone of DNA (Cohen et al., 1990); however, positive interaction of Cr(III) and phosphate has only been shown with mononucleotides containing free phosphate groups (Campomar et al., 1986; Wolf et al., 1989). Another possibility is the N(7) position of guanine, which is a well-established binding site for metals (Marzilli et al., 1980) and has also been suggested for chromium (Tsapakos & Wetterhahn, 1983; Schaaper et al., 1987). In this study the binding of Cr to native double-stranded DNA was compared to that with denatured single-stranded DNA. It was expected that if Cr were reacting at the nucleotide bases, then reaction with single-stranded DNA should provide more accessible bases, and therefore higher levels of Cr/DNA adducts. If binding were occurring only at the phosphate backbone, there should be little or no difference in binding levels. Reactions were carried out as stated above with $K_2Cr_2O_7$ (1.8 mM Cr(VI)) and ascorbic acid (0−18 mM) incubated with single-stranded DNA (1.8 mM DNA-P) in 0.050 M Tris·HCl or 0.10 M HEPES buffer (pH 7.0, 37 °C). Reaction of Cr(VI) and varying ascorbate with denatured CT DNA resulted in the highest levels of binding at the 1:1 reaction ratio (Figure 4) following the same trend as that observed with native DNA. In HEPES buffer the binding level with single-stranded DNA was $(95.8 \pm 9.6) \times 10^{-3}$ Cr/DNA-P, which was 3.3-fold higher than that observed with double-stranded DNA. In Tris·HCl buffer the binding level for the 1:1 Cr(VI) to ascorbate ratio was $(9.7 \pm 1.7) \times 10^{-3}$ Cr/DNA-P, which was 2.6-fold higher than that found with double-stranded DNA. These results are consistent with Cr reacting at the nucleotide bases of DNA.

Chromium Does Not Cross-Link Ascorbate to DNA. The reduction of Cr(VI) by GSH in the presence of CT DNA has been shown to result in GSH−Cr−DNA adducts (Borges & Wetterhahn, 1989). In order to evaluate the analogous role of ascorbate in Cr−DNA adduct formation, the Cr−DNA binding experiments were carried out with $^{14}C(1)$-labeled ascorbic acid. CT DNA (1.8 mM DNA-P) was incubated with Cr(VI) (1.8 mM) and varying ^{14}[C]ascorbic acid (0−9.0 mM) in either 0.10 M HEPES or 0.050 M Tris·HCl buffers at pH 7.0 for 30 min at 37 °C. The presence of Cr(VI) did not result in ascorbate binding to DNA above control levels in either buffer. Thus, it appears that ascorbate is not cross-linked to DNA by chromium.

Formation of pBR322 DNA Single-Strand Breaks. Transient DNA strand breaks have been observed in several in vivo and cell culture systems after Cr(VI) treatment (reviewed in De Flora & Wetterhahn, 1989). The objective in the current study was to evaluate the ability of the reactive intermediates formed upon reaction of Cr(VI) and ascorbate to cause single-strand breaks in plasmid DNA. Solutions of $K_2Cr_2O_7$ (1.8 mM Cr(VI)) and ascorbic acid (0−5.4 mM) were incubated with pBR322 DNA (0.36 mM DNA-P, 82 nM plasmid) in either 0.10 M HEPES or 0.050 M Tris·HCl buffers (pH 7.0, 37 °C) for 30 min at 37 °C. Changes in conformation of the plasmid DNA from the supercoiled (form I) monomer to the nicked circular (form II) or linear (form III) were monitored by gel electrophoresis and quantitated by scanning densitometry. For reaction solutions containing

