EPR Studies of Chromium(V) Intermediates Generated via Reduction of Chromium(VI) by DOPA and Related Catecholamines: Potential Role for Oxidized Amino Acids in Chromium-Induced Cancers

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The reductions of $K_2Cr_2O_7$ by catecholamines, DOPA, DOPA-β,β-d_2, *N*-acetyl-DOPA, α-methyl-DOPA, dopamine, adrenaline, noradrenaline, catechol, 1,2-dihydroxybenzoic acid (DHBA), and 4-*tert*-butylcatechol (TBC), produce a number of Cr(V) electron paramagnetic resonance (EPR) signals. These species are of interest in relation to the potential role of oxidized proteins and amino acids in Cr-induced cancers. With excess organic ligand, all of the substrates yield Cr species with signals at $g_{iso} \sim 1.972$ ($A_{iso}(^{53}Cr) > 23.9 \times 10^{-4}$ cm^{-1}). These are similar to signals reported previously but have been reassigned as octahedral Cr(V) species with mixed catechol-derived ligands, $[Cr^{V}(semiquinone)_2(catecholate)]^+$. Experiments with excess $K_2Cr_2O_7$ show complex behavior with the catecholamines and TBC. Several weak Cr(V) signals are detected after mixing, and the spectra evolve over time to yield relatively stable substrate-dependent signals at $g_{iso} \sim 1.980$. These signals have been attributed to $[Cr(O)L_2]^-$ (L = diolato) species, in which the Cr is coordinated to two cyclized catecholamine ligands and an oxo ligand. Isotopic labeling studies with DOPA (ring or side chain deuteration or enrichment with ^{15}N), and simulation of the signals, show that the superhyperfine couplings originate from the side chain protons, confirming that the catecholamine ligands are cyclized. At pH 3.5, a major short-lived EPR signal is observed for many of the substrates at $g_{iso} \sim 1.969$, but the species responsible for this signal was not identified. Several other minor Cr signals are detected, which are attributed (by comparison with isoelectronic V(IV) species) to Cr(V) complexes coordinated by a single catecholamine ligand (and auxiliary ligands e.g. H_2O), or to $[Cr(O)L_2]^-$ (L = diolato) species with a sixth ligand (e.g. H_2O). Addition of catalase or deoxygenation of the solutions did not affect the main EPR signals. When the substrates were in excess (pH > 4.5), primary and secondary (cyclized) semiquinones were also detected. Semiquinone stabilization by Zn(II) complexation yielded stronger EPR signals ($g_{iso} \sim 2.004$).

Introduction

Chromium(VI) is a common occupational carcinogen,[1] although the main carcinogenic and mutagenic species are proposed to be Cr(IV/V) intermediates formed during the reduction of Cr(VI) to stable Cr(III) compounds. This uptake−reduction model of carcinogenesis[2] involves Cr(VI) entry into cells via anionic transport channels and subsequent intracellular reduction. The interactions of Cr(VI) with ascorbate,[3−5] glutathione,[6−8] and hydrogen peroxide[9,10] have been studied in detail. Several genotoxic Cr(V) species have been detected, and Cr(IV) has been postulated as being present.[8] DNA cleavage resulting from incubation of DNA with Cr(VI) and biological reductants has been postulated to be due to •OH or related radicals from Fenton-type reactions.[5,6,11−13] However, the evidence for the involvement of such species is indirect and has been criticized.[9,14] While there is evidence that there is an oxygen dependence of Cr(VI) genotoxicity, this does not necessarily involve H_2O_2 or •OH radicals.[15,16] Similarly, the presence of long-lived Cr(IV) species in these reactions was shown to be incorrect.[15,17]

Compounds containing the catechol (1,2-benzenediol) group are prevalent in mammalian tissues. Examples include important neurotransmitters (and their precursors), such as dopamine and DOPA, hormones including adrenaline and noradrenaline,[18] and

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the skin pigment, melanin, which is formed by oxidation and polymerization of catecholamines.[19] Other sources of human exposure to catecholic groups involve the oxidation of polyaromatic compounds contained in air pollution and cigarette smoke[20] and the presence of hydroxylated tyrosine residues (DOPA) on proteins damaged by γ-irradiation, UV photolysis, or Fenton-like mechanisms.[21−25]

Several studies have investigated the redox properties of substituted catechols and DOPA derivatives with transition metal ions.[26−34] The $[Cr(quinone)_3]^{3+}$ to $[Cr(catecholate)_3]^{3-}$ series of complexes have been obtained both via direct synthesis from Cr(III) and substituted catechols[28,29] and by reduction of Cr(VI) with catechols.[30] Catecholamine oxidation by Fe(III),[26,27,35,36] Mn(III),[36] and V(IV)[37] has been investigated in relation to the neurotoxicity observed in Parkinson's and Alzheimer's diseases. DOPA and protein-bound DOPA have also been shown to promote DNA damage via reduction of Cu(II) to Cu(I) and radical generation.[38]

While many studies have focused on the reactions of Cr(VI) with a variety of biomolecules, the complexity of the intracellular chemistry requires a knowledge of the reactions of Cr with all potential reductants and ligands that may contribute to its metabolism. In this study the interactions of Cr(VI) with catechol(amines) (Figure 1) have been examined by electron paramagnetic resonance (EPR) spectroscopy, to determine the nature and extent of formation of reactive species. These studies show that both Cr(V) and organic radicals are generated during the reduction of Cr(VI) by free catecholamines. The implications of these findings for Cr(VI)-induced genotoxicity are discussed.

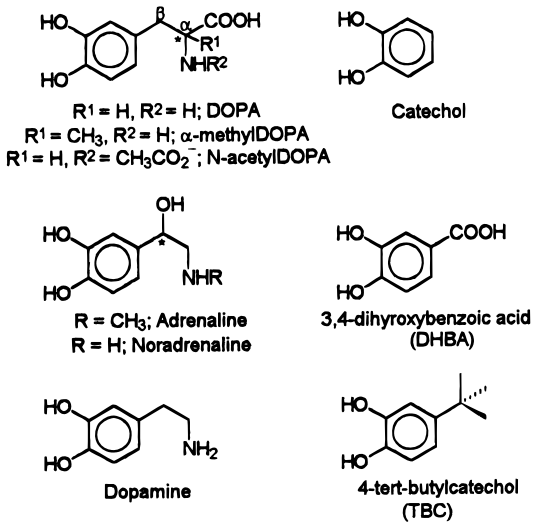

Figure 1. Structures of the catecholamines used during these studies. Chiral centers are indicated by asterisks.

Experimental Section

Materials. The catecholamines D,L-DOPA, D,L-α-methyl-DOPA, dopamine (hydrochloride salt), adrenaline, noradrenaline (all from Sigma), L-DOPA-*ring*-d_3 (Aldrich, 98+ at. %), catechol (Aldrich, 99%), 3,4-dihydroxybenzoic acid (Aldrich, 97%), and 4-*tert*-butylcatechol (Aldrich, 97%) were used as received. D,L-DOPA-β,β-d_2·HBr (CDN Isotopes, 98+ at. %) contained a large proportion of the oxidized compound D,L-dopaquinone-β,β-d_2. This was reduced to DOPA-β,β-d_2 by addition of almost stoichiometric quantities of $NaBH_4$ prior to use. $K_2Cr_2O_7$ (Merck, 99.5%) was used as the source for Cr(VI) without further purification. Catalase (from bovine liver, 1880 U mg^{-1}), tyrosinase (from mushroom, 3400 U mg^{-1}), tyrosine, and *N*-acetyltyrosine were obtained from Sigma; tyrosine-^{15}N (98+ at. %) was purchased from Cambridge Isotope Laboratories, and $ZnSO_4$·$7H_2O$ (99.5%) was from BDH Chemicals. $Na[Cr^{V}O(ehba)_2]$·H_2O was prepared by the method of Krumpolc and Roček.[39] Acetate and phosphate buffers were prepared by dilution of Chelex-treated 1 M CH_3COONa (Merck) or NaH_2PO_4 (Merck) with Milli-Q water, and the pH value was adjusted by addition of glacial acetic acid (Merck, 99.7%) or NaOH (Aldrich, 99.99%), respectively. HEPES buffer (Aldrich, 99%) was prepared in Milli-Q water. Typically, stock solutions were prepared with [catechol(amine)] = 25 ± 1 or 50 ± 1 mM and $[K_2Cr_2O_7]$ = 50 ± 1 mM. Catecholamine solutions were prepared immediately prior to use, to minimize autooxidation.

