Reaction of Chromium(VI) with Glutathione or with Hydrogen Peroxide: Identification of Reactive Intermediates and Their Role in Chromium(VI)-Induced DNA Damage

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The types of reactive intermediates generated upon reduction of chromium(VI) by glutathione or hydrogen peroxide and the resulting DNA damage have been determined. In vitro, reaction of chromium(VI) with glutathione led to formation of two chromium(V) complexes and the glutathione thiol radical. When chromium(VI) was reacted with DNA in the presence of glutathione, chromium-DNA adducts were obtained, with no DNA strand breakage. The level of chromium-DNA adduct formation correlated with chromium(V) formation. Reaction of chromium(VI) with hydrogen peroxide led to formation of hydroxyl radical. No chromium(V) was detectable at 24°C (297 K); however, low levels of the tetraperoxochromium(V) complex were detected at 77 K. Reaction of chromium(VI) with DNA in the presence of hydrogen peroxide produced significant DNA strand breakage and the 8-hydroxydeoxyguanosine adduct, whose formation correlated with hydroxyl radical production. No significant chromium-DNA adduct formation was detected. Thus, the nature of chromium(VI)-induced DNA damage appears to be dependent on the reactive intermediates, i.e., chromium(V) or hydroxyl radical, produced during the reduction of chromium(VI).

Introduction

Epidemiological evidence has clearly demonstrated the carcinogenicity of chromium(VI) compounds (1–5). The ability of chromium(VI) to cause DNA damage has been established through a number of studies, both in vivo and in vitro (6–10). From these studies, it is apparent that metabolic reduction of chromium(VI) is important in chromium(VI)-mediated DNA damage. Cupo and Wetterhahn (6) have shown that the level of chromium(VI)-induced DNA stand breaks in cultured chicken embryo hepatocytes is proportional to the level of glutathione in these cells. In Chinese hamster V-79 cells, Sugiyama et al. (7) established a correlation between chromium(VI)-induced DNA single-strand breaks and the level of a chromium(V) intermediate [possibly a chromium(V)-glutathione complex] detected in these cells. Treatment of the cells with vitamin B₂ prior to chromate treatment led to an increase in DNA single-strand breaks over that observed upon treatment with chromate alone (8), presumably due to an increase in chromium(V)-related hydroxyl radical production. On the other hand, incubation of the V-79 cells with vitamin E, a hydroxyl radical scavenger, prior to chromate treatment led to a decrease in DNA single-strand breaks (9). In animal studies, chromium(VI)-induced DNA damage has been found to be tissue-dependent. DNA-interstrand crosslinks and DNA-protein crosslinks were detected in the livers of chick embryos treated with chromium(VI), whereas in the red blood cells, DNA damage was primarily in the form of strand breaks (10). This may reflect the fact that chromium(VI) reduction occurs by different metabolic pathways in the various tissues.

The purpose of this study was to test the hypothesis that the differences in the types of DNA damage observed in the various cell culture and in vivo systems arise due to different metabolic pathways being involved in chromium(VI) reduction. The study was carried out...
using the reductants glutathione and hydrogen peroxide. Glutathione is the most abundant, naturally occurring, low molecular weight thiol (11) and has been shown to react with chromium(VI) and increase chromium(VI)-induced DNA damage in cell culture (6) and in vitro (12). Hydrogen peroxide is known to react with trace amounts of metal ions such as iron(II) or copper(I) in Fenton-like reactions, giving rise to the very reactive hydroxyl radical, which is known to cause extensive DNA strand breakage in cell culture and in vitro (13–15). Studies by Kawanishi and co-workers (16) showed that reaction of chromium(VI) with hydrogen peroxide led to formation of the tetraperoxochromium(V) species and hydroxyl radical, resulting in DNA strand breakage; no DNA strand breakage was observed when the reductant used was glutathione. We have shown that reaction of chromium(VI) with DNA in the presence of hydrogen peroxide results in formation of DNA single-strand breaks and the 8-hydroxydeoxyguanosine adduct, whereas under identical conditions, the reaction in the presence of glutathione leads to extensive chromium-DNA adduct formation in the absence of significant DNA strand breakage (17). The following is a more detailed study of the relationship between the reactive intermediates generated upon reaction of chromium(VI) with hydrogen peroxide or glutathione and the DNA damage observed in each case.