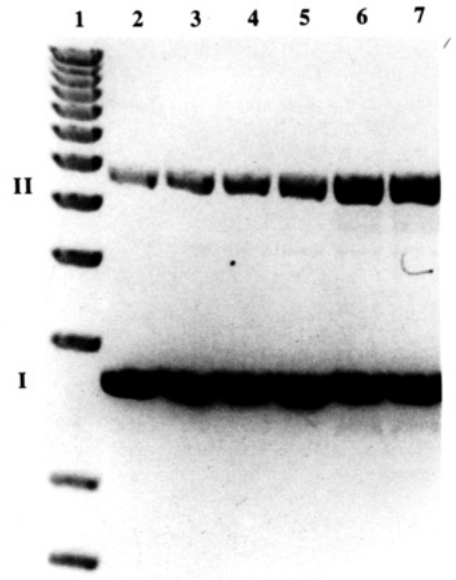

FIGURE 6: Agarose gel showing single-strand breaks (form II) induced in pBR322 DNA (0.36 mM DNA-P) after incubation with $K_2Cr_2O_7$ (1.8 mM Cr(VI)) in the presence or absence of ascorbic acid (1.8 mM) for 30 min at 37 °C in 0.050 M Tris·HCl/25 μM DETAPAC, pH 7.0. Lanes are as follows: 1, MW marker; 2, DNA alone; 3, Cr(VI) alone; 4 and 5, ascorbic acid alone; 6 and 7, Cr(VI) + ascorbic acid.

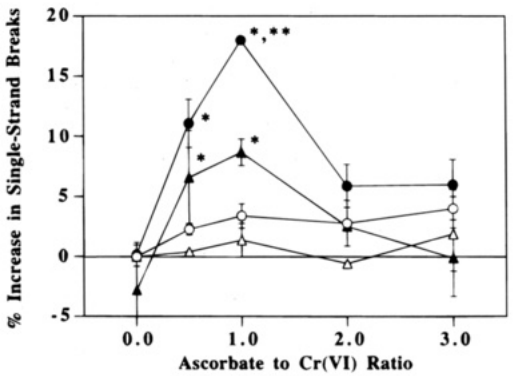

FIGURE 7: Single-strand breaks induced in pBR322 DNA after incubation with $K_2Cr_2O_7$ and ascorbic acid as a function of the ascorbate to Cr(VI) reaction ratio and buffer. pBR322 DNA (0.36 mM DNA-P) was incubated with ascorbic acid (0−5.4 mM) in the presence of 1.8 mM Cr(VI) in 0.10 M HEPES/15 μM DETAPAC buffer (▲); in the absence of Cr(VI) in 0.10 M HEPES/15 μM DETAPAC buffer (△); in the presence of 1.8 mM Cr(VI) in 0.050 M Tris·HCl/25 μM DETAPAC buffer (●); and in the absence of Cr(VI) in 0.050 M Tris·HCl/25 μM DETAPAC buffer (○). Incubations were carried out at pH 7.0, 37 °C, for 30 min. Values plotted are mean ± SD for $n = 3-7$. The symbol * indicates statistical significance between reactions and controls at $P < 0.05$, and ** indicates significance between reactions in HEPES and Tris·HCl at $P < 0.05$ (ANOVA).

both Cr(VI) and ascorbate the highest DNA strand breakage was observed at the 1:1 reaction ratio (Figures 6 and 7). However, the observed buffer effect was the opposite of that seen in the Cr/DNA binding study: reactions in Tris·HCl buffer resulted in higher nicking than reactions in HEPES buffer (Figure 7). Incubation of plasmid DNA with equimolar Cr(VI) and ascorbate resulted in 2-fold higher levels of DNA single-strand breaks in Tris·HCl than HEPES buffer. Interpretation of these results in light of the spectroscopic quantitation of intermediates suggested that these observed DNA single-strand breaks were caused by radicals and not Cr(V) because Cr(V) was not observed in Tris·HCl buffer,

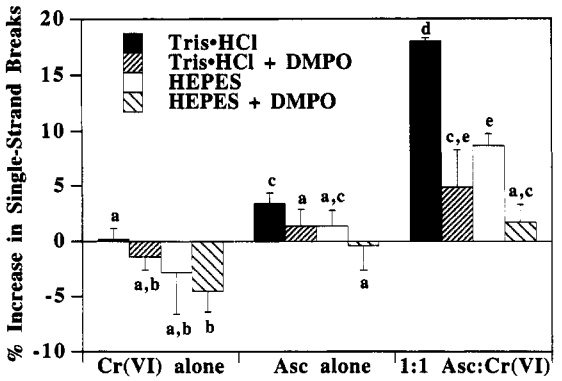

FIGURE 8: Effect of DMPO on single-strand breaks in pBR322 DNA after incubation with Cr(VI) and ascorbate. pBR322 DNA (0.36 mM DNA-P) was incubated with $K_2Cr_2O_7$ (1.8 mM Cr(VI)) and/or ascorbic acid (1.8 mM) in the presence or absence of 0.10 M DMPO in either 0.10 M HEPES/15 μM DETAPAC buffer or 0.050 M Tris•HCl/25 μM DETAPAC buffer at pH 7.0, 37 °C, for 30 min. Values plotted are mean ± SD for $n = 3-7$. Data points having no letters in common (a–e) are significant at $P < 0.05$ (ANOVA).