***Caution**: Cr(VI) is mutagenic and carcinogenic.[1] Cr(V) species[40−42] and the intermediates generated in the reduction of Cr(VI) by catechol(amine)s[43] are capable of cleaving DNA. Appropriate precautions should be taken to avoid skin contact and dust inhalation while handling these chemicals.*

EPR Experiments. A Bruker EMX spectrometer was used for recording X-band EPR spectra from solution samples contained in a Wilmad quartz flat cell. The spectra were calibrated with a Bruker EMX035M NMR gaussmeter in conjunction with an EMX048T Microwave Bridge Controller and an EMX032T Field Controller. EPR spectrometer parameters were typically as follows: central field, 3500−

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3540 G; sweep width, 30−200 G; scans, 2−50; resolution, 1024 points; microwave frequency, ~9.7 GHz; microwave power, 30 mW; receiver gain, 2×10^6; modulation frequency, 100 kHz; modulation amplitude, 0.05−1.0 G; conversion time, 81.92 ms; time constant, 40.96−163.84 ms. EPR spectra were analyzed using WinEPR[44] and simulated using WinSim (Version 0.96, NIEHS).[45] All the spectra presented were filtered with the moving average method over seven experimental data points (WinEPR).[44]

Typical experimental conditions used for acquiring EPR spectra of $K_2Cr_2O_7$ with catecholamines are outlined below. In all cases the ratios are stated as [$K_2Cr_2O_7$]:[catecholamine] (=0.5[Cr(VI)]:[catechol(amine)]). When a 1:1 ratio (with respect to dichromate) was required, concentrations of the substrates were either 5 or 12.5 mM. Excess catecholamine was used in a 2:9 ratio (5 mM:22.5 mM), and with excess $K_2Cr_2O_7$, ratios were typically 8:1 (40 mM:5 mM) or 4:1 (80 mM:20 mM). The enhanced solubility of catechols resulted in slightly altered reactant ratios; for 1:1 ratios, [$K_2Cr_2O_7$] and [catechol] were 5 or 25 mM, but with an excess of either reactant, typically ratios of 10:1 (25 mM:2.5 mM) or 5:1 (25 mM:5 mM) were used. Experiments with additional components were carried out with 100−200 mM Zn(II) or 0.1 mg mL^{-1} catalase. Deoxygenated samples were prepared on a Schlenk line and transferred to the EPR cell under an Ar atmosphere.

In situ generation of DOPA and DOPA-*^{15}N* was achieved by oxidation of tyrosine or tyrosine-*^{15}N* (2.5 mM) in phosphate buffer (50 mM) with tyrosinase (80 U mg^{-1} substrate).[46] The samples were incubated at room temperature for 15 min and became slightly pink. The solutions were then added to $K_2Cr_2O_7$ to give [$K_2Cr_2O_7$]:[tyrosine + oxidized tyrosine] = 10 mM:2 mM. The expected conversion of tyrosine to DOPA was ~50% (by comparison with literature data),[46] yielding a $K_2Cr_2O_7$ excess of ~10:1.

Tyrosine and *N*-acetyltyrosine solutions (2.5 mM) were γ-irradiated (~1000 Gy) in the presence of O_2 with a ^{60}Co source, to generate DOPA and *N*-acetyl-DOPA, respectively.[24] After irradiation, catalase solution (0.01 mg mL^{-1} irradiated solution) was added, to remove radiation-generated H_2O_2. DOPA concentrations were determined by HPLC analysis;[47] [DOPA] = 0.15 mM, and [*N*-acetyl-DOPA] = 0.22 mM.

Determination of [DOPA] by HPLC Analysis. Irradiated tyrosine or *N*-acetyltyrosine solutions (0.9 mL) were analyzed for DOPA as described previously.[47] A gradient of solvent A (100 mM/10 mM sodium perchlorate/sodium phosphate, pH 2.5) in solvent B (80% (v/v) methanol in water) was used. The gradient profile was slightly different from the previous method: isocratic elution with 0% solvent B for 10 min; a gradient of 3% solvent B in 2 min; further elution at 3% solvent B for 6 min; 10% solvent B for 7 min followed by 20% solvent B for 5 min, and then 80% solvent B for 8 min, before reequilibration with 100% solvent A for 10 min.

Results

Reactions of $K_2Cr_2O_7$ with DOPA. When $K_2Cr_2O_7$ (5 mM) was mixed with DOPA (22.5 mM, 2:9) in water (pH ~ 4.5), the color changed from yellow to brown. A strong EPR signal was detected at $g_{iso} = 1.9718$ (line width at full width half-maximum (LW) = 2.2×10^{-4} cm^{-1}), which decayed over ~30 min (Figure 2, Table 1). No ^1H or ^{14}N superhyperfine couplings were observed, but ^{53}Cr hyperfine satellites were detected ($A_{iso} = 24.0 \times 10^{-4}$ cm^{-1}). At early times (<5 min), a very weak signal was also present at $g_{iso} = 1.9800$. With a 1:1 $K_2Cr_2O_7$:DOPA mixture (5 mM:5 mM, or 12.5 mM:12.5 mM for greater signal intensity), the main signals ($g_{iso} = 1.9769$, 1.9725, and 1.9718) were too weak to determine $A_{iso}(^{53}Cr)$ values and decayed within ~15 min. The sample underwent a color change similar to that observed with excess DOPA. The 2:9 and 1:1 ratio experiments in HEPES (pH = 7.0−7.5, 100 mM) or phosphate (pH = 7.0−7.4, 100 mM) buffers yielded similar Cr signals, but with lower intensities than those observed in water.

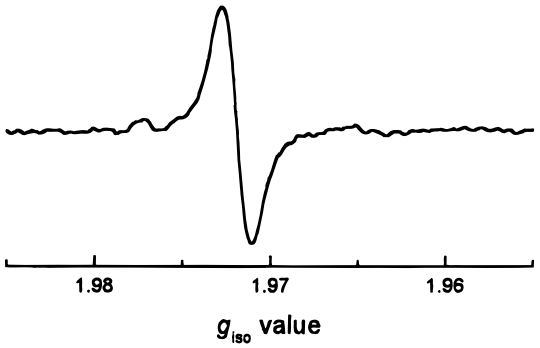

Figure 2. EPR spectrum of the signal at $g_{iso} = 1.9718$ obtained when excess DOPA (20 mM) was mixed with $K_2Cr_2O_7$ (5 mM) in water (pH ~ 4.5). A similar signal was detected at all pH values investigated (pH 3.5−7.5) when DOPA was in excess. Experimental parameters: receiver gain, 2×10^6; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; resolution, 1024; conversion time, 81.92 ms; time constant, 163.84 ms; scans, 2.

With excess $K_2Cr_2O_7$ (40 mM:5 mM, 8:1, Figure 3, Table 1), complex EPR spectra evolved over a period of 30 min. At early times (<5 min), the spectra exhibited very weak signals at $g_{iso} = 1.9739$, 1.9718, and 1.9688 (Figure 3a). Over time (10−20 min) the signals at $g_{iso} = 1.9718$ and 1.9688 disappeared, while that at $g_{iso} = 1.9739$ increased in intensity, and new signals appeared at $g_{iso} = 1.9800$ and 1.9775 (Figure 3b,c). After 30 min, the $g_{iso} = 1.9800$ signal dominated the spectrum, and low-intensity signals remained at $g_{iso} = 1.9775$ and 1.9739 (Figure 3d). The $g_{iso} = 1.9800$ signal grew over the next 2−3 h, and a weak signal was still present after 17 h. In phosphate (pH = 7.0−7.4, 100 mM) or HEPES (pH = 7.0−7.5, 100 mM) buffers, similar signals were observed, but the g_{iso} values and relative signal intensities were altered, especially in HEPES buffer at early reaction times (Figure 4a, Table 1). After ca. 30 min, the signal at $g_{iso} = 1.9800$ ($A_{iso}(^{53}Cr) = 16.3 \times 10^{-4}$ cm^{-1}) was dominant and showed complex superhyperfine coupling. In phosphate buffer (pH = 7.4) with higher reactant concentrations ([$K_2Cr_2O_7$] = 80 mM, [DOPA] = 20 mM, Figure 5a), the relative peak heights of the superhyperfine couplings of the $g_{iso} = 1.9800$ signal changed with time, indicating that it originates from a mixture of at least two Cr(V) species. Under these conditions, a second weak signal was detected with $g_{iso} = 1.9765$, which exhibited a complex superhyperfine coupling pattern that was too weak to simulate accurately. Q-band EPR experiments undertaken to deconvolute the components of the $g_{iso} = 1.9800$ signal were unsuccessful, due to low spectral intensities. Preparation of the Cr(V)−DOPA complexes from ligand-exchange experiments with [$Cr^VO(ehba)_2$]$^-$ ($ehbaH_2$ = 2-ethyl-2-hydroxybutanoic acid) was attempted, but DOPA rapidly reduced the Cr(V) center, showing that ligand exchange was likely to be rate determining in the redox reaction.

