The Generation of DNA Single-Strand Breaks During the Reduction of Chromate by Ascorbic Acid and/or Glutathione *In Vitro*

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The potential role of iron and copper and the involvement of hydroxyl radicals in the DNA cleavage caused by chromate and glutathione (GSH) has been investigated. We have also studied the ability of chromate, on reaction with ascorbate as well as in mixed solutions of ascorbate and GSH, to cause DNA strand breaks. In both fully demetalated and conventional (i.e., metal contaminated) systems, chromate and GSH induced similar numbers of DNA strand breaks. This observation suggests that traces of iron or copper contaminating the reaction mixtures do not play a major role in the DNA cleavage caused by chromate and GSH. A series of hydroxyl radical scavengers exhibited a protective influence on the induction of DNA strand breaks. However, glucose and sucrose, both strong hydroxyl radical scavengers, showed no concentration-dependent inhibition of DNA cleavage. Competition kinetics studies yielded apparent rate constants that were not consistent with hydroxyl radicals being the species responsible for DNA strand breaks. Ascorbate in combination with chromate was also found to induce strand breaks in DNA; this damage could be attributed to reactive intermediates generated during the reduction. When mixed systems of ascorbate and GSH in the presence of chromate were investigated, there were clearly interactions between the two reductants. — Environ Health Perspect 102(Suppl 3):237–241 (1994)

Key words: chromium(VI), DNA strand breaks, ascorbate, glutathione, chromium(VI) reduction, competition kinetics

Introduction

Despite considerable research effort during the last ten years, the mechanisms by which the known human carcinogen chromate can cause DNA impairment and mutations still remain obscure. Chromate is rapidly taken up by mammalian cells and readily reduced by cellular constituents. It is now generally accepted that the intracellular reduction of the metal compound is crucial for the induction of DNA lesions (*1*). On the basis of kinetic studies *in vitro*, it has been suggested that glutathione (GSH), ascorbic acid (AsA), and cysteine are the most important nonenzymatic cellular constituents for the reduction of chromate in the cytoplasm (*2*). However, the relative contribution of these various reductants to the generation of DNA lesions is far from clear. Furthermore, it has as yet not been possible to identify the ultimate carcinogen deriving from chromium(VI) upon reduction.

While the reduction of chromate by GSH has been extensively studied in model systems *in vitro* (*3*) the significance of AsA as an intracellular reductant of chromate has, as yet, received comparatively little attention. Recently, Suzuki and Fukuda (*4*) reported that in the bronchoalveolar lining fluid and the lung parenchyma of rats AsA is more reactive than GSH in the reduction of chromate. In subsequent *in vitro* studies Suzuki (*5*) observed a synergistic effect of AsA and GSH on the rate of reduction of chromate. Later, Standeven and Wetterhahn (*6*) provided evidence suggesting that AsA may be the principal nonenzymatic reductant of chromate in rat liver and kidney. In view of these observations it is now necessary to consider the possibility that reduction by ascorbate mediates the generation of DNA lesions.

In the present article, we report some observations on the induction of DNA strand breaks by chromate and AsA. We also studied the generation of DNA cleavage in the presence of AsA, GSH, and chromate. Initial experiments in our laboratory revealed that AsA itself, in the absence of chromate, can cause DNA strand breaks. The effect is due to the generation of reactive oxygen species during the autoxidation of AsA (i.e., the oxidation of AsA in aqueous solution by dissolved dioxygen) (*7*). The reaction is catalyzed by traces of metal ions, namely copper(II) and iron(III). Ascorbate, in the absence of catalytic metals, is stable even in air-saturated solutions of neutral pH (*8*).

In the light of a controversy concerning the ability of chromate and GSH to induce DNA strand breaks in Fe- and Cu-free systems (*9*) it has also become necessary to reassess the role of traces of catalytic metal ions in the chromate/GSH reaction. While we have observed the induction of single strand breaks in PM2 DNA with chromate and GSH in both phosphate and HEPES buffer (*10*), Aiyar et al. (*11*) using pBR322 DNA, have detected very little, if any, DNA cleavage in a Tris-buffered system. They removed contaminating iron from all solutions while we used all reagents without further purification. The results of subsequent studies carried out in our laboratory (*12*) in an attempt to clarify the situation suggested that Fe could be important in these reactions. Moreover, we observed a protective influence of a series of hydroxyl radical scavengers on the generation of strand breaks by chromate and GSH. Taken together, these observations led us to suggest hydroxyl radicals as the ultimate DNA-cleaving species. However, in an article primarily concerned with studying the effects of Cr binding on the cleavage by restriction endonucleases, the Wetterhahn group recently observed the induction of strand breaks by chromate and glutathione in a fully demetalated system (*13*). The ability of chromate and GSH to cause DNA cleavage seems to be a

