Reactive Oxygen Species Produced from Chromate Pigments and Ascorbate

Yves Lefebvre and Henri Pezerat

Université Pierre et Marie Curie, Laboratoire de Réactivité de Surface et Structure, Paris, France

The reactions of various chromate pigments and ascorbate were investigated by an ESR spin trapping technique. Production of Cr(V) was detected directly and productions of very electrophilic reactive oxygen species (ROS) was detected via the oxidation of formate. We demonstrated previously that both dissolved oxygen and Cr (V) were essential in the production of ROS in this system, and that ROS production was inhibited by catalase. We studied here the effect of solubility of different chromate pigments: sodium, calcium, strontium, basic zinc, basic lead supported on silica, and lead and barium chromates on the production of ROS in buffered medium and cell culture medium (Dublecco's Modified Eagle medium + fetal calf serum). Sodium, calcium, basic zinc, and basic lead chromates were active in the production of ROS in presence of cell culture medium, whereas lead and barium chromates were inactive. — Environ Health Perspect 102(Suppl 3):243–245 (1994).

Key words: chromate, ascorbate, reactive oxygen species, chromium(V), electron paramagnetic resonance, carcinogenesis

Introduction

In a recent article (*1*) we demonstrated that the reaction of soluble Cr(VI) with ascorbate in aqueous aerated medium produces a very electrophilic species of oxygen capable of oxidizing formate to carboxylate radicals. While Cr(V) was essential to observe the oxidative behavior, hydroxyl radicals were not detected in the medium. We suggested that the reactive oxygen species could be a Cr(V)-superoxo complex. These previous findings support classical theories of carcinogenicity implying strongly electrophilic species as DNA-damaging agents (*2*), and give further evidence for the role of oxidative processes in chromium carcinogenesis (*3*). The purposes of the present article are to check if the previously proposed mechanism with soluble chromate can work with less soluble or relatively insoluble chromate pigments used in industry; and, in an attempt to validate this mechanism in the biological environment, to study the effect of a typical cell-culture medium on the kinetics of Cr(V) and reactive oxygen species (ROS) formation. We also report on replacing ascorbate with other reducing agents, the results of which suggest that the production of ROS from Cr(VI) in aerated medium is not "ascorbate dependent."

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Address correspondence to Dr. Henri Pezerat, Université Pierre et Marie Curie, Laboratoire de Réactivité de Surface et Structure, 4 place Jussieu, 75252 Paris, Cedex 05, France. Telephone 33 1 4427 5521. Fax 33 1 4427 6033.

Materials and Methods

We used different chromate pigments possessing moderate to very low solubilities, along with soluble sodium chromate, and tested them for the production of ROS in the presence of ascorbate. (The provenance of these chromate pigments is as follows: $CaCrO_4$: ICN Pharmaceuticals, Plainview, NY; basic zinc chromate: Labosi, Paris, France; lead silicochromate: Société Industrielle du Titane, France; $SrCrO_4$: Ventron, Germany; Na_2CrO_4 and $PbCrO_4$: Merck, Darmstadt, Germany; $BaCrO_4$: Labosi, Paris, France. Detailed analysis and physicochemical characterization of these pigments was given previously (*4*). ROS were detected by the oxidation of formate, and the oxidation product (carboxylate radicals) was quantified by spin-trapping experiments with DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) (*1,5*). L-(+)-ascorbic acid and reduced glutathione (GSH) were from E. Merck (Darmstadt, Germany), NADPH and L-cysteine were from Sigma (St. Louis, MO). Reactions were conducted at 37°C in 100 mM phosphate buffer, pH 7.4, in aerated medium. The phosphate buffer solution and the distilled water used to prepare the other solutions were purified with the aid of Chelex-100 resin (Bio-Rad Laboratories, Richmond, CA) in order to remove trace iron impurities. Cell-culture product Dubelcco's Modified Eagle Medium + 20% fetal calf serum (DMEM/FCS) was kindly supplied by Dr. Z. Elias (INRS, Vandoeuvre-lès-Nancy, France). Electronic spin resonance (ESR) spectra were recorded using a Varian E-3 spectrometer and a flat quartz cell for measurements; results are reported as the mean of the two signal intensities after 10 and 30 min. Reproducibility of measurements is within 10%. We obtained g factors by calibration against DPPH (g = 2.0036).

