Chromium(VI) Reduction by Ascorbate: Role of Reactive Intermediates in DNA Damage *In Vitro*

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Reaction of chromium(VI) with one equivalent of ascorbate was studied by electron paramagnetic resonance spectroscopy in the presence of 0.10 M 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) at room temperature in 0.10 M (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (HEPES) and 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffers (pH 7.0, room temperature). Chromium(V), ascorbyl radical, and carbon-based DMPO-radical adducts were observed. A higher level of Cr(V) was observed in HEPES buffer and a higher level of the DMPO-radical adducts was observed in Tris-HCl buffer. Chromium-DNA binding studies were carried out *in vitro* for calf thymus DNA incubated with Cr(VI) and ascorbate in both buffers at 37°C. Higher Cr-DNA binding was observed in HEPES buffer. DNA strand-break studies were carried out *in vitro* on pBR322 DNA incubated with Cr(VI) and ascorbate in both buffers at 37°C. Higher percent nicking was observed in Tris-HCl buffer. Addition of DMPO decreased nicking levels in Tris-HCl buffer. These results suggest that free radicals are more reactive than Cr(V) in producing DNA strand breaks and that Cr(V) will react with DNA to produce Cr-DNA adducts. The fact that buffer affects the nature of the reactive intermediates produced upon reduction of Cr(VI) may be related to differences in intracellular metabolism of Cr(VI) and resulting DNA damage observed in various cell culture systems and animal tissues *in vivo*. — Environ Health Perspect 102(Suppl 3):21–25 (1994).

Key words: chromium(VI), chromium(V), ascorbate, spin trapping. free radicals, DNA damage

Introduction

Chromium(VI) (Cr(VI) is a known carcinogen (*1*). It causes respiratory cancers in humans and animals, and it is mutagenic and genotoxic (*2*). The mechanism of Cr(VI)-induced carcinogenesis is unclear. The intracellular reduction of Cr(VI) (*3*) can produce many reactive intermediates, including Cr(V), Cr(IV), free radicals, and the final stable product Cr(III). Any of these species could potentially target DNA. Many types of DNA damage have been observed with Cr(VI) *in vivo* and *in vitro*, including interstrand cross-links, DNA-protein cross-links, strand breaks, Cr-DNA adducts, and radical DNA adducts, most notably 8-oxo-deoxyguanosine (*4*). Recent work has shown that ascorbate (vitamin C) is the principal reductant of Cr(VI) in rat kidney, liver, and lung ultrafiltrates (*5,6*). It is not yet fully understood whether the intracellular reduction of Cr(VI) by ascorbate is a toxification or detoxification process, i.e., does ascorbate protect tissues against Cr(VI)-induced DNA damage by reducing it to a substitutionally inert form, Cr(III):

$$2Cr(VI) + 3ascorbate \rightarrow 2Cr(III) + 3dehydroascorbate$$
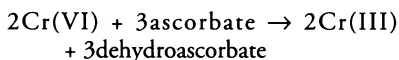
or does this process produce reactive intermediates that are the actual genotoxic species?

The purpose of the current study was to determine the reactive intermediates that are produced upon reduction of Cr(VI) by ascorbate and the extent to which these intermediates, e.g., Cr(V) and free radicals, would react with DNA *in vitro*. Chromium(VI) was reacted with one equivalent of ascorbate in (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (HEPES) or Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer and the intermediates were observed by electron paramagnetic resonance (EPR) spectroscopy. Reactions of Cr(VI) with ascorbate were carried out in the presence of calf thymus DNA or pBR322 DNA to determine the amount of Cr-DNA adducts and single-strand breaks, respectively.

