Chromium(V)−Sialic (Neuraminic) Acid Species Are Formed from Mixtures of Chromium(VI) and Saliva

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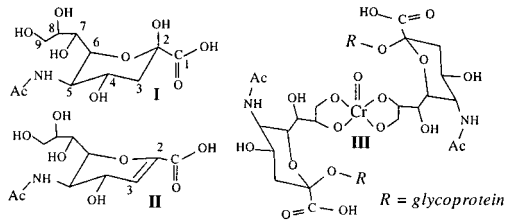
The use of Cr(VI) compounds in leather tanning, pigment production and stainless steel welding can pose serious hazards to workers in these industries, since Cr(VI) is a documented human carcinogen.[1] Epidemiological studies have shown that occupational exposure to Cr(VI) increases the risk of workers developing cancer of the lung and the sinonasal cavity and many countries limit both water soluble and insoluble Cr concentrations in the workplace.[2]

The primary pathways for human uptake of Cr(VI) are inhalation, ingestion, and skin permeation; inhalation of Cr can cause irritation of the nasal mucosa, nasal ulcers, perforation of the nasal septum, and cancer.[2] While the exact mechanisms of Cr(VI)-induced carcinogenicity are not fully understood, there is strong evidence that Cr(V) species, produced from the intracellular reduction of water-soluble (cell-permeable) or insoluble (phagocytosed) Cr(VI) complexes, play a role in damage to DNA and other biomolecules that could initiate cancer if left unrepaired.[3]



To model the species formed in the human respiratory tract after Cr(VI) inhalation, EPR spectroscopy was used to study the reaction between Cr(VI) and human saliva. These mixtures, in the absence of exogenous reductant, exhibit strong EPR signals ascribable to Cr(V) (d^1) species ligated by *N*-acetylneuraminic acid residues (**I**), derived from **I**-terminated salivary glycoproteins. EPR spectra from solutions of Cr(VI) and isolated salivary components, such as mucin (bovine submaxillary mucin, 12% bound **I**), or modified **I** residues (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid; **II**) show similar spectral characteristics to those of Cr(V)-saliva species. Complexes between Cr(V) and **I**-terminated glycoproteins (**III**) are assigned to the dominant EPR signals from mixtures of Cr(VI) and saliva.

The room-temperature X-band EPR spectra from a mixture of whole saliva[4] and Cr(VI) (4 mM) at $t = 5$ min (pH 6.90) shows a signal (line width = 2.2 G) centered at $g_{iso} = 1.9794$ (Figure 1). This spectrum is observed without the addition of exogenous reductant.[5] The spectrum is most consistent with a five-coordinate oxoCr(V) species with oxygen (alcoholato) donors, since the signal line width is too narrow to account for any superhyperfine coupling from nitrogen nuclei (^{14}N $a_{iso} \sim (2.0-2.5) \times 10^{-4}$ cm^{-1}) derived from small molecules and/or salivary proteins.[3b,6] Spin quantitation determined the concentration of the Cr(V)-saliva species to be $\sim 130 \pm 20$ μM, which was maintained over a 24 h period (Figure 1, inset).

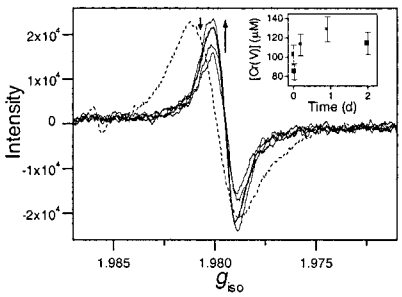


Figure 1. EPR spectra (solid lines) of Cr(V)-saliva species ([Cr(V)] inset) at $t = 4$ min, 30 min, 270 min, 22 h, and 48 h ([Cr(VI)] = 4 mM). The dashed line is the signal from a solution of bovine submaxillary mucin (9 mg mL^{-1}), Cr(VI) (4 mM), and GSH (2 mM).

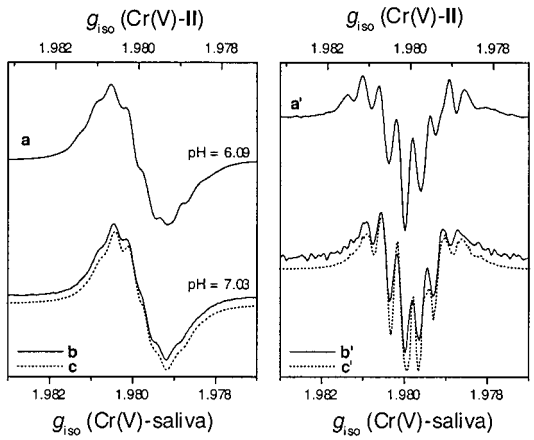


Figure 2. EPR spectra from mixtures of Cr(VI) (40 mM), **II** (100 mM), and glutathione (2 mM) (a) and Cr(VI) and saliva (components $M_r <$ 30 000; [Cr(VI)] = 4 mM) as observed (b) and simulated (c), presented as first (left) and second (right) derivatives.