The $g_{iso} \sim 1.980$ signal was simulated (Figure 5a) using contributions from two overlapping components (Table 2). The first of these at $g_{iso} = 1.9805$, displayed couplings to two equivalent cyclized DOPA ligands, with the two α protons yielding a triplet ($a_{iso} = 0.91 \times 10^{-4}$ cm^{-1}) and the four β protons coupling to give a pseudo-triplet (see Discussion, $a_{iso} = 0.75 \times 10^{-4}$ cm^{-1}). The second component at $g_{iso} = 1.9799$ displayed couplings to four α protons (due to decarboxylation during cyclization of the dopaquinone) with $a_{iso} = 0.81 \times 10^{-4}$ cm^{-1}, and coupling to the β protons again gave a pseudo-triplet but with a reduced coupling constant, $a_{iso} = 0.56 \times 10^{-4}$ cm^{-1}.

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Table 1. Summary of the Major Cr(V) Species Detected during the Reduction of $K_2Cr_2O_7$ by Catecholamines

substrate	react conditions[a]	time/min	g_{iso} value	LW[b]/(10^{-4} cm^{-1})	$A_{iso}(^{53}Cr)$/(10^{-4} cm^{-1})	other a_{iso}/(10^{-4} cm^{-1})
DOPA	4:1 (pH > 4.5)[c]	> 5	1.9805	4.3[d]	16.3	complex
	4:1 (pH > 4.5)[c]	> 5	1.9799	4.3[d]	16.3	complex
	8:1 (pH > 4.5)	> 10	1.9775[e]			
	8:1 (pH > 4.5)	> 10	1.9739[e]			
	2:9 (pH >3.5)	< 30	1.9718	2.2	24.0	unresolved
	2:9 (pH = 7.4)[f]	< 5	1.9690			complex
	2:9 (pH = 3.5)	< 5	1.9689[e]			
	8:1 (pH = 7.4)	< 10	1.9689			
DOPA-β,β-d_2	> 4:1 (pH = 7.4)[g]	> 20	1.9805	4.0[d]	*e*	complex
	> 4:1 (pH = 7.4)[g]	> 20	1.9799	4.0[d]	*e*	complex
	> 4:1 (pH = 7.4)[g]	> 10	1.9775			
	> 4:1 (pH = 7.4)[g]	> 15	1.9741			1.08 d
	> 4:1 (pH = 7.4)[g]	< 10	1.9719	2.4	*e*	
	> 4:1 (pH = 7.4)[g]	< 10	1.9683[e]			
α-Me-DOPA	4:1 (pH = 7.4)[c]	> 5	1.9801	2.5	16.6	complex
	4:1 (pH = 7.4)[c]	> 5	1.9797	2.5	16.6	complex
	4:1 (pH = 7.4)[c]	> 5	1.9740[e]			
	2:9 (pH = 7.4)	< 20	1.9720	2.6	24.4	unresolved
	4:1 (pH = 7.4)	< 10	1.9682[e]			
dopamine	4:1 (pH = 7.4)[c]	> 5	1.9804	4.3[d]	*e*	complex
	4:1 (pH = 7.4)[c]	> 5	1.9799	4.3[d]	*e*	complex
	2:9 (pH = 4.5)	< 20	1.9719	2.4	24.4	unresolved
	8:1 (pH = 4.5)	< 10	1.9686[e]			
adrenaline	4:1 (pH = 6.5)[c]	> 5	1.9796	4.3[d]	*e*	complex
	4:1 (pH = 6.5)[c]	> 5	1.9793	4.3[d]	*e*	complex
	2:11 (pH = 4.5)	< 20	1.9722[e]	2.5		
	2:11 (pH = 4.5)	< 20	1.9690[e]			
	6:1 (pH = 4.5)	< 5	1.9690			
noradrenaline	4:1 (pH = 6.5)[c]	> 5	1.9796	5.6[d]	*e*	complex
	4:1 (pH = 6.5)[c]	> 5	1.9792	5.6[d]	*e*	complex
	8:1 (pH = 4.5)	15−45	1.9738[e]			
	2:9 (pH = 4.5)	< 20	1.9721	2.4	23.5	unresolved
	8:1 (pH = 4.5)	< 10	1.9690[e]			

[a] Ratios stated in the form [$K_2Cr_2O_7$]:[catecholamine], [minor reactant] = 5 mM. [b] LW, line width. [c] Signals measured with higher reactant concentrations, [$K_2Cr_2O_7$] = 80 mM and [catecholamine] = 20 mM. [d] Line width for two overlapping signals. [e] Signal too weak and/or short-lived to measure superhyperfine and/or ^{53}Cr hyperfine couplings. [f] Observed following addition of Zn(II) after all Cr signals have disappeared (>30 min). [g] Substrate generated by reduction of dopaquinone-β,β-d_2; therefore, the exact reactant ratio was not known.

Immediately after mixing the reactants, the solution turned brown and became dark brown/black during the next hour. The UV/visible spectra (Figure S1, Supporting Information) of the solutions exhibited a peak at 380 nm, which is characteristic of both Cr(V) species[48] and dopaquinone,[49] and the background absorbance increased steadily due to the formation of dopachrome[49] and black polymerized materials (which are probably melanin-like pigments).

With DOPA in D_2O (pD = 4.0−5.0), no discernible changes in the g_{iso} values or the superhyperfine couplings were apparent, compared to the H_2O experiments, showing that the superhyperfine couplings were not due to exchangeable protons. Similarly, the correspondence of Cr signals observed with DOPA-*ring*-d_3 in H_2O or D_2O to those with DOPA showed that the superhyperfine couplings for the $g_{iso} \sim 1.980$ signal do not originate from the ring protons.

With DOPA-β,β-d_2, signals were only detected when $K_2Cr_2O_7$ was in excess (80 mM:<20 mM), because of impurities present in the commercial sample (see Experimental Section). The signals had the same g_{iso} values, and kinetic behavior (including changes in the component ratio for the $g_{iso} = 1.9800$ signal over time) as those observed with DOPA, but had a simpler superhyperfine coupling pattern (Figure 5b). The signal was simulated with parameters similar to those for the DOPA signal (Figure 5b, Table 2), but the β protons were substituted with deuterons (i.e. for H, $I = {}^1/_2$, but for D, $I = 1$, and $a_H \sim 6.5a_D$).

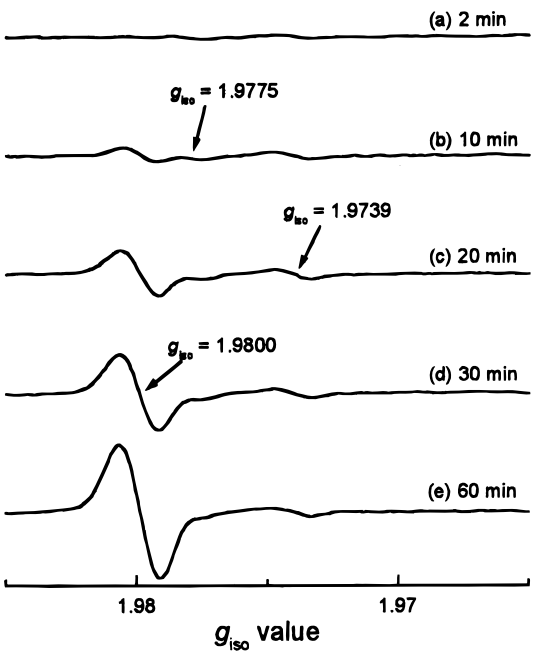

Figure 3. EPR spectra obtained after mixing $K_2Cr_2O_7$ (40 mM) and DOPA (5 mM) in unbuffered aqueous solution (pH ~ 4.5). The spectra show the evolution of the Cr(V) species at different times after mixing; the $g_{iso} \sim 1.980$ signal continues to grow up to 2 h after mixing. Experimental parameters: receiver gain, 2×10^6; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; resolution, 1024; conversion time, 81.92 ms; time constant, 163.84 ms; scans, 2.

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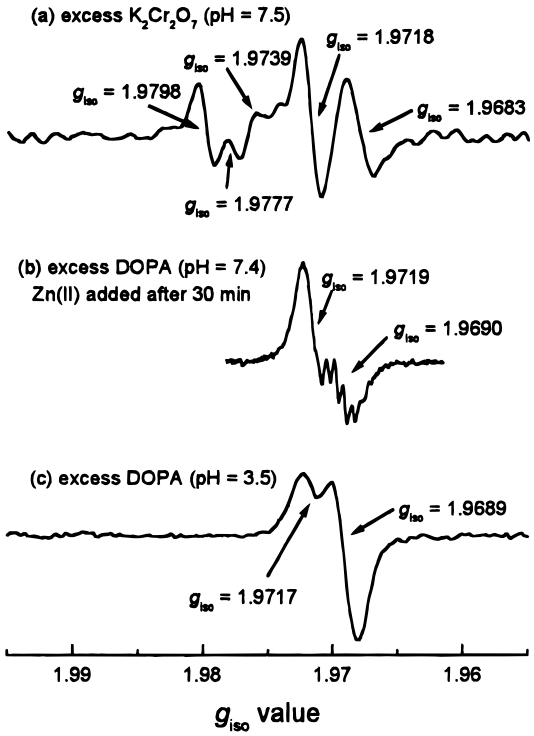

Figure 4. EPR spectra of the Cr(V) species generated: (a) ~5 min after mixing excess $K_2Cr_2O_7$ (40 mM) with DOPA (5 mM) in HEPES buffer (100 mM, pH = 7.5); (b) ~3 min after addition of Zn(II) (200 mM) to the reaction (after all of the Cr(V) signals had disappeared) of $K_2Cr_2O_7$ (5 mM) with excess DOPA (22.5 mM) in phosphate buffer (50 mM, pH = 7.4); and (c) 2 min after mixing $K_2Cr_2O_7$ (5 mM) with excess DOPA (22.5 mM) in acetate buffer (100 mM, pH = 3.5). The spectra clearly show the signals at $g_{iso} \sim 1.969$, as well as species obtained more readily under other conditions. Experimental parameters: receiver gain, 2×10^6; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; resolution, 1024; conversion time, 81.92 ms; time constant, 163.84 ms; scans, 2−10.