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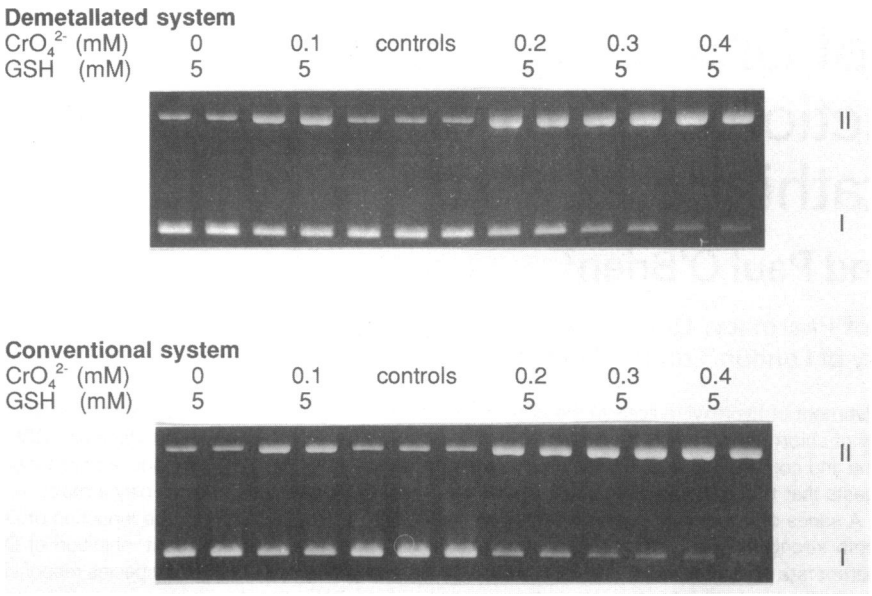

Figure 1. DNA strand breaks induced by chromate and GSH in demetalated and conventional systems. II: nicked, closed circular form of PM2 DNA, I: intact supercoil.

distinct possibility, but more experimental work is needed before firm conclusions can be drawn. Here, we communicate our recent observations that might help to clarify some of the earlier, apparently contradictory, results.

Materials and Methods

All reagents used were of analytical grade. Glutathione, ascorbic acid, diethylendiaminopentaacetic acid (DTPA) and ethidium bromide were from Sigma (Poole, Dorset, UK); bromophenol blue and agarose were from Biorad (Hemel Hempstead, UK). Potassium chromate (K_2CrO_4) was purchased from BDH-Merck (Poole, UK) and PM2 DNA was obtained from Dr. Irene Witte, University of Oldenburg FRG, who isolated it as described by Espejo et al. (*14*).

PM2 DNA (8 µg/ml), in a final volume of 20 µl, was treated with varying concentrations of K_2CrO_4, GSH, and/or AsA in phosphate buffer, 0.1 M, pH 7.0, for at least 3 hr at room temperature (close to 22°C). Where indicated ("demetalated system"), traces of Fe and Cu were removed from buffer solutions by stirring overnight with Chelex 100 resin. To remove metal traces from DNA, it was dialyzed (24 hr) against 1 mM DTPA (dissolved in demetalated phosphate buffer).

The successful removal of catalytic metals from buffer solutions was assessed by using the ascorbate method of Buettner (*8*). The method involves monitoring the autooxidation of ascorbate at 265 nm in the presence of test solution. After treatment with Chelex resin, ascorbate was stable (i.e., loss of absorbance <0.5%) for at least 30 min, indicating that metal ion contamination was minimal. DNA was considered free of metals when ascorbate at concentrations up to 1 mM (in the absence of chromate) did not induce any strand breaks.

Electrophoresis was carried out as described previously (*10,12*).

Results and Discussion

Chromate and Glutathione Cause DNA Strand Breaks in Iron(III)- and Copper(II)-free Systems

In an attempt to probe the involvement of Fe in the generation of DNA strand breaks by chromate and GSH, we studied the reaction in conventional (i.e., metal-contaminated) and exhaustively demetalated solutions. In both systems chromate and GSH induced similar numbers of single-strand breaks in isolated PM2 DNA (Figure 1). The level of cleavage increased with rising concentrations of chromate in phosphate buffer, 0.1 M, pH 7.0, containing 5 mM GSH .