A similar, though broader, EPR spectrum ($g_{iso} = 1.9799$, line width = 3.5 G) was observed from a solution of the sialoglycoprotein, bovine submaxillary mucin (9 mg mL^{-1}), Cr(VI) (4 mM), and glutathione (2 mM) at pH 7.1 (Figure 1).[7] EPR spectra were also acquired from mixtures of Cr(VI) and saliva that had previously been subject to ultracentrifugation, using devices with molecular weight cutoffs of 3, 10, 30, or 100 kDa. While the g_{iso} values of the Cr(V) EPR signals observed from mixtures of Cr(VI) and either whole saliva or the salivary filtrates were similar (again, the spectra were acquired in the absence of exogenous reductant), there were notable spectral differences. First, the EPR spectra of Cr(V)-salivary filtrates (Figure 2b,b′) showed well-resolved ^1H a_{iso} superhyperfine coupling (0.67×10^{-4} cm^{-1}). Second, the intensity of the Cr(V)-salivary filtrate signals was significantly greater ($\sim 3\times$) than signals obtained with whole saliva, under the same instrument conditions. These results were best understood upon consideration of the specific concentration of **I** in whole saliva and in salivary filtrates.

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(4) The whole saliva used in all of these studies was collected from one of the authors (RC; healthy 34 year-old female, blood group B) using salivette tubes (Sarstedt), after a minimum starvation period of 2.5 h.

(5) The most likely candidate acting as the endogenous salivary reductant of Cr(VI) is uric acid, which has been noted as a salivary antioxidant (see: Moore, S.; Calder, K. A. C.; Miller, N. J.; Rice-Evans, C. A. *Free Radical Res.* **1994**, *21*, 417−425).

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(7) In this model system, an exogenous reductant increased the [Cr(V)], as described in other Cr(V)−alcoholato systems, and shows a minor Cr(V)-GSH species at $g_{iso} = 1.9858$ (refs 9 and 11).

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Human saliva and mucosa contain a variety of glycosylated and sialoglycosylated proteins that are terminated by fucose, galactose, and **I** residues.[8a] Of all the salivary sialoglycoproteins (mucins, α-amylase, lactoferrin, sIgA, proline-rich proteins), **I** comprises ∼19% of the total terminating carbohydrate residues.[8a] In addition to sialoglycoproteins, human saliva contains electrolytes, urea, uric acid, and glucose.[8b] Since Cr(V) is known to form complexes with the *cis*-1,2-diolato functionality of D-glucose,[9] the possibility that the Cr(V)-saliva signal was predominantly due to Cr(V)-D-glucose complexes was considered, but discounted on several accounts. First, EPR spectra of Cr(V)-D-glucose species are different from the spectra of Cr(V)-saliva species described here.[9] Second, the Cr(V) EPR signal obtained from an untreated Cr(V)-saliva mixture was similar to that obtained from a mixture in which the small molecule components (M_r < 1 000) had been removed by size exclusion chromatography.[10] Third, the ^{1}H a_{iso} values in the spectra of Cr(V) and salivary filtrates (0.67×10^{-4} cm^{-1}) are indicative of protons that are not part of a cyclically strained molecule. In sugars and cyclically strained analogues, the ^{1}H a_{iso} values are closer to 0.95×10^{-4} cm^{-1}.[9,11] Complexes between Cr(V) and linear ligands having more flexible backbones, such as glycerol or 1,2-ethanediol, yield EPR signals with ^{1}H a_{iso} values of $\sim 0.62 \times 10^{-4}$ cm^{-1}.[12] Although saliva contains D-glucose, this work indicates that Cr(V)-**I** species are present in significantly higher concentrations than Cr(V)-D-glucose species. This may be due to electronic effects reducing the donor ligand strength of the *cis*-1,2-diolato group in D-glucose (due to its proximity to the electron-withdrawing endocyclic oxygen atom),[9] relative to the glycerol-like tail of **I**.