In addition to this signal, ~1 h after mixing, weak signals were detected at $g_{iso} = 1.9775$ and 1.9741. The latter signal displayed some superhyperfine couplings but was too weak to simulate accurately.

With α-methyl-DOPA, g_{iso} values and kinetics were observed in the EPR spectrum similar to those obtained with DOPA, but the superhyperfine coupling pattern was different (Figure 5c). The relative intensities of the superhyperfine components again changed with time, indicating that the signal is due to more than one species. The signal was simulated (Figure 5c) with parameters similar to those employed for DOPA, but the α proton coupling constant was replaced by a small quartet coupling with the methyl protons (Table 2).

Enzymatic generation of DOPA and DOPA-^{15}N in phosphate buffer was achieved in situ by treating tyrosine and tyrosine-^{15}N, respectively, with mushroom tyrosinase.[46] Immediately after mixing the resulting solutions with excess $K_2Cr_2O_7$, weak EPR signals at $g_{iso} \sim 1.980$ appeared, which became more intense over the following hour. After ~30 min, the spectra obtained with DOPA-^{15}N or DOPA were identical; thus, no coupling to the amine nitrogen is observed. No EPR signals were detected from $K_2Cr_2O_7$/tyrosinase controls.

Experiments with DOPA or *N*-acetyl-DOPA (generated by γ-irradiation of tyrosine and *N*-acetyltyrosine, respectively)[38] and excess $K_2Cr_2O_7$ yielded weak signals with DOPA, but none with *N*-acetyl-DOPA. HPLC analysis showed comparable levels of oxidation for both samples, consistent with the differences being due to substrate reactivity and not concentration effects.

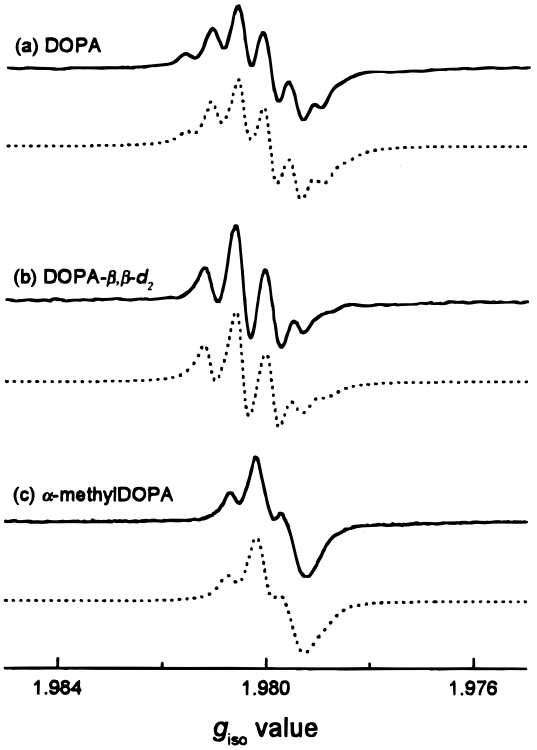

Figure 5. EPR spectra of the signals at $g_{iso} \sim 1.980$ obtained approximately 10−30 min after mixing excess $K_2Cr_2O_7$ with (a) DOPA, (b) DOPA-β,β-d_2, and (c) α-methyl-DOPA ([$K_2Cr_2O_7$] = 80 mM, [catecholamine] = 20 mM). The experimental spectra are shown with solid lines, and the simulated spectra are shown with dotted lines. Experimental conditions: receiver gain, 2×10^6; modulation frequency, 100 kHz; modulation amplitude, 0.1 G; resolution, 1024; conversion time, 81.92 ms; time constant, 40.96 ms; scans, 4−30.

Table 2. Simulation Parameters for the Cr(V) Signals at $g_{iso} \sim 1.980$ Generated during Reaction of Excess $K_2Cr_2O_7$ with DOPA and Its Derivatives

substrate	R[a]	g_{iso}	%	LW[b]	superhyperfine couplings[c]/(10^{-4} cm^{-1})
DOPA	0.990	1.9805	7.5	0.24	a_α 0.91 (2H); a_β 0.75 (t)[d]
		1.9799	92.5	0.51	a_α 0.81 (4H); a_β 0.56 (t)[d]
DOPA-β,β-d_2	0.978	1.9805	32.1	0.39	a_α 1.00 (2H)
		1.9799	67.9	0.59	a_α 0.78 (4H)
α-methyl-DOPA	0.995	1.9801	16.4	0.25	a_β 0.94 (t);[d] a_{Me} 0.14 (6H)
		1.9797	83.6	0.42	a_α 0.46 (2H); a_β 0.65 (t);[d] a_{Me} 0.15 (6H)

[a] *R*, correlation coefficient. [b] Line width (LW) is calculated assuming that the line shapes are 100% Lorentzian. [c] See eq 3 for the key to the origin of the couplings; t, triplet. [d] Triplet coupling is due to two equivalent pairs of β protons and is equal to $a_{\beta 1} + a_{\beta 2}$ (see text).

In addition to the observed Cr(V) EPR signals, weak organic signals at $g_{iso} \sim 2.004$ were detected during DOPA oxidation (in H_2O, HEPES, or phosphate buffers, pH = 4.0−7.5), especially when [DOPA] ≥ [$K_2Cr_2O_7$]. The stabilization of semiquinones (SQ) by complexation to Zn(II) (100−200 mM) enhances detection of SQ radicals by EPR spectroscopy[19,50,51] and was used to confirm their presence in the $K_2Cr_2O_7$/DOPA reactions. With [$K_2Cr_2O_7$]:[DOPA] ratios of 1:1 and 2:9, a strong ZnSQ signal was observed at $g_{iso} = 2.0039$ with complex multiplet splitting patterns (Figure S2a, Supporting Information), but when $K_2Cr_2O_7$ was in excess (8:1), no ZnSQ signal was observed. The ZnSQ signals were simulated satisfactorily with

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Table 3. Summary of the Organic Semiquinones Detected during the Reduction of $K_2Cr_2O_7$ by Excess Catecholamines in the Presence of Zn(II) (100−200 mM)

substrate[a]	assignmt (rel [SQ])	superhyperfine couplings[b]/(10^{-4} cm^{-1})
DOPA	primary (44%)	a_3 0.22 (1H); a_5 3.58 (1H); a_6 0.62 (1H); a_β 5.36 (d)[c]
	secondary (56%)	a_3 0.23 (1H); a_5 3.57 (1H); a_6 0.63 (1H); a_β 0.75 (1H)
DOPA-*ring*-d_3	primary (47%)	a_3 0.05 (1D); a_5 0.54 (1D); a_6 0.09 (1D); a_β 5.38 (d)[c]
	secondary (53%)	a_3 0.04 (1D); a_5 0.56 (1D); a_6 0.07 (1D); a_β 0.68 (1H)
α-methyl-DOPA	primary (47%)	a_3 0.32 (1H); a_5 3.80 (1H); a_6 0.66 (1H); a_β 4.76 (d)[c]
	secondary (53%)	a_3 0.31 (1H); a_5 3.82 (1H); a_6 0.66 (1H); a_β 1.19 (1H)
dopamine	primary (100%)	a_3 0.18 (1H); a_5 3.62 (1H); a_6 0.62 (1H); a_β 3.12 (2H)
adrenaline[d]	cyclized (100%)	a_N 4.15 (1N); a_{NMe} 4.78 (3H); a_α 0.85 (2H)
noradrenaline[d]	primary (12%)	a_3 0.36 (1H); a_5 3.53 (1H); a_6 0.65 (1H); a_β 3.04 (1H)
	cyclized (88%)	a_N 3.28 (1N); a_{NH} 2.98 (1H); a_α 1.17 (2H)
catechol	primary (97%)	a_3,a_6 0.45 (2H); a_4,a_5 3.65 (2H)
	4-hydroxy (3%)	a_3,a_6 0.42 (2H); a_5 2.98 (1H)
DHBA	primary (80%)	a_3 0.22 (1H); a_5 3.19 (1H); a_6 0.84 (1H)
	6-hydroxy (20%)	a_3 0.22 (1H); a_6 0.51 (1H)
TBC[e]	primary (100%)	a 3.6 (broad doublet)