Reactive Intermediates Observed by EPR Spectroscopy

EPR spectroscopy was used to determine the reactive intermediates formed from the reduction of Cr(VI) by ascorbate. EPR spectra were acquired at room temperature for a 1:1 stoichiometric ratio of Cr(VI) (from $K_2Cr_2O_7$) to sodium ascorbate at 9.0 mM, 3.6 mM, or 1.8 mM Cr(VI) and sodium ascorbate with or without 0.10 M 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) in either 0.10 M HEPES (pH 7.0, room temperature) or 0.05 M Tris-HCl (pH 7.0, room temperature) buffers. Reactions at 1.8 mM Cr(VI) and ascorbate were run with and without 1.8 mM calf thymus DNA. Chromium(V) and carbon-based free-radical adducts of DMPO were observed under these conditions (Figure 1). In HEPES buffer, Cr(V) had a g value of 1.980 and $\Delta H = 1.173$ G (where ΔH is the line width measured between the maximum and minimum of the first derivative spectral peak); and in Tris-HCl buffer a much weaker Cr(V) signal was detected at $g = 1.978$. These intermediates were best observed at high concentrations. Spectra at the lower concentrations used for DNA studies showed the same features, with differences consisting of an overall decrease in
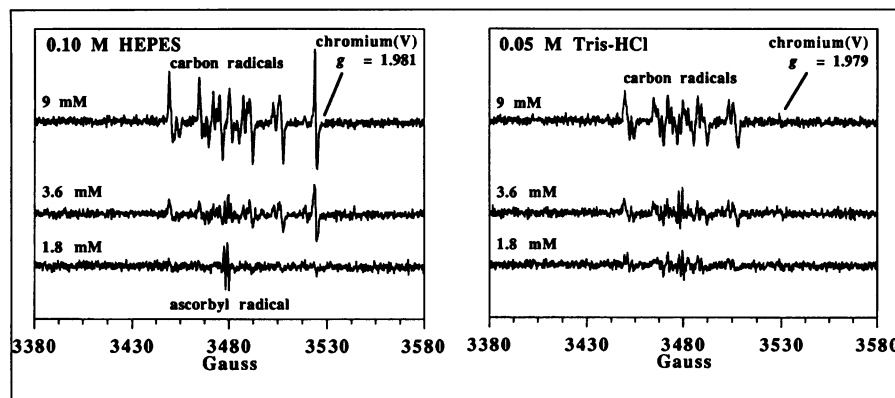
Figure 1. EPR spectra of intermediates formed during reaction of Cr(VI) and ascorbate in HEPES versus Tris-HCl buffers. (A) Cr(VI) (9.18, 3.63, or 1.82 mM, from $K_2Cr_2O_7$) was reacted with sodium ascorbate (9.00, 3.63, or 1.82 mM) in 0.10 M HEPES buffer (pH 7.0, room temperature) in the presence of 0.10 M DMPO. (B) Cr(VI) (9.00, 3.60, or 1.80 mM) was reacted with sodium ascorbate (8.24, 3.80, or 1.80 mM) in 0.05 M Tris-HCl buffer (pH 7.0, room temperature) in the presence of 0.10 M DMPO. Spectra at 1.8 mM Cr(VI) and ascorbate were acquired in the presence of 1.8 mM calf thymus DNA. Trace iron was removed from all buffer and ascorbate solutions by treatment with Chelex 100 resin. Ascorbate concentrations were determined by UV/Vis spectroscopy (265 nm, $\varepsilon = 14500$ M^{-1} cm^{-1} (23)). DMPO solutions were purified by charcoal filtration (24). Solutions were made immediately before use. Reactions were initiated by addition of Cr(VI) and spectra were acquired beginning at 80 sec reaction time and signal averaged over nine scans. EPR parameters are as follows: 100 kHz field modulation, 1.0 G modulation amplitude, 1×10^5 receiver gain, 9.772–9.769 microwave frequency, 3430–3530 sweep width, and 21 sec scan time. The g values were calculated from calibration against DPPH (g = 2.0036). Reactions were run in duplicate.
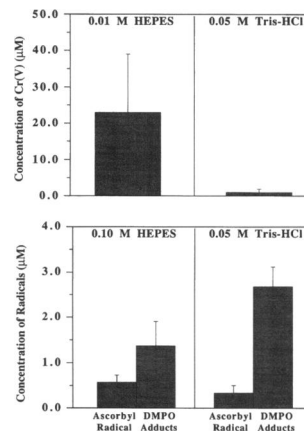

Figure 2. Concentration of (A) Cr(V), and (B) ascorbate radical and DMPO-radical adducts, produced upon reaction of Cr(VI) (1.8 mM) with ascorbate (1.8 mM) in HEPES versus Tris-HCl buffers determined by EPR spectroscopy. Experimental conditions are given in Figure 1 caption. Calculations are described in text; values on the graph are averages ± SD. (n = 6 for HEPES data; n = 4 for Tris-HCl data).