**Reaction of Chromium(VI) with Hydrogen Peroxide: Reactive Intermediates and Resulting DNA Damage**

**Reactive Intermediates**

The reduction of chromium(VI) by hydrogen peroxide in 25 mM Tris-HCl, pH 8.0, 24°C (297 K) was investigated by electron paramagnetic resonance (EPR) spectroscopy at 297 K in the presence of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Figure 1 shows that reaction of chromium(VI) (27 mM) with hydrogen peroxide (27 mM) in the presence of DMPO (0.10 M) led to formation of the DMPO-hydroxyl radical adduct \( g = 2.0044, \alpha^I = \alpha^H = 14.9 \text{ G} \), in the absence of detectable chromium(V). Hydroxyl radical production has been reported in earlier studies of the reaction of chromium(VI) with hydrogen peroxide (16,17). In addition, Kawanishi et al. (16) detected the tetraperoxochromium(V) complex \( g = 1.9735, \Delta H = 5.0 \text{ G} \), using concentrations of chromium(VI) and hydrogen peroxide which were higher [40 mM chromium(VI), 400 mM hydrogen peroxide] than those employed in our study. Formation of the DMPO-hydroxyl radical adduct increased as the concentration of chromium(VI) was increased (Fig. 2), suggesting that chromium(VI) is required to react with hydrogen peroxide in more than a catalytic amount in order to produce hydroxyl radical to a significant extent. No chromium(V) was detected even at the highest concentration (27 mM) of chromium(VI) employed in the reaction (Fig. 1).

Although the level of chromium(V) produced upon reaction of chromium(VI) with hydrogen peroxide was too low to be detected by EPR spectroscopy at room temperature (297 K), EPR spectroscopic studies at 77 K showed that reaction of chromium(VI) with hydrogen peroxide led to formation of the tetraperoxochromium(V) complex. Reaction of chromium(VI) (1.8 mM) with hydrogen peroxide (18 mM) gave rise to a weak chromium(V) EPR signal as shown in Figure 3. Upon increasing the chromium(VI) concentration to 18 mM, the signal showed distinct axial symmetry \( g_1 = 1.983, \Delta H = 8.0 \text{ G} \), calculated \( g_a = 1.972 \), and, based on earlier studies (18), was identified as the tetraperoxochromium(V) complex (Fig. 3). In the absence of hydrogen peroxide, no chromium(V) signal was observed even at the highest concentration of chromium(VI) employed (Fig. 3). The time course of formation and decay of the tetraperoxochromium(V) EPR signal showed a steady increase in intensity over the
Asthe
mium(VI)
DNA
chromium(V)
peroxide
the
crease
chromium(V)

FIGURE 5.4, a
guanosine(8-OH-dGuo)
hydroxyl
chromium(VI)
tion
observed
increase
conditions,
in
calf
0.00036 ± 0.0003 was used as a reference standard.

Reaction of the plasmid pBR322 DNA (0.10 mM) with chromium(VI) (1.8–18 mM) in the presence of hydrogen peroxide (18 mM) led to nicking of the supercoiled (form I) plasmid DNA backbone, as shown by the appearance of the nicked circular (form II) and linear (form III) plasmid. Densitometric determination of the extent of chromium(VI)-induced DNA nicking in the presence of hydrogen peroxide showed that as the concentration of chromium(VI) was increased, supercoiled (form I) DNA (~ 65% in control samples) was increasingly converted to nicked circular (form II) and, subsequently, linear (form III) DNA (Fig. 6). The level of form II DNA increased from a control level of 35% to a maximum of 84% at a chromium(VI) concentration of 5.4 mM, followed by a decrease due to subsequent double strand nicking at the higher chromium(VI) concentrations (10.8 and 18 mM), giving rise to the linear form III DNA (Fig. 6). At the two highest concentrations of chromium(VI) employed, the supercoiled Form I plasmid DNA was almost completely converted to forms II and

first 7 min of reaction, followed by a more gradual increase between 7 and 30 min into the reaction (Fig. 4). As the concentration of chromium(VI) was increased from 1.8 mM to 18 mM, the time course of formation of chromium(V) followed the same trend, but the maximal chromium(V) intensity increased (Fig. 4).