[a] All signals were observed at $g_{iso} = 2.0039$, except with TBC. [b] See eq 3 for the key to origin of the couplings; d, doublet. [c] Doublet coupling equal to $a_{\beta 1} + a_{\beta 2}$ (see text). [d] For enhanced solubility, the sample was at pH ∼ 5.5−6.5, rather than pH = 7.4 for other substrates. [e] No Zn(II) added—signal intense without stabilization; $g_{iso} = 2.0043$.

two species that were present in the same ratio (Table 3), even when Zn(II) was added at different times after mixing, although the overall signal intensity reduced as the [SQ] decreased. With excess DOPA-*ring*-d_3 or α-methyl-DOPA, the ZnSQ signals were simplified (Figure S2b,c, Supporting Information), and the simulations were consistent with the ZnSQ assignment for ring-protonated DOPA (Table 3). Addition of Zn(II) to the $K_2Cr_2O_7$/DOPA (2:9) reaction mixture after all of the Cr signals had disappeared (∼30 min) regenerated two intense Cr(V) signals, which both decayed within 10 min. The longer-lived signal (g_{iso} = 1.9719) was identical to that observed after mixing $K_2Cr_2O_7$ with DOPA (2:9). The second species ($g_{iso} = 1.9690$) was very short-lived (<5 min) and displayed well-resolved superhyperfine couplings to the ligand (Figure 4b). Experiments with DOPA-*ring*-d_3 resulted in a different superhyperfine coupling pattern of the $g_{iso} = 1.9690$ signal, showing that some of the couplings are due to ring protons.

When $K_2Cr_2O_7$ and DOPA (1:1, 5 mM) were mixed in acetate buffer (pH = 3.5), a very weak Cr signal was detected at $g_{iso} \approx$ 1.97 after 5 min, which disappeared at longer times. At higher concentrations (12.5 mM), the solution immediately became black and no EPR signals were observed. With excess DOPA (2:9), the spectra contained two signals at $g_{iso} = 1.9717$ and 1.9689 (Figure 4c), which did not exhibit any superhyperfine coupling to the ligands even at low modulation amplitudes (<0.2 G). The latter signal was dominant at early times (<2 min) but decayed within 6 min, while the former signal was longer-lived and decayed over 15 min. No organic radicals were observed at these pH values. With excess $K_2Cr_2O_7$ (8:1), signals were detected at $g_{iso} = 1.9690$ and 1.9735, which were weaker than with excess DOPA. Both signals disappeared within 10−15 min, without the formation of any further EPR-active species.

Similar experiments with DOPA were performed in the presence of catalase (0.1 mg mL^{-1}, in water or phosphate buffer) or under deoxygenated conditions. No spectral or kinetic differences were observed above the level of the noise for the Cr or ZnSQ signals, compared to spectra from air-equilibrated samples. These results show that H_2O_2 and O_2 are not required to form the major Cr(V) complexes.

Reactions of $K_2Cr_2O_7$ with Dopamine, Adrenaline, or Noradrenaline. The EPR signals detected with excess catecholamines (in H_2O, pH = 4.0−5.0) were intense and similar to those with DOPA. With excess dopamine (2:9), the solution turned red/brown within a few seconds. A strong EPR signal (with no superhyperfine couplings) was detected at $g_{iso} = 1.9719$ (LW = 2.4×10^{-4} cm^{-1}; $A_{iso}(^{53}Cr) = 24.4 \times 10^{-4}$ cm^{-1}), which decayed over a period of 15−20 min. At early times (<15 min), an additional weak signal at $g_{iso} = 1.9789$ and a relatively strong organic signal at $g_{iso} = 2.0045$ (quartet, $a_{iso} \sim 2.8 \times 10^{-4}$ cm^{-1}) were observed. The experiments with adrenaline (2:11) and noradrenaline (2:9) gave similar EPR signals (Table 1), and the solutions turned vibrant red and red/brown, respectively. With adrenaline, the signal at $g_{iso} = 1.9722$ had a shoulder at g_{iso} = 1.9690, but both signals were too weak and short-lived to determine $A_{iso}(^{53}Cr)$ values. With noradrenaline, the g_{iso} = 1.9721 signal ($A_{iso}(^{53}Cr) = 23.5 \times 10^{-4}$ cm^{-1}) was moderately intense at early times (<10 min). Only very weak organic signals were detected with adrenaline or noradrenaline, but, in the presence of Zn(II) (200 mM), strong ZnSQ signals at $g_{iso} \sim$ 2.0040 were observed with all three substrates. The ZnSQ spectra for dopamine (Figure S2d, Supporting Information) were simulated using literature values[52] for the superhyperfine couplings for the primary ZnSQ complex (Table 3). For adrenaline (Figure S2e, Supporting Information) and noradrenaline (Figure S2f, Supporting Information), the ZnSQ spectra were simulated using parameters consistent with the presence of both primary and secondary (cyclized) ZnSQ complexes (Table 3).[53,54] Similar Cr(V) and organic signals were observed for all three substrates when mixed in a 1:1 (12.5 mM) ratio with $K_2Cr_2O_7$ (in H_2O, pH 4.0−5.0), but the spectra were much weaker.

The EPR spectra acquired with excess $K_2Cr_2O_7$ and the catecholamines (in H_2O, pH = 4.0−5.0) mirror the behavior observed with DOPA (Table 1), but the signals were weaker and slightly shifted. With dopamine (8:1), a weak signal was detected at $g_{iso} = 1.9686$, which decayed within 10 min with concomitant growth of new signals at $g_{iso} \sim 1.980$ and 1.9721. The $g_{iso} \sim 1.980$ signal gained intensity over the following 20 min, and another weak signal appeared at $g_{iso} = 1.9737$ over a period of 30 min. The intensity of the $g_{iso} \sim 1.980$ signal remained almost unaltered up to 80 min, at which time the other signals had all decayed. With adrenaline (6:1) and noradrenaline (8:1), weak signals were observed at $g_{iso} \sim 1.969$ and 1.979 at

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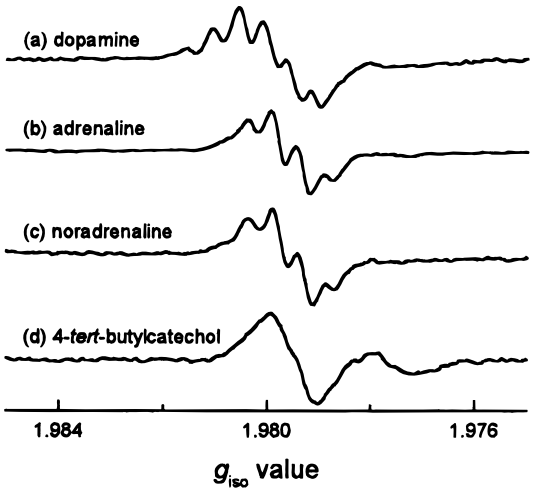

Figure 6. EPR spectra of the signals at $g_{iso} \sim 1.980$ obtained approximately 10−30 min after mixing excess $K_2Cr_2O_7$ with various catecholamines ([$K_2Cr_2O_7$] = 80 mM, [catecholamine] = 20 mM). Experimental conditions: receiver gain, 2×10^6; modulation frequency, 100 kHz; modulation amplitude, 0.1 G; resolution, 1024; conversion time, 81.92 ms; time constant, 40.96 ms; scans, 4−30.

early times (<5 min). The spectra with noradrenaline contained extra signals at $g_{iso} = 1.9727$ and 1.9760, which decayed within 5 min. In both cases, the signal at $g_{iso} \sim 1.9795$ became more intense over time and was still present after 320 min. With both substrates, a signal was also observed at $g_{iso} = 1.9737$ between 15 and 45 min. At higher reactant concentrations (80 mM:20 mM) and pH ∼ 6.5, the $g_{iso} \sim 1.980$ signals were more intense and the superhyperfine coupling patterns were resolved with all three substrates (Figure 6a−c). With dopamine, the observed couplings were almost identical to those obtained with DOPA (Figure 5a), whereas the signals from adrenaline and noradrenaline were simpler and almost identical to each other (Figure 6). No organic signals were detected with any of the catecholamines at any time after mixing when $K_2Cr_2O_7$ was in excess.