signal intensity and a slightly higher relative signal intensity for ascorbate radical, and higher amounts of carbon-based DMPO-radical adducts in Tris-HCl vs HEPES buffer. Concentrations of intermediates were estimated from signal intensities ($I\Delta H^2$, where I is the signal intensity measured as peak to trough height from the baseline of the first derivative spectra) by comparison to signal intensities of standards of known concentration, K_3CrO_8 for Cr(V) (7) and TEMPO• (8) for ascorbate radical and DMPO-radical adducts. At the 1.8 mM concentration of Cr(VI) and ascorbate, the Cr(V) signal was 20-fold higher in HEPES buffer than in Tris-HCl buffer (Figure 2A). This difference in relative signal intensity, as well as the Cr(V) g value, has been reported previously for reactions of Cr(VI) with ascorbate (9,10) and is assumed to result from reaction of chromium with the Tris-HCl buffer. HEPES buffer did not react with chromium on this time scale as evidenced by the identical EPR spectra for the same reaction of Cr(VI) with ascorbate carried out in phosphate, dimethylarsenate (cacodylate) and HEPES buffers (10). Three DMPO-radical adducts were distinguished with g values and hyperfine coupling constants estimated from simulations as follows: 1, g=2.007, A_H = 25.7 G, A_N = 15.5, G; 2, g=2.007, A_H = 18.8 G, A_N = 15.5 G; and 3, g=2.007, A_H = 22.8 G, A_N = 15.8 G. The relative hyperfine splittings $A_H > A_N$ identify the DMPO-adducts as

being derived from carbon-based radicals (11). These carbon radicals are assumed to arise from fragmentation of ascorbate under these reaction conditions. The hydroxyl radical adduct of DMPO was not observed (12). There was 2-fold more ascorbate radical detected for the reaction of Cr(VI) with ascorbate in HEPES than Tris-HCl buffer and 2-fold less DMPO-radical adducts produced by the reaction in HEPES buffer than Tris-HCl buffer (Figure 2B). There was 2-fold more DMPO-radical adduct than ascorbate radical produced upon reaction of Cr(VI) with ascorbate in HEPES buffer and 8-fold more in Tris-HCl buffer. The Cr(V) and DMPO-adduct signals were not affected by the presence of DNA, and DMPO had no effect on the signal intensity of Cr(V) (data not shown).

These EPR experiments demonstrate that the reduction of Cr(VI) by ascorbate in HEPES or Tris-HCl buffers results in various amounts and types of Cr(V) species and varying amounts of radical species. This allowed us to study the reactivity of Cr(V) with DNA, and to distinguish between Cr(V) and free radicals as being responsible for Cr-DNA adducts in calf thymus DNA and/or single-strand breaks in plasmid DNA (below).

Chromium-DNA Adducts Resulting from Reduction of Cr(VI) by Ascorbate

The formation of Cr-DNA adducts was measured after reacting Cr(VI) (1.8 mM,

from $K_2Cr_2O_7$) with ascorbic acid (1.8 mM) in the presence of native (double-stranded) or denatured (single-stranded) calf thymus DNA (1.8 mM). Since EPR spectroscopy (above) had shown a strong buffer dependence on the level of Cr(V), the reactions were carried out in both 0.05 M Tris-HCl buffer (pH 7.0, 37°C) and 0.10 M HEPES buffer (pH 7.0, 37°C) to determine the role of Cr(V) in Cr-DNA binding. Experiments carried out with double-stranded vs single-stranded calf thymus DNA allowed for evaluation of a preference for chromium to bind to the nucleotide base rather than the sugar–phosphate backbone of DNA.

No Cr-DNA binding was detected upon reaction of calf thymus DNA with Cr(VI) in the absence of ascorbate. Reactions of DNA with Cr(VI) in the presence of ascorbate resulted in higher Cr-DNA binding in HEPES relative to Tris-HCl, with a 6.9-fold difference for native calf thymus DNA and a 9.3-fold difference for denatured DNA (Figure 3). There was 3.3-fold higher binding of chromium to single-stranded DNA than native DNA in HEPES buffer, and 2.4-fold higher binding to denatured DNA in Tris-HCl buffer.

The higher Cr-DNA binding in HEPES compared to Tris-HCl buffer correlates with the higher relative level of the Cr(V) intermediate produced during the reduction of Cr(VI) by ascorbate in HEPES. The higher Cr-DNA binding observed with single-stranded vs double-stranded

DNA is consistent with previous work (*13*) that suggested chromium is bound covalently to the nucleotide bases, not simply interacting electrostatically with the anionic sugar–phosphate backbone.