DNA Damage

Reaction of calf thymus DNA (1.8 mM) with chromium(VI) (1.8–18 mM) in the presence of hydrogen peroxide (18 mM) showed no significant binding (p > 0.05) of chromium to DNA at the lower concentrations (1.8, 5.4, and 10.8 mM) of chromium(VI) (data not shown). At the highest concentration (18 mM) of chromium(VI), only a slight increase (1.4-fold) in DNA-bound chromium was observed over control incubations consisting of DNA and chromium(VI) in the absence of hydrogen peroxide (Table 1). However, significant formation of one of the major hydroxyl radical adducts of DNA, 8-hydroxydeoxyguanosine (8-OH-dGuo) was observed (Table 1). Formation of the 8-OH-dGuo adduct increased as the concentration of chromium(VI) was increased from 1.8 mM (8.4-fold increase over basal 8-OH-dGuo) to 18 mM (37.2-fold increase over basal 8-OH-dGuo) (Fig. 5). Under identical conditions, reaction of chromium(VI) with DNA in the absence of hydrogen peroxide led to no detectable increase in 8-OH-dGuo levels over the basal level observed in the calf thymus DNA used in this study (Fig. 5).
III (Table 1), with the form III DNA increasing to a maximum (18%, from a control level of 0%) at the highest concentration (18 mM) of chromium(VI) employed (Fig. 6).

Thus, our study of the reaction of chromium(VI) with DNA in the presence of hydrogen peroxide showed that production of hydroxyl radical correlated with significant formation of the 8-OH-dGuo adduct and the generation of DNA single-strand breaks. A low level of chromium-DNA adducts was detected only at the highest concentration (18 mM) of chromium(VI) employed in the study, which was also the chromium(VI) concentration at which maximal tetraperoxochromium(V) production was observed. Similar results have been reported by us in our earlier study (17). These observations are consistent with those reported by Kawanishi et al. (16), who incubated chromium(VI) with a $^{32}$P-labeled DNA fragment in the presence of hydrogen peroxide and showed that hydroxyl radical-associated DNA strand breaks were produced.

**Reaction of Chromium(VI) with Glutathione: Reactive Intermediates and Resulting DNA Damage**

**Reactive Intermediates**

The reduction of chromium(VI) (2.7 mM) by glutathione (8.1–27 mM) in 25 mM Tris-HCl, pH 8.0, 24°C

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**Table 1. Chromium(VI)-induced DNA damage in the presence of either hydrogen peroxide (H$_2$O$_2$) or glutathione (GSH): Cr-DNA adducts, 8-hydroxydeoxyguanosine (8-OH-dGuo) adduct, and single-strand breaks.**

| Reaction | Cr-DNA adducts, nmole Cr/nmole DNA-P $\times 10^3$ | 8-OH-dGuo adduct, nmole 8-OH-dGuo/nmole DNA-P $\times 10^3$ | Single-strand breaks, % (form II + form III)$^a$ |
|----------|---------------------------------|------------------|-------------------|
| Cr$^{VI}$ + H$_2$O$_2$ + DNA$^b$ | 306.0 ± 33.0 (1.4)$^{d,*}$ | 99.6 ± 24.3 (26.9)$^{d,*}$ | 98.9 ± 0.7 |
| Cr$^{VI}$ + DNA | 224.0 ± 30.0 | 3.7 ± 0.9 | 38.1 ± 0.6 |
| H$_2$O$_2$ + DNA | 7.1 ± 4.3 | 3.4 ± 2.0 | 35.5 ± 3.9 |
| Cr$^{VI}$ + GSH + DNA$^b$ | 2325 ± 829 (66.1)$^{d,*}$ | 3.7 ± 0.6 (1.2)$^{d}$ | 31.2 ± 1.3 |
| Cr$^{VI}$ + DNA | 35.2 ± 1.7 | 3.1 ± 2.2 | 33.0 ± 0.7 |
| GSH + DNA | 3.5 ± 0.2 | 2.4 ± 0.4 | ND$^f$ |

$^a$Values are means ± SD of two to three determinations.