Reactions of $K_2Cr_2O_7$ with Catechol, 3,4-Dihydroxybenzoic acid (DHBA), or 4-*tert*-Butylcatechol (TBC). When $K_2Cr_2O_7$ was mixed with excess organic substrates (1:10 for catechol, and 2:9 for DHBA and TBC) in water (pH = 4.0−5.0), relatively intense Cr(V) signals were observed with all three substrates early in the reaction (<20 min). With catechol, a signal at $g_{iso} \sim 1.972$ ($A_{iso}(^{53}Cr) = 24.2 \times 10^{-4}$ cm^{-1}; LW = 2.4×10^{-4} cm^{-1}) comprised of a constant ratio (for at least 30 min) of two components at 1.9722 and 1.9705 (Table 4). A similar signal was detected with TBC at $g_{iso} = 1.9716$ ($A_{iso}(^{53}Cr) = 24.5 \times 10^{-4}$ cm^{-1}; LW = 1.8×10^{-4} cm^{-1}, Table 4), together with a broad underlying signal at $g_{iso} = 1.9716$. With DHBA, a weaker signal was detected at $g_{iso} = 1.9720$ (LW = 2.5×10^{-4} cm^{-1}), but the ^{53}Cr hyperfine coupling was not observed (Table 4). No superhyperfine couplings were detected for the $g_{iso} \sim 1.972$ signals with any of the substrates. The Cr(V) signals detected with catechol and TBC were stable for at least 60 min, but with DHBA, the signals decayed within 20 min. Additional weak signals were detected with catechol (<15 min) at $g_{iso} = 1.9792$ and 1.9769 and with DHBA at $g_{iso} = 1.9784$ (<15 min). Organic signals ($g_{iso} \sim 2.004$) were detected with all three substrates, which were enhanced by Zn(II) stabilization (except with TBC, where the signal was very intense without Zn(II) stabilization). The superhyperfine splitting patterns were consistent with the presence of SQ (Table 3). Control samples containing Zn(II) and catechol alone yielded much weaker ZnSQ signals, showing that only slow ligand autooxidation occurs. Experiments with 1:1 ratios (in H_2O, pH 4.0−5.0) yielded similar, but much weaker, signals.

The Cr(VI)/catechol reaction in HEPES buffer (pH = 7.5) yielded a strong SQ signal at $g_{iso} = 2.0043$ (without Zn(II) stabilization). Initially, no Cr signals were detected, but after 10 min, a weak signal was observed at $g_{iso} = 1.9720$ (LW = 2.2×10^{-4} cm^{-1}). Over an hour, the intensity of the Cr signal steadily increased, and the organic signal decayed to ∼40% of its initial intensity. A second Cr signal at $g_{iso} = 1.9705$ was detected as a shoulder on the $g_{iso} = 1.9720$ signal, consistent with the data obtained in water. In acetate buffer (pH = 3.5) with excess catechol (1:5), a relatively strong signal was observed at $g_{iso} = 1.9693$ after 2 min, and a weak signal at $g_{iso} = 1.9722$. Both signals decayed within 10 min, and no SQ signals were detected. Similar, but much weaker, signals were observed with 1:1 ratios (in HEPES and acetate buffers).

With excess $K_2Cr_2O_7$ (10:1 or 5:1 with catechol, and 8:1 with DHBA and TBC) in water (pH = 4.0−5.0), no EPR signals were detected with catechol, and only weak, short-lived (<10 min) signals at $g_{iso} = 1.9719$ and 1.9791 were observed with DHBA. This was despite characteristic color changes that indicated reactions had occurred. By contrast, the reaction of excess $K_2Cr_2O_7$ with TBC yielded EPR spectra similar to those obtained with DOPA under the same conditions. At early times (<5 min), several weak signals were detected at $g_{iso} = 1.9681$, 1.9796, 1.9775, and 1.9739 (Table 4). The signal at $g_{iso} = 1.9681$ disappeared rapidly (<5 min), while those at $g_{iso} = 1.9739$ and 1.9775 became weaker over 30 min, but did not decay completely. The signal at $g_{iso} = 1.9796$ was more intense after 60 min and was investigated further at higher concentrations (80 mM:20 mM) in phosphate buffer (pH = 7.4). Unlike the DOPA signal ($g_{iso} = 1.9800$), the superhyperfine couplings were not fully resolved for the $g_{iso} = 1.9796$ signal (Figure 6d), though evidence was obtained (from the second derivative) for the presence of two species at $g_{iso} = 1.9797$ and 1.9793. No SQ signals were detected with these substrates when $K_2Cr_2O_7$ was in excess. Similar experiments in HEPES buffer (pH = 7.5) with catechol yielded weak signals, but the spectra were the same as those with excess catechol, with additional short-lived (<5 min) signals at $g_{iso} = 1.9767$ and 1.9789. No Cr(V) or SQ signals were detected for the catechol experiments in acetate buffer (pH = 3.5).

Discussion

An important contrast between the results presented here and those from previous studies of Cr(III/VI) reactions with substituted catechols[28−30] is that the catecholamines have side chains which are capable of cyclization, and the quinones are susceptible to further oxidation. In fact, the quinone forms of catecholamines are known to cyclize rapidly to generate secondary cyclized semiquinones, cyclized quinones (known as aminochromes), and eventually indolic and polymeric materials.[55] The semiquinones of DOPA, dopamine and the adrenaline derivatives can also undergo ring hydroxylation at the six position,[46] which prevents cyclization and leads to the formation of two possible quinones—ortho and para. The currently accepted DOPA oxidation cycle is complex (Scheme 1),[55] and many of these compounds (and those from the related catecholamines) are potential ligands for Cr.

The complexity of this chemistry results in the formation of a number of Cr(V) species, as well as organic radicals. The

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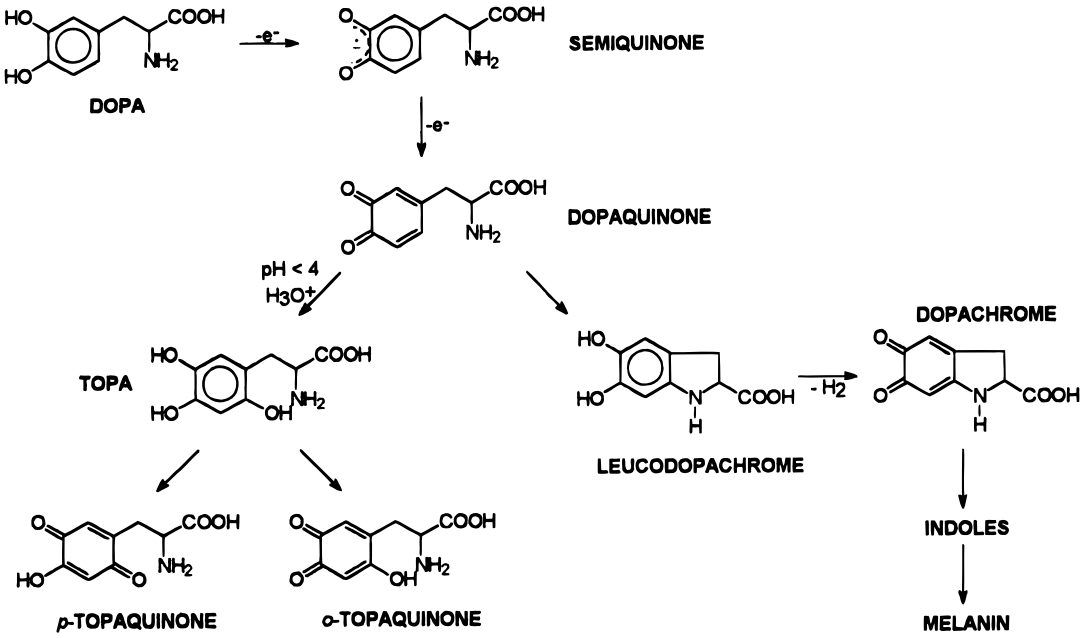
Table 4. Summary of the Major Cr(V) Species Detected during the Reduction of $K_2Cr_2O_7$ by Substituted Catechols

substrate	reacn conditions[a]	time/min	g_{iso} value	line width/(10^{-4} cm^{-1})	A_{iso}(^{53}Cr)/(10^{-4} cm^{-1})	other a_{iso}/(10^{-4} cm^{-1})
catechol	5:1 (pH = 7.5)	< 5	1.9789[b]			
	5:1 (pH = 7.5)	< 5	1.9767[b]			
	1:10 (pH > 4.5)	> 5	1.9722	2.4	24.2	unresolved
	1:5 (pH = 3.5)	< 10	1.9722			
	1:10 (pH > 4.5)	> 5	1.9705[b]			
	1:5 (pH = 3.5)	< 10	1.9693[b]			
DHBA	8:1 (pH = 4.5)	< 10	1.9791[b]			
	2:9 (pH = 4.5)	< 20	1.9784[b]			
	2:9 (pH = 4.5)	< 20	1.9720[b]	2.5		unresolved
TBC	4:1 (pH = 7.4)[c]	> 5	1.9797	2.1[d]	16.2	unresolved
	4:1 (pH = 7.4)[c]	> 5	1.9793	2.1[d]	16.2	unresolved
	8:1 (pH = 4.5)	< 30	1.9775[b]			
	8:1 (pH = 4.5)	< 30	1.9739[b]			
	2:9 (pH = 4.5)	> 5	1.9716	1.8	24.5	unresolved
	8:1 (pH = 4.5)	< 5	1.9681[b]			

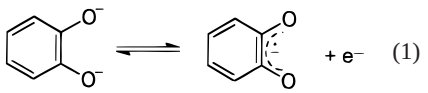
[a] Ratios stated as $[K_2Cr_2O_7]$:[catechol], [minor reactant] = 5 mM except with catechol (1:10), where $[K_2Cr_2O_7]$ = 2.5 mM. [b] Signal too weak and/or short-lived to measure superhyperfine and ^{53}Cr hyperfine couplings. [c] Spectra at higher reactant concentrations; $[K_2Cr_2O_7]$ = 80 mM and [TBC] = 20 mM. [d] Line width for two overlapping signals.