Single-Strand Breaks in Plasmid DNA Resulting from Reduction of Cr(VI) by Ascorbate

Single-strand breakage by the Cr(V) and free radical intermediates was determined using supercoiled pBR322 DNA. Reaction of 1.8 mM Cr(VI) (from $K_2Cr_2O_7$) with 1.8 mM sodium ascorbate in the presence of 1.0 µg pBR322 DNA (0.36 µM DNA-P) with 15 µM diethylenetriaminepentaacetic acid (DETAPAC) in either 0.10 M HEPES or 0.05 M Tris-HCl buffers (pH 7.0, 37°C) resulted in significant nicking of plasmid DNA relative to control reactions (Figure 4). Reaction of pBR322 DNA with Cr(VI) in the presence of ascorbate showed 2-fold higher nicking in Tris-HCl buffer relative to reactions in HEPES buffer. Chromium(VI) or ascorbate alone did not cause any DNA single-strand breakage. Addition of 0.10 M DMPO dramatically decreased the extent of nicking of pBR322 DNA produced in reactions with Cr(VI) in the presence of ascorbate in Tris-HCl buffer (Figure 4). This is consistent with the higher levels of free radicals observed in Tris-HCl buffer by EPR spectroscopy.

Chromium(V) Produces Cr-DNA Adducts and Carbon-based Radicals Produce Single-strand Breaks

Comparison of the Cr-DNA binding and plasmid nicking experiments to the intermediates detected in EPR spectra suggests that Cr(V) produces Cr-DNA adducts and that the carbon-based radicals are responsible for DNA single-strand breaks. The higher binding of Cr to calf thymus DNA in HEPES vs Tris-HCl buffer is proportional to the increased levels of Cr(V) in HEPES observed by EPR. If Cr(V) were the more reactive species in the plasmid nicking study, the nicking should be higher in HEPES buffer relative to Tris-HCl; but the opposite was observed. The 1.6-fold higher nicking in Tris-HCl vs HEPES is consistent with the 2-fold higher level of DMPO-adducts in Tris-HCl buffer detected by EPR. This buffer effect can also be used to argue against reactivity of ascorbate radical, since ascorbate radical was observed at higher concentration in HEPES vs Tris-HCl
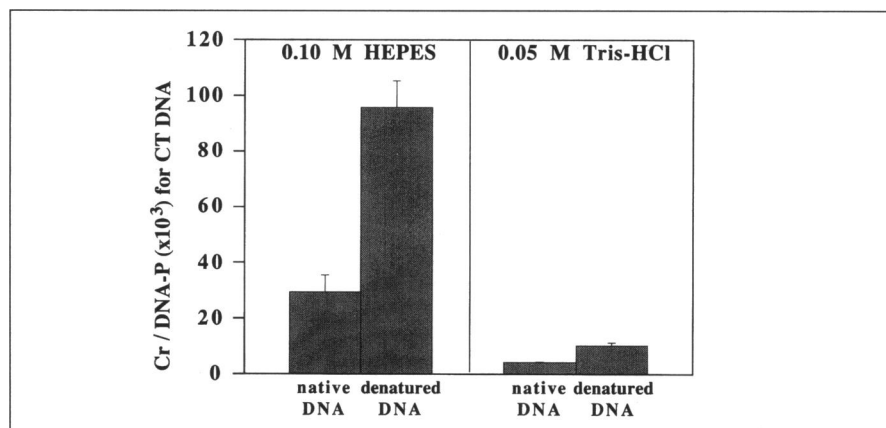

Figure 3. Levels of chromium bound to double-stranded vs single-stranded calf thymus DNA produced upon reaction of DNA with Cr(VI) in the presence of ascorbate in HEPES vs Tris-HCl buffers. Native (DS) or denatured (SS) calf thymus DNA (1.8 mM DNA-P) was incubated with Cr(VI) (1.8 mM, from $K_2Cr_2O_7$) and ascorbic acid (1.8 mM) in either 0.05 M Tris-HCl buffer (pH 7.0, 37°C) or 0.10 M HEPES buffer (pH 7.0, 37°C). All buffers and ascorbate solutions were treated with cation exchange resin AG50W X 8 to remove trace iron. Ascorbate concentrations were measured as stated in Figure 1 caption. Incubations were carried out at 37°C for 30 min. NENsorb chromatography and dialysis was used to remove non-covalently bound chromium from DNA (*25*). The amount of chromium bound to a sample of DNA was measured by atomic absorption spectroscopy. DNA concentrations per sample were determined by the diaminobenzoic acid (DABA) fluorometric assay (*26*). The amount of chromium measured in controls lacking DNA was subtracted from the amount of chromium in reaction samples before calculation of binding ratios. Values are the averages of binding ratios ± SD. (n=16 for reactions in HEPES buffer, n=10 for reactions in Tris-HCl buffer, p= 0.01 for all data.)