whereas 2-fold more carbon-based DMPO radical adducts were observed in Tris•HCl buffer than in HEPES buffer. If Cr(V) were causing DNA single-strand breaks, higher activity should have been observed in HEPES buffer. This hypothesis was explored by determining the effect of the spin trap DMPO on DNA single-strand breaks. Addition of 0.10 M DMPO to the 1:1 reaction ratio resulted in quenching of single-strand breaks in plasmid DNA in both HEPES and Tris•HCl buffers (Figure 8). The spin trap decreased plasmid relaxation in reaction solutions, as well as in controls of 1.8 mM Cr(VI) or 1.8 mM ascorbate alone. Addition of DMPO to the 1:1 Cr(VI) to ascorbate reaction resulted in a 4-fold decrease in DNA single-strand breaks in Tris•HCl buffer and a 5-fold decrease in strand breaks in HEPES buffer. This result suggested that radicals formed during the reaction of Cr(VI) with ascorbate were the major cause of single-strand breaks in plasmid DNA, since the level of Cr(V) was unaffected by DMPO (Figure 2).

DISCUSSION

EPR and UV/visible spectroscopies have been used to determine the relative amounts of Cr(V), Cr(IV), and Cr(III) species, and free radicals formed from reaction of Cr(VI) with different amounts of ascorbate. The most Cr(IV) and Cr(III) were observed under conditions of excess ascorbate, whereas chromium(V) and carbon-based free radicals were observed under substoichiometric conditions of 0.5:1 and 1:1 ascorbate to Cr(VI). The coordination of the final Cr(III) product(s) varied with the concentration of ascorbate. UV/vis spectroscopy suggested that Cr(III) was coordinated by ascorbate when ascorbate was in excess, and was likely coordinated by DHA and/or DKG when ascorbate was the limiting reactant.

Reaction of Cr(VI) with ascorbate in the presence of CT DNA resulted in Cr covalently bound to DNA. The highest levels of Cr bound to DNA were observed for reaction conditions that produced the most Cr(V), which were low ratios of ascorbate to Cr(VI). This interpretation was further supported by the buffer effect because Cr(V) was not stable in Tris•HCl buffer and Cr–DNA binding was decreased in Tris•HCl relative to HEPES buffer. Preincubation of Cr(VI) with ascorbate to produce the final Cr(III) product(s) prior to reaction with DNA also resulted in loss of Cr binding to DNA. This also suggested that Cr(V) was more reactive toward DNA than Cr(III).

The observation that more Cr was bound in reactions carried out with single-stranded DNA *vs* double-stranded DNA suggested that Cr was reacting at the nucleotide base rather than at the phosphate backbone. Although a previous study (Salnikow et al., 1992) that analyzed the digestion products of Cr-bound DNA after treatment of intact cells with Cr(VI) concluded that the final Cr(III) was coordinated to the phosphate backbone only, many other previous studies have demonstrated a specificity for Cr binding to guanine-rich nucleic acids, which supports the hypothesis that at least partial Cr binding occurs at the nucleotide base. Treatment of homopolyribonucleotides with Cr(VI) in the presence of NADPH and rat liver microsomes produced Cr-bound polymers with Cr-binding levels for poly(G) higher than those of poly(A), poly(C), or poly(U) (Tsapakos & Wetterhahn, 1983). Reaction of Cr(VI) with GSH in the presence of DNA polynucleotides resulted in higher Cr binding for polydeoxynucleotides that contained guanine, and 10-fold higher levels for poly(dG) than poly(dT) and poly(dA) (Borges & Wetterhahn, 1989). Chromium(III) bound to double-stranded pSV2neoTS plasmid DNA was found to induce polymerase arrest one nucleotide upstream of guanine, suggesting chromium binding specifically to guanine bases (Bridgewater et al., 1994). In contrast, both tight binding sites and nonspecific binding of chromium(III) were found with single-stranded M13mp2 DNA, resulting in increased processivity of DNA polymerase, increased bypass of oxidative DNA lesions, and increased mutagenesis in *E. coli* (SOS^-) host cells (Snow, 1994).