$^b$Numbers represent total percent form II (nicked circular) + form III (linear) pBR322 DNA obtained due to single-strand breakage. The pBR322 DNA used in these experiments consisted of 64.1 ± 1.9% form I (supercoiled), 38.9 ± 1.0% form II (nicked circular), and no significant form III (linear) DNA, as determined by densitometry.

$^c$Complete reaction consisted of 18.0 mM Cr$^{VI}$ (0.9 mM Na$_2$Cr$_2$O$_7$·2H$_2$O), 18.0 mM H$_2$O$_2$ and 1.8 mM DNA-P in 25 mM Tris-HCl (for calf thymus DNA experiments) or 0.1 mM DNA-P 50 mM Tris-HCl (for pBR322 DNA experiments), pH 8.0, 37°C. Calf thymus DNA was used to measure Cr-DNA adducts and 8-OH-dGuo adduct; pBR322 DNA to measure single-strand breaks.

$^d$Numbers in parentheses represent fold increase over control reactions carried out in the absence of H$_2$O$_2$ or GSH.

$^e$Complete reaction consisted of 1.8 mM Cr$^{VI}$ (9.0 mM Na$_2$Cr$_2$O$_7$·2H$_2$O), 18.0 mM GSH and 1.8 mM DNA-P in 25 mM Tris-HCl (for calf thymus DNA experiments) or 0.1 mM DNA-P 50 mM Tris-HCl (for pBR322 DNA experiments), pH 8.0, 37°C. Calf thymus DNA was used to measure Cr-DNA adducts and 8-OH-dGuo adduct; pBR322 DNA to measure single-strand breaks.

$^f$Densitometry not determined, but no detectable strand breakage observed (17).  

*p < 0.01 versus control.
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Figure 5. Level of the 8-hydroxydeoxyguanosine adduct produced upon reaction of calf thymus DNA with chromium(VI) in the presence of hydrogen peroxide. The calf thymus DNA used in all reactions was treated for iron removal as previously described (17), so that <1 µM Fe was present in the reaction solutions. Calf thymus DNA (1.8 mM) was incubated in the presence (△) or absence (●) of 18.0 mM hydrogen peroxide and 0, 1.8, 5.4, 10.8, or 18.0 mM chromium(VI) (0, 0.9, 2.7, 5.4, or 9.0 mM Na\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}, respectively), in 25 mM Tris-HCl, pH 8.0, 37°C for 30 min. Samples were repeatedly dialyzed and ethanol-precipitated as described earlier (17). The 8-hydroxydeoxyguanosine adduct (8-OH-dGuo) was determined by HPLC analysis with electrochemical detection (LCED) after exhaustive digestion of the DNA (50–140 µg) to nucleosides, as previously described (17–19). DNA concentration was assayed using a modification of the microfluorometric technique of Kissane and Robins (10), as previously described (14). The control 8-OH-dGuo/DNA ratio (2.68 ± 1.26 × 10\textsuperscript{-5} nmole 8-OH-dGuo/nmole DNA-P) represents the basal level of adduct present in samples consisting of calf thymus DNA alone. Results are the mean ± SD of three to six determinations.

Figure 6. Level of DNA strand breakage produced upon reaction of pBR322 plasmid DNA with chromium(VI) in the presence of hydrogen peroxide. Trace amounts of iron were removed from the plasmid DNA as described earlier (17). pBR322 plasmid DNA (0.10 mM DNA-P) was incubated with 18.0 mM hydrogen peroxide, and 0, 1.8, 5.4, 10.8, or 18.0 mM chromium(VI) (0, 0.9, 2.7, 5.4 or 9.0 mM Na\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}, respectively), in 50 mM Tris-HCl, pH 8.0, 37°C for 30 min. Samples were loaded directly onto a 0.7% agarose gel containing 0.5 µg/mL ethidium bromide, subjected to electrophoresis at 4 V/cm for 4 to 5 hr, and photographed under short wave ultraviolet light using Polaroid 55 film. DNA strand breakage was quantitated by densitometry of the photograph negatives using a Helena Quick-Scan R & D densitometer connected to a Hewlett-Packard 3396A integrator. (●) form I (supercoiled) DNA; (△) form II (nicked circular) DNA; (□) form III (linear) DNA. Results are the mean ± SD of two determinations.