Results and Discussions

The production of ROS and Cr(V) from chromate pigments and ascorbate is presented in Table 1. To probe the reactivity of these solid chromate pigments, we chose to work at low concentration in ascorbate (1 mM), because an excess of this reagent would simply lead to the disappearance of any ROS eventually formed. We also studied the effect of the DMEM/FCS culture medium, which is known to increase the solubility of chromate pigments (*4*). An ESR signal of intensity under 100 arbitrary units (a.u.) in $[DMPO–COO^-]^\bullet$ spin adducts is considered nonsignificant; intensity between 100 and 500 a.u., moderate; and intensity over 500 a.u., high. An intensity of 1000 a.u. corresponds approximately to 4×10^{18} spins L^{-1} (*5*). Only the very insoluble $PbCrO_4$ and $BaCrO_4$ do not produce ROS detectable in significant amounts. While soluble Na_2CrO_4 produces the higher intensity signal, all moderately soluble chromates, including $SrCrO_4$, produce detectable ROS in the presence of the biological medium. The difference between basic lead silicochromate and lead chromate is noteworthy, underlining the role of the different physicochemical properties of

Table 1. Comparison of the $[DPMO–COO^-]^•$ and Cr(V) signal intensitites in the presence or absence of DMEM/FCS medium after reaction of various chromates with ascorbate.

	Signal intensity, (arbitrary units)			
	With DMEM/FCS		Without DMEM/FCS	
Chromate	$[DMPO–COO^-]^•$	Cr(V)	$[DMPO–COO^-]^•$	Cr(V)
Na_2CrO_4	880	10	600	25
$CaCrO_4$	460	20	570	10
Basic zinc chroamte[a]	310	250	250	90
Basic lead silicochromate	425	25	550	10
$SrCrO_4$	90	0	165	0
$PbCrO_4$	10	0	0	0
$BaCrO_4$	5	0	0	0
Without chromate	10	0	0	0
Without ascorbate	0	10	0	0

[a]"Zinc yellow." Solid 10 mg except for soluble Na_2CrO_4 used as 1 mM. Ascorbate 1 mM, phosphate buffer, pH 7.4, 100 mM sodium formate 1 M, DMPO 50 mM. DMEM/FCS used as 25% of the reaction mixture. Results represent the mean of signal intensities after 10 and 30 min reaction at 37°C. A result is considered significant in $[DMPO–COO^-]^•$ if the intensity is >100 u.

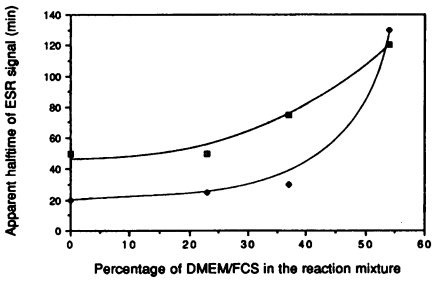

Figure 1. Influence of DMEM/FCS medium on the stabilities of $[DMPO–COO^-]^•$ and Cr(V) radicals formed during the reaction of soluble chromate(VI) with ascorbate at 37°C. (■), $[DMPO–COO^-]^•$; (◆). Cr(V). Conditions: Na_2CrO_4 10 mM, ascorbate 10mM, phosphate buffer, pH 7.4, 100mM, NaHCOO 1mM, DMPO 50 mM, and DMEM/FCS as indicated.

these minerals. The overall effect of DMEM/FCS is not clear from results presented in Table 1, probably because it acts simultaneously in different ways, including increasing chromate solubility (increasing ROS), reducing Cr(VI) (increasing ROS), complexing Cr(V), and reducing ROS.

In general DMEM/FCS is a weakly complexing and reducing medium, and its effect is not very apparent at low Cr(VI) and ascorbate concentrations. Results with 10 mM soluble sodium chromate and 10 mM ascorbate (instead of 1 mM) reveal somewhat lower ROS but higher Cr(V) production in the presence of DMEM/FCS; however, almost all moderately to poorly soluble chromate pigments show no ROS production in the presence of 10 mM ascorbate (data not shown).

It seems that basic zinc chromate yields significantly more Cr(V) than any other chromate (Table 1), irrespective of the presence of DMEM/FCS. It is possible that the Zn(II) ion thus has a stabilizing effect on Cr(V). This may help explain the synergistic effect between Cr(VI) and Zn(II) observed for SHE cell transformation (*6*).

Kinetic results of the reaction between soluble chromate with ascorbate, in presence of various DMEM/FCS concentrations, are presented in Figure 1. The halftimes reported in ordinate are apparent, because they represent an equilibrium between formation and disappearance of the radical species. At 0% DMEM/FCS, we observe a $T_{1/2}$ of 50 min for the spin adduct $[DMPO–COO^-]^•$, which agrees reasonably well with the value of 60 min obtained by Zalma (*7*). We observed for Cr(V) a $T_{1/2}$ of 20 min, indicating that it may form a complex with the ascorbate radical produced during this reaction (*1,8*).