buffer. It would be expected that ascorbate radical would be less reactive with DNA than the carbon-based radicals since ascorbate radical is too stable to react with DMPO.

The Cr-DNA binding results presented here are consistent with previous work that showed a correlation between Cr-DNA adducts and Cr(V) produced from the reaction of Cr(VI) with dithiothreitol, β-mercaptoethanol, cysteine, and glutathione (*13*). While the role of free radicals in DNA cleavage has been established above and elsewhere (*14*), the reactivity of Cr(V) with DNA in terms of producing single strand breaks is less clear. Farrell et al. (*15*) have reported that the Cr(V) complex of 2-ethyl-2-hydroxybutanoate caused strand breaks in pUC9 DNA at pH<5; however, instability of the Cr(V) complex at higher pH prevented the same study at physiologic pH. Kortenkamp et al. (*16*) observed strand breaks in bacteriophage PM2 DNA after incubation with a Cr(V) species prepared from the reduction of Cr(VI) by glutathione; however, this nicking was shown later to be dependent on contaminating iron (*17*). A different reactivity toward DNA for Cr(V) vs free radicals was observed for the reduction of Cr(VI) by gluthathione in the presence or absence of H_2O_2 (*18*) where single-strand breaks were only observed in the presence of H_2O_2 under conditions which were shown to generate hydroxyl radical, •OH. In the absence of H_2O_2, the Cr(V) that was generated upon reaction of Cr(VI) with glu-

tathione produced Cr-DNA adducts only. Under our experimental conditions the free radicals were more reactive than Cr(V) in the production of single-strand breaks, since the level of strand breakage correlated with level of free radicals but not with Cr(V), and since DMPO, a radical trap that did not affect Cr(V), decreased the level of strand breakage. However, the lack of Cr(V)'s reactivity was not determined.