Scheme 1. DOPA Oxidation Pathways Including Many Transient Intermediates

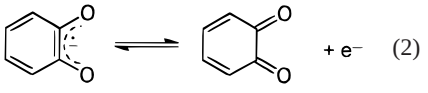


chemistry is affected by the pH value and reactant ratios, but consistent trends are observed throughout the series of catecholamines. Despite such complexity, only a few intense signals were observed, and the identities of the species giving rise to the major EPR signals are discussed below.

***g*$_{iso}$ ~ 1.972 Signals.** A seven-membered series of $[CrL_3]^n$ (L = catecholate, semiquinone or quinone; $n = 3+ \rightarrow 3-$) complexes have previously been synthesized by reaction of Cr(III) with various catechols, followed by multistep oxidation and reduction reactions.[28,29] The oxidation state of the Cr in this redox series is ambiguous, since the ligands can be in their catecholate, semiquinone, or quinone forms (eqs 1 and 2).



(1)



(2)

Theoretical studies[31] predicted that the monoanionic and monocationic species have single unpaired electrons, and both types of complex have been observed by EPR spectroscopy, with signals at $g_{iso} \sim 1.972$. No superhyperfine couplings to catecholic protons were detected in either complex at room temperature, but the superhyperfine couplings observed in low-temperature EPR studies[56] (248−273 K) confirmed that the complexes contain three catecholic ligands. The ^{53}Cr hyperfine satellite couplings were large (A_{iso}(^{53}Cr) = 21.2−25.6 × 10^{-4} cm^{-1})[28,29] and were attributed to the single unpaired electrons occupying metal-centered molecular orbitals. The line widths of the EPR signals allow the complexes to be distinguished, since monocationic species typically have LW ~ 2.8 × 10^{-4} cm^{-1} and the monoanionic complexes have LW ~ 9.2 × 10^{-4} cm^{-1}.[28] On this basis, the signals observed at $g_{iso} \sim 1.972$ in the present study are assigned to the monocation, as they have large ^{53}Cr hyperfine satellite couplings, and typically the LW = 2.3−2.8 × 10^{-4} cm^{-1}. The spectra reported here are almost identical to those reported during the Cr(VI) oxidation of 3,5-

(56) Solodovnikov, S. P.; Sarbasov, K.; Tumanskii, B. L.; Prokof'ev, A. I.; Vol'eva, V. B.; Bubnov, N. N.; Kabachnik, M. I. *Izv. Akad. Nauk. SSSR, Ser. Khim.* **1984**, *8*, 1789−1794.

di-*tert*-butylcatechol,[30] but the signals were previously assigned to the monoanionic complex. The assignment in those studies was based on the size of the ^{53}Cr couplings (which tend to be slightly larger for the monoanions), but the line widths of the signals were not considered.[30] However, the majority of the literature data are reported in organic solvents, where significant variations in both the line widths and ^{53}Cr hyperfine couplings are observed depending on the solvent and the ligands.[28−30,34] While this makes definitive assignment of the complexes somewhat ambiguous, especially with the transient water-soluble species that were detected with DOPA and the other catecholamines, it is unlikely that air-sensitive monoanions are produced in the oxidizing environment of the Cr redox chemistry. Although both the monoanionic and monocationic complexes were previously assigned as Cr(III) complexes,[28,29,34] the observed Cr signals are typical of Cr(V) species. Further evidence for Cr(V) structures, with some delocalization, was obtained from the pre-edge peak and the edge energy in the XANES spectrum of the monoanionic Cr complex with three DTBC ligands.[57] This would require two of the ligands to be oxidized by one electron, and the cationic complex could be written as $[Cr^{V}(SQ)_2(catecholate)]^+$. A labile Cr(V) structure is supported by the rapid conversion of $[Cr^{V}(SQ)_2(catecholate)]^+$ to $[Cr^{V}O(catecholate)_2]^-$ as the reaction with excess Cr(VI) proceeds. Such behavior would not be expected for an inert Cr(III) complex but is consistent with a weakly oxidizing Cr(V) complex, as further reaction with DOPA would be expected to occur relatively slowly.

There is a linear correlation between the EPR parameters for isoelectronic Cr(V) and V(IV) complexes.[58] The parameters for the $[V(catecholate)_3]^{2-}$ do not accurately predict the $[Cr(catecholate)_3]^-$ parameters,[59] indicating that the monoanionic complex also has electron delocalization over the Cr center and two ligands (as shown by the theoretical studies[31] and XANES[57]), but is best described as a Cr(V) complex and not a Cr(III) one.

An important contrast between the results presented here and those carried out previously is the longevity of the $g_{iso} \sim 1.972$ signals. Previous studies showed that the $g_{iso} \sim 1.972$ signals persisted for at least an hour,[29,30] as was observed here with catechol and TBC. The signals obtained with DOPA and analogues were only visible for 10−30 min, depending on the pH value and reactant ratios. This was due to cyclization and further reactions of the ligands, since the brown and red/pink colors of aminochromes were clearly visible during the reactions. The UV/visible spectra contained a peak that grew in at 480 nm (characteristic of aminochromes),[49] and the steady increase in background absorbance as the reaction proceeded is ascribed to melanin formation.[46,60]

***g*$_{iso}$ ∼ 1.980 Signals.** The g_{iso} values and ^{53}Cr coupling constants of these signals are consistent with the five-coordinate Cr(V) bis(diolato) complexes observed by direct reduction of Cr(VI) (e.g. with ascorbic and quinic acids),[3,61] by ligand-exchange experiments of $[Cr^{V}O(ehba)_2]^-$ with diols (e.g., ethanediol),[58,62,63] or reduction of Cr(VI) with glutathione in the presence of saccharides (e.g., glucose).[64] These Cr(V) signals typically have $g_{iso} \sim 1.980$ and ^{53}Cr hyperfine couplings of $14.8-15.7 \times 10^{-4}$ cm^{-1}. Additional evidence for the structures (Figure 7) of these species is obtained from the linear relationship of EPR parameters for Cr(V) complexes and isoelectronic V(IV) complexes.[58] This relationship predicts $g_{iso} \sim 1.980$ and $A_{iso}(^{53}Cr) \sim 17 \times 10^{-4}$ cm^{-1},[59] which are in good agreement with the observed Cr(V) parameters. This is supported by the simulations of superhyperfine couplings that require two catechol ligands per complex and the observation that as the ligand is consumed, the intensity of the tris(catechol) species decreases relative to the bis(catechol) signal. The peak at 380 nm in the UV/visible spectrum obtained with excess Cr(VI) and DOPA is characteristic of Cr(V) species,[48] but this is not definitive evidence since dopaquinone also has a peak at 380 nm.[49]

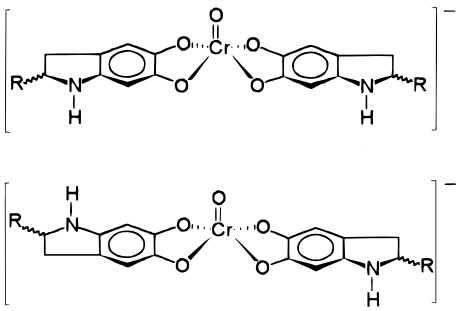


Figure 7. Proposed structural isomers responsible for the signals at $g_{iso} \sim 1.980$ when excess $K_2Cr_2O_7$ was mixed with DOPA (R = COOH or H, depending on whether decarboxylation occurs during dopaquinone cyclization). It should be noted that the carbon adjacent to the nitrogen atom is a chiral center (when R = COOH); therefore, further stereoisomers of these complexes also exist.

The intense EPR signals at $g_{iso} \sim 1.980$ observed with DOPA, the other catecholamines, and 4-*tert*-butylcatechol, compared to their absence and transient existence with catechol and DHBA, respectively, suggest that the bulky side chain is required to stabilize five-coordinate Cr(V). This may act to protect the Cr(V) center from attack by other ligands such as water or H_2O_2 (generated during catecholamine oxidation),[65] which could displace the catechol ligands from the metal center. Peroxo species generated via reaction of Cr(V/VI) with H_2O_2 can only be minor components of the system, as catalase addition and deoxygenation have no effect on the main EPR signals.