DNA Damage

Reaction of calf thymus DNA (1.8 mM) with chromium(VI) (1.8 mM) in the presence of glutathione resulted in significant binding of chromium to DNA (Fig. 9). The level of chromium-DNA adducts increased as the concentration of glutathione (5.4–18 mM) was increased (Fig. 9), and the increase was particularly dramatic at the highest concentration (18 mM) of glutathione employed [66-fold higher than that seen in the reaction of chromium(VI) with DNA alone (Table 1)]. In the absence of glutathione, no significant chromium-DNA adduct formation was detected (Fig. 9). No 8-OH-dGuo adduct formation above the basal level was observed in the reaction of chromium(VI) with calf thymus DNA in the presence of glutathione, nor was there any indication of significant DNA strand breakage when chromium(VI) was reacted with pBR322 DNA in the presence of glutathione (Table 1).

These results indicate that the generation of chromium(V) intermediates during reaction of chromium(VI)
with DNA in the presence of glutathione leads to chromium-DNA adduct formation. The involvement of chromium-glutathione complexes in binding to DNA has been established by Borges and Wetterhahn (24), who obtained evidence that reaction of chromium(VI) with DNA in the presence of glutathione led to formation of DNA-chromium-glutathione adducts. Since the level of chromium-DNA adducts showed an exponential dependence on glutathione concentration, it is likely that the $g = 1.996$ chromium(V) species, whose formation increased in an exponential manner with increasing glutathione concentration (Fig. 8A), is more reactive toward DNA than the $g = 1.986$ chromium(V) species. However, both species are involved in binding to DNA, since chromium-DNA binding was detected even under conditions where no significant $g = 1.996$ chromium(V) complex formation was observed.

Although these chromium(V) complexes have not been fully characterized, some suggestions as to their nature have been made (19, 25). Goodgame and Joy (19) detected similar chromium(V) EPR signals at $g = 1.995$ and at $g = 1.985$ upon reaction of chromium(VI) with glutathione and suggested that the $g = 1.985$ complex may be a 1:1 glutathione complex of chromium(V), while the $g = 1.995$ complex may contain two molecules of glutathione coordinated to chromium(V). O'Brien and Ozolins (25, 26) have conducted preliminary studies on the isolation and characterization of the $g = 1.996$ chromium(V) complex and have reproducibly obtained a formulation involving four molecules of glutathione coordinated to chromium(V). They suggested that glutathione is bound to chromium(V) through its sulphydryl and carboxylate groups and that two of the glutathione molecules may be bound to chromium(V) in the oxidized form, giving the formula Na$_2$Cr(GSH)$_2$GSSG-8H$_2$O (26). Our study provides no information about the coordinating atoms of glutathione to chromium(V), or whether the thiol is present in oxidized or reduced form. However, the small line-width ($\Delta H = 1.2$ G) of the $g = 1.986$ chromium(V)

Figure 7. EPR spectrum at 297 K of chromium(V) species and DMPO-glutathione thiol radical adduct resulting from reaction of chromium(VI) with glutathione in the presence of DMPO. Incubation mixture contained chromium(VI) (2.7 mM, 1.35 mM Na$_2$CrO$_4$-2H$_2$O), 27.0 mM glutathione, and 0.10 M DMPO in 25 mM Tris-HCl, pH 8.0 at 297 K for 30 min. The reaction was initiated by addition of glutathione and its EPR spectrum recorded at 297 K as described in Figure 1. The EPR spectrum was recorded beginning at 1 min from the start of the reaction and represents an average of 90 scans (21 s/scan).