When present as the terminating residue of carbohydrate chains of salivary glycoproteins, **I** is linked to the penultimate sugar residue via the 2′ position leaving the glycerol-like, 6′-triol substituted tail of the molecule free for metal binding. EPR spectra from solutions of Cr(VI) and **II** (Figure 2a,a′) are very similar to those of the Cr(V)-saliva species. Since there is no tertiary hydroxy acid in **II** for Cr(V) binding, it is a good model for the glycerol-like tail motif of **I**-terminated glycoproteins. The Cr(V)-saliva signal (Figure 2b,b′) was simulated (Figure 2c,c′) as an overlapping quintet ($g_{iso} = 1.9799$, ^{1}H $a_{iso} = 0.68 \times 10^{-4}$ cm^{-1}; 13.4%), sextet ($g_{iso} = 1.9798$, ^{1}H $a_{iso} = 0.59 \times 10^{-4}$ cm^{-1}; 23.4%), and septet ($g_{iso} = 1.9797$, ^{1}H $a_{iso} = 0.69 \times 10^{-4}$ cm^{-1}; 63.2%). These Cr(V) species are consistent with coordination to 8,9- and/or 7,8-diolato groups of the 2′-bound **I** residues (**III**). Coordination by two **I** (or two **II**) residues via the 8,9- or the 7,8-diolato groups would yield a septet or a quintet EPR signal, respectively, since the protons in these complexes are magnetically equivalent. A mixed-ligand species, with an 8,9- and a 7,8-diolato donor from **I**, would give rise to a sextet EPR signal. It is likely that the dominant Cr(V) signal observed in mixtures of Cr(VI) and saliva involve coordination of small **I**-terminated glycoprotein units (**III**, M_r *R* is small), rather than from completely unhydrolyzed **I**-terminated glycoproteins, since the latter would result in a broader Cr(V) signal, similar to that observed for the sialylated Cr(V)-bovine submaxillary mucin (M_r ∼1 MDa) signal. Complexes between Cr(V) and small **I**-terminated glycoproteins are best modeled by Cr(V)-**II** complexes, as evidenced by the similarity between the EPR spectra of Cr(V)-**II** and Cr(V)-salivary filtrates, at similar pH values (Figure 2). Also, the concentration of a Cr(V) complex with two free **I** residues coordinated via the hydroxy acid moiety is very small, since Cr(V)-bis-(1,2-**I**) has an EPR singlet with a lower g_{iso} value than **III**,[13] which would be evident in the signal from the Cr(VI)-saliva reaction. The distinct EPR signals for the Cr(V)-**I** linkage isomers[13] are similar to those of Cr(V)-quinic acid species[11] and to signals from ligand-exchange reactions between parent Cr(V)-bis-hydroxy acid species (which have EPR singlets at $g_{iso} \sim 1.9785$[3b]) and excess 1,2-diolato ligands.[12]

Table 1. Cr(V)/Saliva Mixtures: [Cr(V)], [**I**], and [Protein]

M_r fraction	[protein] (mg mL^{-1})	[**I**] (mg mL^{-1})	[**I**] specific	[Cr(V)] (mM)
whole	1.065	0.058	0.054	0.130
<100 000	0.274			0.223
<30 000	0.127	0.012	0.094	0.383
<10 000	0.060	0.023	0.383	0.370
<3 000	0.065	0.034	0.523	0.393

The concentration of total (bound and unbound) **I** in the saliva used in these experiments was estimated by using the Warren−Aminoff method[14] as 58.2 μg mL^{-1} (188 μM), which agrees with literature reports (57 μg mL^{-1}).[15] In addition, the specific concentrations of **I** ([**I**]/[protein]) in the salivary filtrates were determined (Table 1). These results show that as the total concentration of salivary protein decreases in the salivary filtrates from 100, 30, and 10 to 3 kDa, the specific concentration of **I** increases. This observation further substantiates the notion that the Cr(V)-saliva species are stabilized by ligands derived from **I**-terminated salivary glycoproteins, since the increase in the specific concentration of **I** maps onto the increase in the intensity of the Cr(V) signal obtained from mixtures of Cr(VI) and salivary filtrates.

The nature of the biological damage potentially caused by Cr(V)-**I**-terminated glycoprotein species is not clear; however, it is possible that damage may occur via DNA cleavage and/or the oxidation of alternative biomolecules.[3] Chromium(VI) has been shown to be genotoxic and cytotoxic in human gastric mucosa cells[16] and more recent work has shown that Cr(V) cleaves α_1-acid glycoprotein (a sialoglycoprotein).[17] Currently we are seeking to obtain further details of the structure of Cr(V)-**I**-terminated glycoprotein complexes and to investigate the potential biological damage caused by these species.

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(10) The intensity of the signal was somewhat diminished, which was most likely a result of simultaneously removing endogenous salivary small-molecule reductants.

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