While increasing the DMEM/FCS concentration, we observed a gradual augmentation of the $T_{1/2}$ values, which became much more pronounced as the concentration of 50% was reached. $T_{1/2}$ for Cr(V) increased more steadily than for $[DMPO–COO^-]^•$, indicating complexation of Cr(V) by DMEM/FCS. The kinetics of ROS and Cr(V) production are sufficiently slow to permit their study in biological systems. At 100% DMEM/FCS, we can extrapolate that both $T_{1/2}$ values will reach many hours.

Table 2. $[DMPO–COO^-]^•$ and Cr(V) production from soluble chromate and various biologic reductants.

Reducing agent	Signal intensity, (arbitrary units) $[DMPO–COO^-]^•$	Cr(V)	*g*[Cr(V)]
GSH	220	200	1.988
NADPH	320	6500	1.982
Cysteine	20	0	—

[a]Conditions: Na_2CrO_4 10 mM, phosphate buffer, pH 7.4 100mM, formate 1 mM, DMPO 50 mM. GSH 30 mM, or NADHPH 10 mg (≈5 mM), or cysteine 10 mM. Results represent the mean of signal intensities after 10 and 30 min reaction at 37°C. Control experiments (without chromate or without reductant) were performed and yielded insignificant values.

The reaction of chromate with ascorbate was chosen because it reacts rapidly and gives a good yield of ROS, and also because ascorbate is an important reductant of chromate in biologic systems (*9*). However, other biologic reductants can lead to ROS in the presence of chromate in aerated medium. Table 2 suggests that ROS are also produced by a reaction of Cr(VI) with glutathione, γ-L-glutamyl-L-cysteinylglycine (GSH) and β-nicotinamide adenine dinucleotide phosphate (NADPH). Both reactions lead to Cr(V) long-lived complexes (*10,11*). However, the reaction of cysteine with chromate, which produces only short-lived Cr(V) (*12*), does not lead to formate oxidation in our experimental conditions. These results suggest a correlation between the production of ROS in these reactions and the ability to stabilize Cr(V), such stabilization being provided whether by the reductants or their conjugated oxidized form. It seems reasonable to envisage an interaction between paramagnetic O_2 and Cr(V), and then appearance of ROS. For ascorbate, we can suggest the following reactions:

$$Cr(VI)+HA^- \rightarrow Cr(V)\ldots(A^{\bar{•}})+H^+ \quad [1]$$

$$O_2+Cr(V)\ldots(A^{\bar{•}}) \rightarrow Cr(V)\ldots(O_2^{\bar{•}})+A \quad [2]$$

$$Cr(V)\ldots(O_2^{\bar{•}}) \leftrightarrow Cr(VI)\ldots(O_2^{2-}) \quad [3]$$

where HA^- = ascorbate, $A^{\bar{•}}$ = ascorbate radical, A = dehydroascorbate.

We suggest the formation of a $Cr(V)\ldots(O_2^{\bar{•}})$ complex in the Cr(VI)-ascorbate reaction because we did not observe •OH in this case (*1*). For GSH and NADPH, the mechanism is still to be elucidated and may be different than the ascorbate mechanism. •OH formation is very possible with GSH and NADPH, and may involve the reaction of H_2O_2 with Cr(V) in a Fentonlike reaction (*13*).

Conclusions

The reaction of ascorbate with various chromate pigments produces ROS as evidenced by formate oxidation in aqueous solution at 37°C. The ROS production seems closely related to the solubility of the pigments. The presence of a cell-culture medium (DMEM/FCS) has a measurable effect in terms of kinetics of Cr(V) and carboxylate radical production. This is important because the observed half-lives in presence of DMEM/FCS are of sufficient magnitude to allow biologic manifestations (eventually cancer) to occur.

The reduction of Cr(VI) leading to ROS requires that some degree of stabiliza-

tion of the produced Cr(V) occurs. The reduction by cysteine, which does not provide such stabilization, logically does not produce ROS capable of oxidizing formate. Another condition for ROS production would be that the Cr(V) ligands possess some degree of lability, but it is generally recognized that both Cr(VI) and Cr(V) complexes are subject to ligand exchange reactions (*14,15*), thus permitting the redox couple Cr(VI)/Cr(V) to act as a catalyst.

This article supports mechanistic considerations relative to the appearance of cancer from chromate exposure. It rests on classic theories of carcinogenicity that imply strongly that electrophilic species (including ROS) cause primary DNA damage. The origin of ROS is molecular oxygen, not hydrogen peroxide. We consider dissolved oxygen activation mechanisms very important for cancer-causing oxidative damage because oxygen is present in every cells of the body, whereas H_2O_2 is produced only in very few cells like macrophages. For chromates, ROS production originating from dissolved oxygen may arise from a variety of biologic reductants, including ascorbate, glutathione, and NADPH.

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