Reaction of Cr(VI) with ascorbate in the presence of pBR322 DNA resulted in conversion of supercoiled (form I) to nicked-circular (form II) DNA. The highest levels of DNA single-strand breaks were observed when ascorbate was the limiting reactant. This result was consistent with reactivity of either Cr(V) or carbon-based radicals toward plasmid DNA. It was concluded that for these conditions the carbon-based radicals were more reactive than Cr(V) toward DNA single-strand breaks for the following reasons: (i) 2-fold higher levels of DNA single-strand breaks were observed in Tris•HCl *vs* HEPES buffer, and EPR studies showed 2-fold higher levels of carbon-based radicals in Tris•HCl *vs* HEPES buffer, but higher levels of Cr(V) in HEPES *vs* Tris•HCl buffer; and (ii) addition of DMPO to reaction solutions resulted in quenching of DNA single-strand breaks, and EPR studies showed that DMPO reacted with radicals but not Cr(V). Carbon-based radicals have been found to produce single- and double-strand breaks in plasmid DNA (Leite & Augusto, 1989; Iheanacho et al., 1991; Hiramoto et al., 1993) and have been proposed in the mechanism of DNA damage of the enediyne antitumor agents neocarzinostatin and calicheamicin (Dedon & Goldberg, 1992). The mechanism of Cr-induced single-strand breaks is unknown. The C1′, C4′, and C5′ positions of deoxyribose have been found to be the most susceptible to H atom abstraction by radicals in the initial step leading to DNA strand breaks by neocarzinostatin and calicheamicin (Dedon & Goldgberg, 1992). However, DNA strand breakage by neocarzinostatin and calicheamicin has been shown to be dependent on oxygen (Dedon & Goldberg, 1992). The effect

of dioxygen on Cr-induced single-strand breaks was not determined in our studies. However, the formation of Cr(V) and carbon-based radicals from the reaction of Cr(VI) and ascorbate was found to be independent of oxygen, hydroxyl radical was not observed as a product of the reaction, and the carbon radicals were trapped by formate in the absence of oxygen (Stearns & Wetterhahn, 1994). Although the single-strand breaks induced by neocarzinostatin and calicheamicin were oxygen dependent (Dedon & Goldberg, 1992), other carbon-based radicals have been shown to cleave plasmid DNA anaerobically (Augusto et al., 1984; Iheanacho et al., 1991; Hiramoto et al., 1993). Thus, oxygen may not be required for strand breaks induced by carbon-based radicals in the reaction of plasmid DNA with Cr(VI) and ascorbate. The formation of carbon-based radicals during the reduction of Cr(VI) in vivo has yet to be established, and DNA single-strand breaks may be a lesion of lesser importance than Cr−DNA adducts in Cr(VI)-induced carcinogenesis since they are more easily repaired (Hamilton & Wetterhahn, 1986).

This current model in which Cr reacts with DNA at the oxidation state of Cr(V) prior to reduction to the substitutionally inert Cr(III) is supported by previous studies. The reaction of CT DNA with Cr(VI) and the thiols glutathione, cysteine, β-mercaptoethanol, and dithiothreitol resulted in Cr−DNA binding that correlated with levels of Cr(V) (Borges et al., 1991; Aiyar et al., 1991). Reaction of Cr(VI) with glutathione in the presence of plasmid DNA led to strand breaks only in the presence of hydrogen peroxide, with the concomitant formation of hydroxyl radical (Aiyar et al., 1990).