There is evidence to suggest that Cr(V) and radical intermediates will produce similar types of DNA damage in *in vivo* systems. Chromium(V) has been shown to have a significant lifetime *in vivo* (*19,20*). Chromium(V) has been detected by EPR in chick embryo red blood cells (*19*) and liver (*20*) after *in vivo* treatment of chick embryos with Cr(VI). The Cr(V) signal in liver reached a maximum at 70 min after treatment and was detectable for 240 min. The Cr(V) signals observed *in vivo* had g values of 1.981, 1.984-5, and 1.987 (*19,20*). The Cr(V) signal with g=1.980 observed in this study of Cr(VI) with ascorbate in HEPES buffer is similar to the Cr(V) signal with g=1.981 observed *in vivo* in chick embryo red blood cells. The Cr(V) species formed *in vivo* (*20*) was detectable in liver cells at a lower dose of Cr(VI) than was necessary to detect Cr(V) in red blood cells. This difference in the levels of Cr(V) correlates with the DNA damage observed in chick embryo liver and blood cells. In chick embryo liver, Cr(VI) treatment induced DNA cross-links but not strand
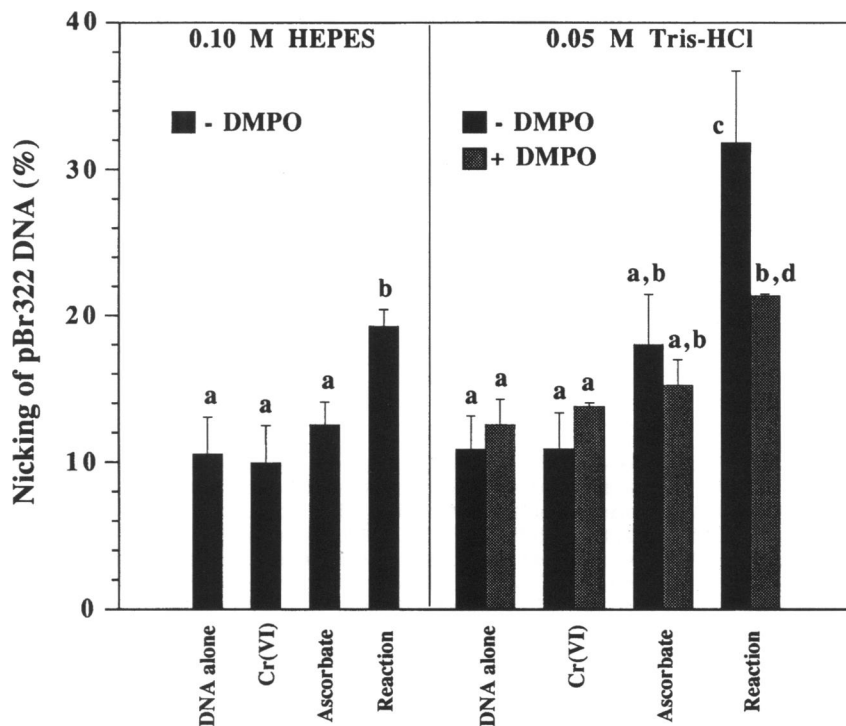
Figure 4. Levels of nicking of pBR322 DNA upon reaction with Cr(VI) in the presence of ascorbate in HEPES versus Tris-HCl buffers. Reaction mixtures (10 μl) contained 1.0 μg pBR322 DNA (0.36 μM DNA-P), 1.8 mM Cr(VI) (from $K_2Cr_2O_7$), 1.8 mM sodium ascorbate and 15 μM DETAPAC in either 0.10 M HEPES or 0.05 M Tris-HCl buffers (pH 7.0, 37°C). Control mixtures contained Cr(VI) with DNA or ascorbate with DNA. Reactions were incubated for 30 min at 37°C and were terminated by cooling in an ice bath. Loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) was added to each reaction at 2 μl/10 μl reaction volume. Reactions were analyzed by agrose gel electrophoresis on 0.7% agrose gels containing 0.5 μg/ml ethidium bromide, against 0.8 μg of 1.0 kB Lamda molecular weight marker DNA. Samples were electrophoresed at 120 V (4 V/cm) for 8 to 12 hr. Gels were destained in water for at least 2 hr, and photographs were taken with Polaroid 55 film under ultraviolet light. Gel band intensities representing supercoiled and nicked plasmid DNA were determined with a Helena Laboratories Quick Scan R&D Densitometer and HP3396A Integrator. Multiple comparisons of data groups were accomplished by a one-way analysis of variance (ANOVA) and evaluated by a paired, two-tailed t-test. Data points having no letters (a–d) in common are significant at $p<0.05$.

with Cr(VI) reduced the amount of Cr(V) formed as an intermediate relative to the final Cr(III) product. They also found pretreatment with ascorbate increased the amount of DNA-protein cross-links and decreased the chromate-induced alkali-labile sites. In contrast to the observations presented here and elsewhere (18,21), Sugiyama et al. concluded that Cr(V) was responsible for the alkali-labile sites and that Cr(III) caused the DNA-protein cross-links. However, it has been shown above that radicals and Cr(V) can be formed concomitantly; but the presence of radicals was not explored in the study of Sugiyama et al. (22). More importantly, no comparison can be made between these studies (21,22) of the relative concentrations of reducing agents in chick embryo cells vs V-79 cells.

The experiments described here present a model system that may be useful in understanding the reduction of Cr(VI) by ascorbate *in vivo*. Just as the level of reactive intermediates and resulting DNA damage differ in HEPES and Tris-HCl buffers, tissue-specific cellular components affect the formation of reactive intermediates and DNA damage. The significance of these *in vitro* results is shown by the correlation to the *in vivo* chick embryo experiments (19–21), in which the DNA damage in different tissues was observed to be consistent with the presence or absence of Cr(V) and radicals. A broader picture is emerging that suggests a role for Cr(V) as the ultimate genotoxic form of chromium, but also suggests that different reducing environments will affect the formation and stability of reactive intermediates, thus producing varying types of DNA damage in different tissues or assay systems.

breaks, whereas in chick embryo red blood cells Cr(VI) induced DNA strand breaks in the absence of cross-links (21). This is consistent with the *in vitro* Cr(V) and radical reactivity presented in this work. Sugiyama et al. (22) have reported that pretreatment of Chinese hamster V-79 cells with ascorbate followed by incubation

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