These findings clearly indicate that traces of Fe or Cu contaminating the buffers or bound to DNA do not play a major role in the DNA cleavage caused by chromate and GSH. Our results are in agreement with a recent report by the Wetterhahn group that strand breaks are formed in a demetalated system (*13*), an observation that contradicts earlier reports by the same group (*11,15*).

The demonstration that there is no specific need for Fe or Cu in this reaction makes it necessary to reassess our earlier suggestion (*12*) of a thiyl radical/superoxide anion/hydrogen peroxide-mediated pathway leading to the generation of hydroxyl radicals as the ultimate DNA-cleaving agent. This suggestion was based on observations of a concentration-dependent depression of the level of DNA cleavage by benzoate and formate upon addition to chromate and GSH. Consequently, we extended these studies and explored the influence of a wider range of hydroxyl radical scavengers.

The results confirm our earlier findings (*12*). We observed a concentration-dependent inhibition of DNA degradation with sodium formate and sodium benzoate. Dimethyl sulfoxide (DMSO) and mannitol also showed a concentration-dependent inhibitory effect. To our surprise, glucose, a strong hydroxyl radical scavenger, exhibited no protective influence on the induction of strand breaks by chromate/GSH at concentrations of up to 10 mM. Sucrose showed effects very similar to those of glucose (data not shown). Table 1 summarizes typical results observed conventional, non-demetalated systems (for results with DMSO, see P. O'Brien and A. Kortenkamp, in this issue). Essentially the same results were obtained in systems with exhaustively demetalated DNA and buffer solutions (data not shown).

Competition Kinetics

The somewhat unexpected results with glucose and sucrose prompted us to probe further the involvement of hydroxyl radicals in the chromate/GSH system by using the method of competition kinetics. This method has been widely used in the determination of rate constants of radicals. It utilizes the fact that any hydroxyl radicals (•OH) generated in the present reaction mixtures should react with DNA to form a strand break. Any other molecule S added that is capable of reacting with •OH should

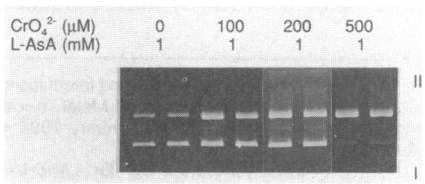

Figure 2. DNA strand breaks caused by chromate and ascorbate. II: nicked, closed circular form of PM2 DNA, I: intact supercoil.

Table 1. Inhibitory effect of formate, benzoate, mannitol, and glucose on DNA strand breaks generated by chromate (0.2 mM) and glutathione (5 mM).

	Number of strand breaks			
Scavenger, mM	Formate	Benzoate	Mannitol	Glucose
0	0.70	0.66	0.67	0.83
1.0	0.53	0.20	0.60	0.67
2.5	0.36	0.16	0.58	0.85
5.0	0.33	0.11	0.48	0.60
7.5	0.22	0.13	0.42	0.84
10	0.11	0.04	0.40	0.26

compete with DNA for •OH, thereby reducing the level of DNA cleavage. The extent of competition, and hence the degree of protection against strand scission will depend on the rate constant for reaction of S with •OH and its concentration relative to DNA. Since the rate constants for reaction of all the above scavengers with •OH are known (*16*), analysis of the data obtained (Table 1) should allow calculation of a consistent value for the rate of reaction of DNA with •OH. Furthermore, the rate constant calculated should agree with the literature value 0.8×10^9 M^{-1} s^{-1} (*17*) for the rate of reaction of •OH with DNA-P.

The changes in the number of chromate/GSH-induced DNA strand breaks in the presence of varying concentrations of •OH scavengers can be expressed by

$$(N_o/N)-1 = k_s[S]/k_{DNA}[DNA]$$

in which N_o and N are the numbers of strand breaks in the absence or presence of scavenger, respectively; [S] and [DNA] are the concentration of scavenger and DNA-P, respectively; and k_s and k_{DNA} are the bimolecular rate constants for the reactions of •OH with scavenger and DNA, respectively. Thus, a plot of $(N_o/N)-1$ versus the concentration of scavenger should be linear with an intercept of 1 and give as the slope the ratio $k_s/k_{DNA}[DNA]$. The rate constant for the reaction of •OH with PM2 DNA can then be calculated from known values of k_s. As shown in Table 2, consistent values for k_{DNA} are not obtained. Furthermore, far higher concentrations of scavengers are needed to inhibit the DNA cleavage induced by chromate and GSH than would be expected for a reaction involving free hydroxyl radicals. Consequently, the apparent values of k_{DNA} exceed diffusion limit.