The Cr(V) complex of 2-ethyl-2-hydroxybutyric acid (EHBA) has been shown to produce plasmid relaxation at pH 3.8 (Farrell et al., 1989). A mechanism was suggested that involved decay to Cr(IV), which was proposed to be more reactive than Cr(V) (Barr-David et al., 1992). DNA cleavage has also been proposed to result from Cr(V) produced in vivo (Farrell & Lay, 1992). However, our current results do not support this hypothesis. There are several possible explanations for the difference in reactivity between the "Cr(V) ascorbate" complex at pH 7.0 and $[Cr(V)O(EHBA)_2]^-$ at pH 3.8. The first is the effect of pH on the Cr redox potential. The Cr(V)EHBA complex has been shown to undergo two different decay pathways depending on pH (Krumpolc & Roček, 1985). At high pH Cr(V)EHBA undergoes disproportionation to give 2Cr(VI) + Cr(III). At low pH decay occurs through ligand oxidation, demonstrating a greater oxidizing strength of Cr(V) at acidic pH. The ligand will affect the redox potential as well. The redox potential of Cr(V)EHBA has been shown to correlate with the EPR ^{53}Cr hyperfine splitting (Judd, 1992), where a larger splitting was indicative of a more easily reduced Cr(V). The Cr(V) EHBA complex in acetic acid gave $A^{53}_{Cr} = 18.5$-(3) G (Judd, 1992) whereas that of the "Cr(V) ascorbate" at pH 7.0 was 17.8(1) G (Stearns & Wetterhahn, 1994). It is likely as well that the reaction of Cr(V) with DNA is influenced by the coordinating ligand. The EHBA ligand may be more resistant to oxidation than the DNA sugar moiety; thus Cr(V)EHBA coordination would be followed by oxidative strand breakage, whereas with the "Cr(V) ascorbate" complex the ascorbate may be oxidized preferentially resulting in Cr(III) bound to uncleaved DNA.

Relevance to Cr(VI) Metabolism in Vivo. The concentration of 1.8 mM Cr(VI) used in this study resulted in Cr(V) concentrations that approached the lower limit of detection for Cr(V) by EPR spectroscopy, but this concentration and the Cr(VI) to ascorbate reaction ratios were deemed to be in the physiologically relevant range on the basis of intracellular Cr and ascorbate concentrations measured in vivo after treatment with nontoxic doses of Cr(VI). Chromium intracellular concentrations have been measured at 1.2 mM in CHO cells treated with 1.6 $\mu g/cm^2$ particulate $PbCrO_4$ for 2 h (Wise et al., 1993). Treatment of rats with 10 mg/kg Na_2CrO_4 resulted in Cr levels of 0.55 mM in rat kidney 2 h post-treatment (Standeven & Wetterhahn, 1991b) and Cr levels of 0.5−0.7 mM in rat liver 4 h post-treatment (Misra and Wetterhahn, unpublished results). Cr levels of 0.1−0.2 mM were measured in red blood cells and liver of 14 day old chick embryos treated with 0.1 mmol/kg body weight Cr(VI) for 2 h (Hamilton & Wetterhahn, 1986). In vivo ascorbate concentrations have been measured at ~1 mM in both 14 day old chick embryo liver and red blood cells (Misra et al., submitted for publication) and in rat liver and kidney (Standeven & Wetterhahn, 1992).

Formation of Cr(V) in cell culture and animal studies has been correlated with Cr(VI)-induced DNA damage. In CHO cells treated with Na_2CrO_4 formation of Cr(V) was observed by EPR spectroscopy and was linked to alkali-labile sites, whereas DNA−protein cross-links were proposed to correlate with Cr(III) (Sugiyama et al., 1991). It should be noted that a distinction is made in the present study between DNA single-strand breaks and alkali-labile sites whereas the lesions were equated in the previous study (Sugiyama et al., 1991). A Cr−DNA adduct at the N-7 position of purines may lead to an alkali-labile site through enhanced hydrolysis of the *N*-glycosidic bond (Schaaper et al., 1987), but would not necessarily produce a strand break at pH 7.0.

In the chick embryo in vivo model Cr(V) has been observed by EPR spectroscopy in liver after treatment with a nontoxic dose of $Na_2Cr_2O_7$ (Liebross & Wetterhahn, 1992). Chromium(V) was observed in red blood cells only at a higher toxic dose of Cr(VI). Chromium(VI)-induced DNA damage has been measured in the chick embryo system. DNA−protein cross-links and Cr−DNA adducts were observed in embryo liver after a nontoxic dose of Cr(VI); however, in red blood cells the nontoxic dose of Cr(VI) produced DNA single-strand breaks and 8-oxo-2′-deoxyguanosine in the absence of Cr−DNA adducts (Hamilton & Wetterhahn, 1986; Misra et al., submitted for publication). This is consistent with the current study correlating Cr(V) with Cr−DNA adducts, not DNA single-strand breaks.