Figure 8. (A) Intensity of chromium(V) EPR signals produced upon reaction of chromium(VI) with glutathione in the presence of DMPO. Chromium(VI) (2.7 mM, 1.35 mM Na$_2$CrO$_4$-2H$_2$O) was incubated with 8.1 mM, 16.2 mM, or 27.0 mM glutathione and 0.10 M DMPO in 25 mM Tris-HCl, pH 8.0, 297 K. Reactions were initiated by the addition of glutathione, and EPR spectra recorded as described in Figure 1. The EPR spectra were recorded beginning 1 min from the start of each reaction and represent an average of 90 scans (21 s/scan). The intensity of each chromium(V) signal was measured by double-integration analysis of the first derivative spectra. Results are the mean ± SD of two determinations. (B) Intensity of DMPO-glutathione thiol radical adduct EPR signal produced upon reaction of chromium(VI) with glutathione in the presence of DMPO. Reactions were carried out as described in (A) and their EPR spectra recorded at 297 K as described in Figure 1. The EPR spectra were recorded beginning 1 min from the start of each reaction and represent an average of 90 scans (21 s/scan). For the DMPO-GS-radical adduct, the intensity of the second line (the first line being at lowest field) of the four-line signal was measured by the double-integration analysis of the first derivative spectra. Results are the mean ± SD of two determinations.
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Reactive Intermediates As Mediators of Chromium(VI)-Induced DNA Damage

We have shown that the nature and extent of chromium(VI)-induced DNA damage is strongly dependent on the nature and extent of reactive intermediates produced upon reaction of chromium(VI). Our earlier study (17) demonstrated that the nature of the reactive intermediates formed upon reaction of chromium(VI) with a reductant was critical in determining the type of DNA damage observed. Thus, reaction of chromium(VI) with glutathione led to formation of chromium(V) complexes as the major DNA damaging species, whereas, under identical conditions, reaction of chromium(VI) with hydrogen peroxide resulted in generation of hydroxyl radical as the primary DNA damaging agent (17). This study has also established the importance of the reduction pathway in determining the nature of chromium(VI)-induced DNA damage (Fig. 10). Reaction of chromium(VI) (primarily chromate at pH 8.0) with hydrogen peroxide produces hydroxyl radical as the significant DNA damage agent, with relatively little chromium(V) formation. The hydroxyl radical reacts with DNA, resulting in formation of DNA strand breaks and the 8-hydroxydeoxyguanosine adduct. Very little chromium-DNA binding is detected. On the other hand, reaction of chromium(VI) with glutathione gave rise to two chromium(V) species and the glutathione thiyl radical. Formation of chromium(V) correlated with the binding of chromium to DNA, resulting in the formation of DNA-chromium-glutathione crosslinks, which have been identified in an earlier study (24). No thiyl radical-mediated DNA strand breaks were detected.

In this study we have shown that, in addition to the nature of the reactive intermediate [i.e., chromium(V) or hydroxyl radical] being important in determining the nature of chromium(VI)-induced DNA damage, the relative amount of reactive intermediate generated correlated with the extent of observed DNA damage.

EPR signal suggests that it is likely to be a square pyramidal oxochromium(V) complex coordinated to two molecules of glutathione (27). The g = 1.996 chromium(V) species, which is detected only at the highest molar ratio of glutathione per chromium(VI) employed in this study [10 glutathione (27.0 mM) per chromium(VI) (2.7 mM)(Fig. 8A)] may be formed by the binding of one or two additional molecules of glutathione to the g = 1.986 chromium(V) complex. The increase in g value from 1.986 to 1.996 indicates the binding of additional sulfur atoms of chromium(V) (28).