We conclude that simple Fenton chemistry does not explain the ability of chromate and GSH to induce single-strand breaks in DNA and that Fe or Cu is not necessary for this process. At this stage, we can only speculate which species are responsible for the DNA damage. Hypervalent Cr species known to be generated during reduction by GSH include several chromium(V) [Cr(V)] complexes. Both Lay and co-workers (*18*) and we (*10*) found evidence that Cr(V) complexes can cause DNA single-strand breaks. Thus, mechanisms involving the direct reaction of a high oxidation state complex with DNA, as suggested by Lay, are worth serious consideration. Furthermore, there is now evidence for some accumulation of Cr(IV) during the reaction of chromate with GSH (*19*), and chromium(IV) complexes are known to cause C—C bond scission (*20*). A further possibility includes the reaction of hypervalent Cr with molecular oxygen to form some reactive DNA cleaving species (P. O'Brien and A. Kortenkamp, this issue).

Another factor, which might help explain the conflicting results concerning the ability of chromate and GSH to cause DNA cleavage, is the nature of the buffering agent. We showed earlier (*10*) that the buffering agent present in the incubation mixtures exhibits a strong influence on the level of strand breaks induced by chromate and GSH. When HEPES buffer was used instead of phosphate buffer, higher chromate concentrations were required to produce the same level of DNA strand breaks. The use of Tris seems to be problematic; it interferes with the reduction of chromate by AsA (*21*). We are exploring the effect of buffering agents in greater detail.

Induction of DNA Strand Breaks by Chromate in Combination with Ascorbate and Glutathione

Chromate and AsA, in the absence of traces of catalytic metal ions, caused DNA single-strand breaks. Figure 2 shows the results of an experiment in phosphate-buffered solution containing 1 mM AsA. With rising concentrations of chromate, the level of strand breaks increased and reached a plateau as the ratio chromate:AsA approached 1. In the presence of 100 μM chromate, the number of strand breaks was still significantly higher than in the absence of chromate. These results indicate that chromate, upon reduction by AsA, has the potential to generate species that cleave DNA and lend further support to the idea that the reduction of chromate is a crucial step in the generation of DNA lesions. It remains to be seen whether this also applies to the induction of DNA-protein cross-links.

Suzuki has reported that the rate of reduction of chromate by AsA increases with rising concentrations of AsA *in vitro* (*5*). We conducted experiments using chromate and AsA at concentrations similar to those chosen by Suzuki and observed a decrease in the level of DNA cleavage with increasing concentrations of AsA (Figure 3). These results could be indicative of an inverse relationship between the rate of reduction and the generation of DNA damage: the slower the reduction, the greater the damage. Alternatively, the effect could be due to an efficient scavenging of the cleaving species by an excess of AsA. The observation of a protective effect with increasing amounts of AsA clearly indicates that the DNA damage is caused by a species generated during the reduction of chromate by AsA rather than by the final product of the reaction. If the final product were responsible for the DNA impairment, rising amounts of AsA should have left the level of strand breaks unchanged.

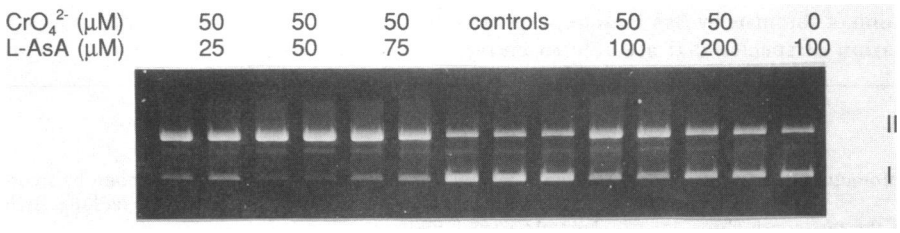

Figure 3. The influence of varying amounts of ascorbate on the DNA cleavage induced in the presence of constant amounts of chromate. II: nicked, closed circular