CONCLUSIONS

To summarize, reduction of Cr(VI) by ascorbate under physiological conditions produced Cr(V) and carbon-based radicals as intermediates that reacted with DNA to produce Cr−DNA adducts and DNA single-strand breaks, respectively. The spectroscopic studies showed that the reactive intermediates produced during the reaction of Cr(VI) with ascorbate were dependent on the concentration of reducing agent relative to Cr(VI). Application of this observation to in vivo experiments suggests that the type and extent of Cr(VI)-induced DNA damage in tissues and cell lines will be a function of the concentration of intracellular reducing

agents and will therefore differ between tissues and cell lines with different concentrations of reductants. Effects of the addition or depletion of intracellular reducing agents should be evaluated in terms of changes relative to basal levels. These tissue-specific effects of intracellar reductant concentrations may lead to two pathways of chromium(VI)-induced DNA damage in vivo, namely, a direct pathway of DNA lesions mediated by reactive chromium intermediates, and an indirect oxidative pathway mediated by free radicals.

REFERENCES

Aiyar, J., Berkovits, H. J., Floyd, R. A., & Wetterhahn, K. E. (1990) *Chem. Res. Toxicol. 3*, 595−603.

Aiyar, J., Berkovits, H. J., Floyd, R. A., & Wetterhahn, K. E. (1991) *Environ. Health Perspect. 92*, 53−62.

Augusto, O., Faljoni-Alário, A., Leite, L. C. C., & Nóbrega, F. G. (1984) *Carcinogenesis 5*, 781−784.

Barr-David, G., Hambley, T. W., Irwin, J. A., Judd, R. J., Lay, P. A., Martin, B. D., Bramley, R., Dixon, N. E., Hendry, P., Ji, J.-Y., Baker, R. S., & Bonin, A. M. (1992) *Inorg. Chem. 31*, 4906−4908.

Borges, K. M., & Wetterhahn, K. E (1989) *Carcinogenesis 10*, 2165−2168.

Borges, K. M., Boswell, J. S., Liebross, R. H., & Wetterhahn, K. E. (1991) *Carcinogenesis 12*, 551−561.

Bridgewater, L. C., Manning, F. C. R., Woo, E. S., & Patierno, S. R. (1994) *Mol. Carcinog. 9*, 122−133.

Buettner, G. R. (1988) *J. Biochem. Biophys. Methods 16*, 27−40.

Buettner, G. R., & Oberley, L. W. (1978) *Biochem. Biophys. Res. Commun. 83*, 69−74.

Campomar, J. A., Fiol, J. J., Terron, A., & Moreno, V. (1986) *Inorg. Chim. Acta 124*, 75−81.

Cohen, M., Latta, D., Coogan, T., & Costa, M. (1990) in *Biological Effects of Heavy Metals* (Foulkes, E. C., Ed.) pp 40−42, CRC Press, Boca Raton, FL.

Connett, P. H., & Wetterhahn, K. E. (1983) *Struct. Bonding 54*, 93−124.

Connett, P. H., & Wetterhahn, K. E. (1985) *J. Am. Chem. Soc. 107*, 4282−4288.

Cupo, D. Y., & Wetterhahn, K. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6755−6759.

Dalal, N. S., Millar, J. M., Jagadeesh, M. S., & Seehra, M. S. (1981) *J. Chem. Phys. 74*, 1916−1923.

Dedon, P. C., & Goldberg, I. H. (1992) *Chem. Res. Toxicol. 5*, 311−332.

De Flora, S., & Wetterhahn, K. E. (1989) *Life Chem. Rep. 7*, 169−244.

De Flora, S., Bagnasco, M., Serra, D., & Zanacchi, P. (1990) *Mutat. Res. 238*, 99−172.

Dixon, D. A., Sadler, N. P., & Dasgupta, T. P. (1993) *J. Chem. Soc., Dalton Trans.*, 3489−3495.

Egan, T. J., Barthakur, S. R., & Aisen, P. (1992) *J. Inorg. Biochem. 48*, 241−249.

Farrell, R. P., & Lay, P. A. (1992) *Comments Inorg. Chem. 13*, 133−175.

Farrell, R. P., Judd, R. J., Lay, P. A., Dixon, N. E., Baker, R. S. U., & Bonin, A. M. (1989) *Chem. Res. Toxicol. 2*, 227−229.

Goodgame, D. M. L., & Joy, A. M. (1987) *Inorg. Chim. Acta 135*, 115−118.