No hydroxyl radical production was observed upon reaction of chromium(VI) with glutathione, which correlated with lack of detection of the 8-OH-dGuo adduct in DNA reacted with chromium(VI) in the presence of glutathione. In addition, no DNA strand breakage was detected, an observation which was consistent with earlier studies (16,17). However, Kortenkamp et al. (12) reported DNA strand breakage in PM2 phage DNA that had been reacted with chromium(VI) in the presence of glutathione. Their studies were carried out in HEPES or phosphate buffers (12). Since Tris is known to be a radical scavenger (29), it is possible that, under our conditions, the amount of glutathione thiyl radical available to react either directly with DNA or with oxygen to give hydroxyl radical was insufficient to cause extensive DNA damage. The explanation that a relatively low amount of glutathione thiyl radical may be available to react with DNA in our study is consistent with the observation that the maximum DMPO-glutathione thiyl radical adduct intensity observed (Fig. 8B) was 3-fold less than the DMPO-hydroxyl radical adduct intensity observed upon reaction of the lowest concentration (2.7 mM) of chromium(VI) with hydrogen peroxide (Fig. 2) and 7-fold less than the DMPO-hydroxyl radical adduct intensity observed at the highest concentration (27 mM) of chromium(VI) reacted with hydrogen peroxide (Fig. 2).

Figure 9. Level of chromium-DNA adducts produced upon reaction of calf thymus DNA with chromium(VI) in the presence of glutathione. Calf thymus DNA (1.8 mM; treated for iron removal as described in Figure 3) was incubated in the presence (●) or absence (○) of 1.8 mM chromium(VI) (0.9 mM Na,CrO\textsubscript{4}·2H\textsubscript{2}O) and 0, 5.4, 10.8, or 18.0 mM glutathione in 25 mM Tris-HCl, pH 8.0, 37°C for 30 min. Samples were repeatedly dialyzed and ethanol-precipitated as described earlier (17). Chromium was determined by atomic absorption spectroscopy using a wavelength of 357.9 nm on a Thermo Jarrell Ash Video 22 atomic absorption spectrophotometer equipped with a Model 686 furnace atomization unit, as described earlier (17), and DNA concentration was assayed using a modification of the microfluorometric technique of Kissane and Robins (49), as previously described (47). Samples consisting of DNA and no chromium gave a background (“control”) chromium/DNA ratio of 2.74 ± 1.56 × 10\textsuperscript{-11} nmole Cr/nmole DNA-P and therefore represents the detection limit of the instrument. Results are the mean ± SD of three to six determinations.

The increase in g value from 1.986 to 1.996 indicates the binding of additional sulfur atoms of chromium(V) (28).
Thus, when the reaction of chromium(VI) with glutathione was carried out in a glutathione concentration-dependent fashion, the amount of chromium(V)-glutathione complex formation increased as the concentration of glutathione in the reaction was increased. Consequently, reaction of chromium(VI) with DNA in the presence of glutathione resulted in chromium-DNA adducts, whose formation increased as the concentration of glutathione was increased. When the reaction of chromium(VI) with hydrogen peroxide was studied in a chromium(VI) concentration-dependent fashion, the amount of hydroxyl radical adduct generated increased as the concentration of chromium(VI) was increased. Consequently, reaction of chromium(VI) with DNA in the presence of hydrogen peroxide gave rise to the 8-hydroxydeoxyguanosine adduct and DNA strand breaks, whose formation increased as the concentration of chromium(VI) was increased. Kawanishi et al. (16) obtained similar results, showing that reaction of chromium(VI) with hydrogen peroxide resulted in formation of hydroxyl radical which was responsible for DNA strand breakage. They proposed that reaction of chromium(VI) with hydrogen peroxide produced the tetraperoxochromium(V) species which participated in a superoxide-driven, catalytic, Fenton-type reaction to produce hydroxyl radical as shown (16):

\[
\begin{align*}
2\text{Cr}^{VI}O_2^- + 9\text{H}_2\text{O}_2 + 2\text{OH}^- & \rightarrow 2\text{Cr}^{VI}(\text{O}_2)_{4^-}^n + 10\text{H}_2\text{O} + \text{O}_2^- \quad (1) \\
2\text{Cr}^{VI}(\text{O}_2)_{4^-}^n & \rightarrow 2\text{Cr}^{VII}O_2^2^- + 2\text{O}_2 + 2\text{O}_2^- \quad (2) \\
2\text{H}_2\text{O}_2 + 2\text{O}_2^- & \rightarrow 2\cdot \text{OH} + 2\text{OH}^- + 2\text{O}_2 \\
\end{align*}
\]