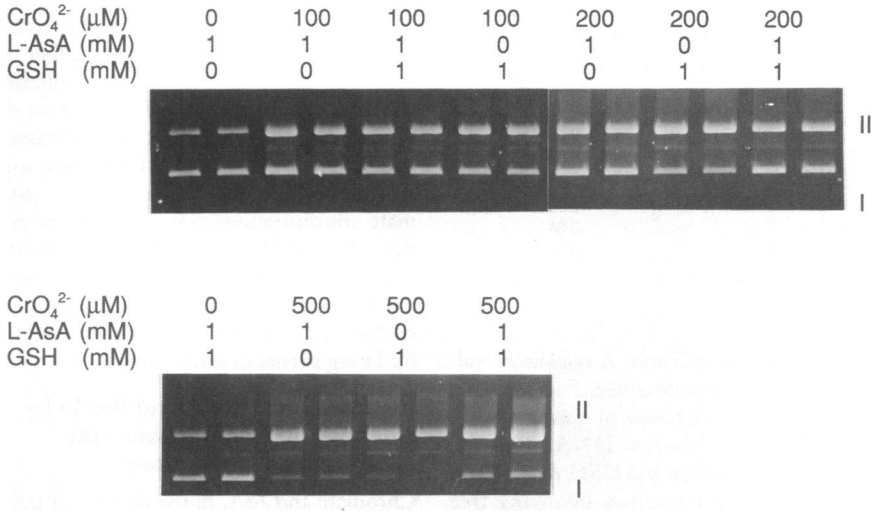

Figure 4. DNA strand breaks generated by chromate and mixed solutions of ascorbate and glutathione. II: nicked, closed circular form of PM2 DNA, I: intact supercoil.

We started exploring the DNA damaging effects of chromate in mixed solutions of AsA and GSH. Glutathione, when administered separately, appeared to be more effective than AsA in generating DNA strand breaks in the presence of identical amounts of chromate. As shown in Figure 4, samples containing chromate (100, 200, 500 μM), but with GSH (1 mM) instead of AsA, yielded slightly higher levels of strand breaks. The effect of mixed systems on the induction of DNA strand breaks varied with the concentration of chromate in the samples. Upon addition of AsA (1mM) to solutions containing GSH (1 mM) and relatively small amounts of chromate (100 μM, 200 μM) the number sof strand breaks returned to the levels observed with chromate and AsA alone (Figure 4). With relatively high amounts of chromate (500 μM) however, the combination of AsA and GSH exhibited a slightly protective influence on the generation of strand breaks; the cleavage observed was less pronounced than with AsA and chromate alone.

Table 2. Values for the apparent rate constant k_{DNA} for the reaction of hydroxyl radical with DNA calculated from the results shown in Table 1.

Scavenger	k_{DNA}	k_s
	$M^{-1}s^{-1}$	$M^{-1}s^{-1}$
Formate	4.9×10^{16}	3.2×10^9
Benzoate	3.2×10^{15}	5.9×10^9
Mannitol	4.8×10^{15}	1.0×10^9

Values for k_{DNA} shown compare to a literature value of 0.8×10^9 $M^{-1}s^{-1}$ (*17*). The other constants are taken from Dorfman and Adams (*16*).

Conclusions

Our efforts to define which DNA damaging species are generated upon reduction of chromate by GSH now lead us to conclude that ·OH are not the cause for the strand scission observed in PM2 DNA. The process does not depend on the presence of Fe or Cu, and simple Fenton chemistry can not explain the experimental results. It seems to us that the direct interaction of some hypervalent Cr species with DNA, probably involving the participation of molecular oxygen, is worth serious consideration.

We have demonstrated that the reduction of chromate by AsA leads to the generation of species that are able to cleave DNA. The final product of the reaction is not involved in the induction of these effects. The ultimately active species generated in such reaction mixtures are not yet known; however it is likely that they are different from the species present in solutions containing chromate and GSH.

The study of systems in mixed solutions of AsA and GSH has yielded results that are indicative of an inverse relationship between rate of reduction and generation of DNA damage. Another important factor is the influence of scavenging properties of AsA or GSH when present in excess. More work is needed to probe this suggestion further.

Although the levels of AsA and GSH in the human lung are not precisely known, available data for rats suggest that the cytoplasmic levels of both reductants are equimolar and fall in the millimolar range (*4*). Considerable amounts of Cr can accumulate inside cells after exposure to chromate. Sehlmeyer et al. (*22*) have shown that after the exposure of V79-cells to 10 μM chromate the total intracellular, cytosolic, Cr concentrations can be in the millimolar range. Under similar conditions Cr levels in nuclei can be even higher than in the cytosol. The concentrations of chromate, AsA, and GSH used in our experiments are similar to levels that might be attained within cells; our observations are of relevance to the *in vivo* situation. However, it is too early for firm conclusions because the relative contribution of the extracellular reduction of chromate in lung tissue and the influence of intracellular compartmentalization is not known. Suzuki and Fukuda (*4*) have reported that no other effective reductant apart from AsA exists in the alveolar-lining fluid of rats. Thus, in the earlier periods of exposure to chromate, extracellular reduction may be the overall determining step. After depletion of extracellular AsA, intracellular reductions may become more important. However, more information is needed to delineate between these two routes of chromate reduction.

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