Hamilton, J. W., & Wetterhahn, K. E. (1986) *Carcinogenesis 7*, 2085−2088.

Hiramoto, K., Johkoh, H., Sako, K.-I., & Kikugawa, K. (1993) *Free Radical Res. Commun. 19*, 323−332.

Hneihen, A. S., Standeven, A. M., & Wetterhahn, K. E. (1993) *Carcinogenesis 14*, 1795−1803.

Iheanacho, E. N., Sarel, S., Samuni, A., Avramovici-Grisaru, S., & Spira, D. T. (1991) *Free Radical Res. Commun. 11*, 307−315.

International Agency for Research on Cancer (IARC) (1987) *IARC Monogr. Eval. Carcinog. Risk Chem. Hum. Suppl. 7*, 165−168.

Judd, R. J. (1992) Ph.D. Dissertation, University of Sydney.

Kissane, J. M., & Robins, E. (1958) *J. Biol. Chem. 233*, 184−188.

Krumpolc, M., & Roček, J. (1985) *Inorg. Chem. 24*, 617−621.

Leite, L. C. C., & Augusto, O. (1989) *Arch. Biochem. Biophys. 270*, 560−572.

Léonard, A., & Lauwerys, R. R. (1980) *Mutat. Res. 76*, 227−239.

Liebross, R. H., & Wetterhahn, K. E. (1992) *Carcinogenesis 13*, 2113−2120.

Lin, X., Zhuang, Z., & Costa, M. (1992) *Carcinogenesis 13*, 1763−1768.

Marzilli, L. G., Kistenmacher, T. J., & Eichhorn, G. L. (1980) in *Nucleic Acid−Metal Ion Interactions* (Spiro, T. G., Ed.) pp 186−219, Wiley & Sons, New York.

Salnikow, K., Zhitkovich, A., & Costa, M. (1992) *Carcinogenesis 13*, 2341−2346.

Sambrook, J., Fritsch, E. F., & Maniatis, T., Eds. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schaaper, R. M., Koplitz, R. M., Tkeshelashvili, L. K., & Loeb, L. A. (1987) *Mutat. Res. 177*, 179−188.

Snow, E. T. (1994) *Environ. Health Perspect. 102* (Suppl. 3), 41−44.

Standeven, A. M., & Wetterhahn, K. E. (1989) *J. Am. Coll. Toxicol. 8*, 1275−1283.

Standeven, A. M., & Wetterhahn, K. E. (1991a) *Carcinogenesis 12*, 1733−1737.

Standeven, A. M., & Wetterhahn, K. E. (1991b) *Pharmacol. Toxicol. 68*, 469−476.

Standeven, A. M., & Wetterhahn, K. E. (1992) *Carcinogenesis 13*, 1319−1324.

Stearns, D. M., & Wetterhahn, K. E. (1994) *Chem. Res. Toxicol. 7*, 219−230.

Stearns, D. M., Courtney, K. D., Giangrande, P. H., Phieffer, L. S., & Wetterhahn, K. E. (1994) *Environ. Health Perspect. 102* (Suppl. 3), 21−25.

Sugiyama, M., Ando, A., Furuno, H., Furlong, N. B., Hidaka, T., & Ogura, R. (1987) *Cancer Lett. 38*, 1−7.

Sugiyama, M., Tsuzuki, K., & Ogura, R. (1991) *J. Biol. Chem. 266*, 3383−3386.

Suzuki, Y., & Fukuda, K. (1990) *Arch. Toxicol. 64*, 169−176.

Tan, J. D., Hudson, S. E., Brown, S. J., Olmstead, M. M., & Mascharak, P. K. (1992) *J. Am. Chem. Soc. 114*, 3841−3853.

Tolbert, B. M., & Ward, J. B. (1982) in *Ascorbic Acid: Chemistry, Metabolism and Uses* (Seib, P. A., & Tolbert, B. M., Eds.) pp 116−118, Advances in Chemistry Series 200, American Chemical Society, Washington, DC.

Tsapakos, M. J., & Wetterhahn, K. E. (1983) *Chem.-Biol. Interact. 46*, 265−277.

Wise, J. P., Orenstein, J. M., & Patierno, S. R. (1993) *Carcinogenesis 14*, 429−434.

Wolf, T., Kasemann, R., & Ottenwälder, H. (1989) *Carcinogenesis 10*, 655−659.

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