The above mechanism is a metal-catalyzed reaction in which tetraperoxochromium(V) is the catalyst which is continuously recycled [Eqs. (1) and (2)] and undergoes a Fenton-type reaction with hydrogen peroxide in a manner analogous to that of the ferrous and cuprous ions (14,15). Eq. (3) is the Haber-Weiss reaction representing the combination of superoxide and hydrogen peroxide to generate hydroxyl radical. For the reaction of tetraperoxochromium(V) with hydrogen peroxide to proceed in a catalytic fashion, it is clear that Eqs. (1) and (2) should occur at comparable rates. Our study shows a chromium(VI) concentration dependence of tetraperoxochromium(V) and hydroxyl radical production, suggesting that the reaction of chromium(VI) with hydrogen peroxide [Eq. (1)] is relatively slow. Thus, EPR studies of the reaction of the chromium(VI) with hydrogen peroxide at 77 K showed small but detectable levels of a tetraperoxochromium(V) EPR signal \( g_1 = 1.950, g_t = 1.983, g_{sw} = 1.972 \) similar to that reported by Dalal et al. (18) \( g_1 = 1.9445, g_t = 1.9862, g_{sw} = 1.9712 \) whose formation increased as the chromium(VI) concentration in the reaction was increased. In addition, the tetraperoxochromium(V) complex may be directly involved in hydroxyl radical production without regeneration of chromium(VI), in the following reaction proposed by Kawanishi et al. (16):

\[
\begin{align*}
\text{Cr}^{VII}(\text{O}_2)_{4^-}^n + n\text{O}_2^- + n\text{H}^+ & \rightarrow \text{Cr}^{VII}(\text{O}_2)_{4^-}^n(\text{O})_{w}^n + n\cdot \text{OH} + n\text{O}_2 \\
(n = 1-4) \\
\end{align*}
\]

This would then explain the chromium(VI) concentration dependence of hydroxyl radical production and suggests that reaction of chromium(VI) with hydrogen peroxide does not proceed in a catalytic Fenton-type reaction involving the tetraperoxochromium(V) intermediate. We have investigated the possibility that the tetraperoxochromium(V) intermediate may participate in a reductant-mediated Fenton-type reaction by addition of the reductant glutathione to the reaction of chromium(VI) with hydrogen peroxide. The order of addition of glutathione and hydrogen peroxide to chromium(VI) was found to be of importance, and the results will be discussed elsewhere (30).

It is possible that singlet oxygen may be the species involved in mediating the strand breakage detected upon reaction of chromium(VI) with DNA in the presence of hydrogen peroxide. Kawanishi et al. (16) used the singlet oxygen spin trap 2,2,6,6-tetramethyl-4-piperidone (TEMPO) and detected singlet oxygen in the reaction of chromium(VI) with hydrogen peroxide. They proposed that singlet oxygen was responsible for oxidation of the guanine residues on DNA, but that hydroxyl radical was primarily responsible for deoxyribose phosphate backbone breakage (16). However, Yamamoto et al. (31) have shown that reaction of the cobalt(II) ion with DNA in the presence of hydrogen peroxide produces singlet oxygen as the major species responsible for DNA cleavage.

The results obtained in this study, taken in conjunction with the cellular studies performed by other workers, offer strong evidence in favor of reactive interme-
diates such as chromium(V) complexes, hydroxyl radical and thyl radical being determinants of the nature of chromium(VI)-induced DNA damage. The types of DNA damage we have discussed in this study may be critical to the mutagenic and carcinogenic action of chromium(VI), e.g., misreading of DNA templates has been shown to occur at 8-hydroxydeoxyguanosine and at adjacent residues (32), while the stable DNA-chromium-glutathione adducts may crosslink the DNA extensively (24, 33–36), giving rise to lesions that are difficult to repair. The reduction pathway for chromium(VI) may therefore be a key determinant of the types of resulting DNA lesions and, consequently, of carcinogenic transformation of cells that are exposed to this carcinogen.

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