The $g_{iso} \sim 1.980$ signals observed in previous studies of Cr(V) bis(diolato) complexes exhibited complex superhyperfine couplings, due to the protons attached to the carbon atom adjacent to the coordinated oxygen atoms.[3,61,62,64] While the $g_{iso} \sim 1.980$ signals observed with DOPA and the catecholamines also exhibit superhyperfine coupling, they lack such protons. The isotopic labeling studies show that the superhyperfine couplings originate from the α and β protons of the catecholamine side chains. No couplings to exchangeable, ring, or nitrogen atoms are apparent. The complexity of the superhyperfine coupling patterns observed with the other catecholamines and TBC are consistent with this hypothesis, as DOPA and dopamine have two β protons, whereas adrenaline and noradrenaline have only one β proton and TBC has no protons at the equivalent carbon. The $g_{iso} \sim 1.980$ signal in the DOPA spectrum has been successfully simulated with two overlapping species (Figure 5, Table 2), both of which have two cyclized ligands. In one of the species, the ligands have been decar-

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(64) Irwin, J. A. Ph.D. Thesis, University of Sydney, Australia, 1998.
(65) Herlinger, E.; Jameson, R. F.; Linert, W. *J. Chem. Soc., Perkin Trans. 2* **1995**, 259−263.

boxylated during cyclization (as proposed previously during DOPA oxidation).[66] The change in the signals over time could not be simulated by simply changing the ratio of the two signals; therefore, it is likely that new species are generated as further ligand oxidation occurs. These simulations are consistent with the spectra observed from DOPA-β,β-d_2, and α-methyl-DOPA, where the β and α proton couplings are replaced by deuteron or small quartet couplings (from the methyl protons), respectively (Figure 5, Table 2). Although the signals have been simulated satisfactorily with the parameters given for the two species shown in Figure 7, contributions from other species cannot be discounted. The structures of any such additional species are unclear at present but must display coupling to the α and/or β protons of the ligands. The presence of at least two species with dopamine is probably due to geometrical isomers of the complexes, and with adrenaline and noradrenaline, adrenolutin (where the β-OH has been oxidized to a ketone) may be an additional ligand.

The observation that the superhyperfine couplings originate from the α and β protons, rather than the ring protons, is good evidence that the ligands are cyclized. The Zn(II)-stabilized SQ (see later) exhibit superhyperfine couplings to the ring protons in the open chain form, but to the α and β protons (and the amine nitrogen) in the cyclized form, and similar electron density patterns would be expected for the Cr complexes. The absence of any coupling to the amine nitrogen in the Cr complex can be rationalized, since the amine is probably deprotonated in the complex, thus shifting electron density away from the nitrogen and reducing the magnitude of the coupling. With TBC as the ligand, coupling to the ring protons might be expected (as cyclization cannot occur), but these are probably too small to resolve.

Reactions with *N*-acetyl-DOPA, where no EPR signals were observed, provide further evidence that cyclization of the catecholamines is required to stabilize the species responsible for the $g_{iso} \sim 1.980$ signals. The *N*-acetyl group slows cyclization of DOPA[55] and presumably the five-coordinate Cr(V) species is less stable due to reduced steric hindrance with uncyclized DOPA. In addition, the reduced cyclization rate may allow competitive hydroxylation of the catechol ring, generating 3,4,6-trihydroxyphenylalanine (TOPA) (Scheme 1).[46,55] TOPA quinones do not cyclize,[46,55] and the stable products derived from further oxidation of TOPA are *o*- and *p*-quinones.[46,55] These are poorer donors and are unable to form the species giving rise to the $g_{iso} \sim 1.980$ signal. Furthermore, at low pH values, the cyclization of dopaquinone is much slower than at neutral pH, as the rate is inhibited by protonation of the terminal amino group.[55] This is consistent with the results obtained in acetate buffer (pH = 3.5), where the $g_{iso} \sim 1.980$ signal was not observed. Thus, cyclization of the catecholamines may favor the formation of the Cr(V) species by preventing *p*-quinone generation, as well as by increasing steric hindrance around the metal center.

The observation that the $g_{iso} \sim 1.980$ signal is only detected with excess $K_2Cr_2O_7$ is consistent with a Cr(V) center bound to a cyclized DOPA moiety. The excess $K_2Cr_2O_7$ should ensure that DOPA is oxidized rapidly to its more stable cyclized oxidation products (dopachrome, indoles, melanin), but without complete reduction of Cr(VI) to Cr(III). Thus, when DOPA oxidation has gone to completion, Cr(IV/V/VI) will remain and the cyclized DOPA products will stabilize the Cr(V) that is present. This suggestion is supported by the detection of cyclized SQ, even when the ligand is in excess (see below).

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$g_{iso} \sim 1.969$ Signals. The low g_{iso} values of these complexes, compared to those reported for other Cr(V) species with oxygen donors[58] are most consistent with six-coordinate Cr(V) species or Cr(V)−Cr(VI) dimers.[67] Unfortunately, these signals were too short-lived to determine the presence of superhyperfine couplings or the magnitude of the ^{53}Cr couplings. These species were observed mostly at low pH values, suggesting they are stabilized by acidic conditions but are probably short-lived intermediates in the reactions at higher pH values. This pH dependence suggests that the species contain protonated ligands, although the complexity of the chemistry precluded its experimental verification.

Signals were also detected at $g_{iso} \sim 1.969$ when Zn(II) was added to a mixture if DOPA was in excess, and all of the transient EPR signals had decayed (>30 min). In this case, the signal was again short-lived (<5 min) but was sufficiently intense to observe superhyperfine coupling to the ligands. The alterations to the superhyperfine coupling pattern when DOPA-*ring*-d_3 was used showed that coupling to ring protons is involved for this complex, but the number of DOPA ligands present could not be determined. The instability of the complex has prevented full characterization, but it is probably generated by breakdown of EPR-silent Cr(V) dimers or EPR-silent monomers (e.g., $[Cr(SQ)(catecholate)_2]^0$), by competitive coordination to Zn(II). Alternatively, it may be generated by reaction of Cr with SQs newly generated by Zn(II) assisted comproportionation reactions (see later).

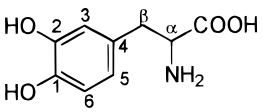
Other Cr Signals. Many weak Cr signals were observed during the reactions of the catecholamines with $K_2Cr_2O_7$, but in most cases they were too weak, or unstable, to enable full characterization. Signals were detected at $g_{iso} = 1.9775$ with DOPA and derivatives in low concentrations over several hours and at early times with TBC. Comparison of these g_{iso} values with those predicted ($g_{iso} = 1.9773$) from the equivalent V(IV) complexes[58] suggests that the $g_{iso} = 1.9775$ signal is due to species where only one catechol group is coordinated to the Cr center (with OH^- and H_2O as the remaining ligands).[59] Further signals at $g_{iso} \sim 1.974$ were present in most cases when $K_2Cr_2O_7$ was in excess and remained at low concentrations for long periods. Superhyperfine couplings were observed for these signals, but the exact coupling patterns were not sufficiently resolved to determine whether one or two catechol ligands were present. Finally, weak signals at $g_{iso} \sim 1.976$ were often detected with excess $K_2Cr_2O_7$ and in the reaction with DOPA (pH = 7.4). The signal had superhyperfine couplings similar to those of the more intense signal observed at $g_{iso} \sim 1.980$, which suggests that it is due to a Cr(V) complex with at least one cyclized DOPA moiety.

The complexity of catecholamine oxidation is still not fully understood but provides a wide variety of ligands that can coordinate with Cr(V) during the redox reactions. It is likely that the majority of the weak Cr signals observed during the reactions are due to interactions of Cr(V) centers with one or two catecholamine ligands in various oxidation states, with auxiliary ligands (e.g. H_2O) completing the coordination sphere where necessary.

Organic Signals. Semiquinones have been detected by EPR spectroscopy during the oxidation of catecholamines to quinones via one-electron oxidation of the precursor.[50,53,68−70] Zinc(II) and other diamagnetic metal ions (Mg^{2+}, Cd^{2+}, Ba^{2+}) can stabilize SQ by complexation with the metal, resulting in more

(67) Farrell, R. P.; Lay, P. A.; Levina, A.; Maxwell, I. A.; Bramley, R.; Brumby, S.; Ji, J. Y. *Inorg. Chem.* **1998**, *37*, 3159−3166.

intense, longer-lived signals, which facilitate spectral analysis.[50,54,68−71] The observed superhyperfine couplings are attributed to both ring protons, and with catecholamines, the β-methylene protons of the side chain (assignment of couplings is numbered relative to the diol group as shown in eq 3).[68] The

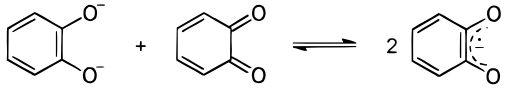


(3)

ring protons give doublets, with splittings: $a_3 \sim 0.4 \times 10^{-4}$ cm^{-1}, $a_5 \sim 3.4 \times 10^{-4}$ cm^{-1}; and $a_6 \sim 0.8 \times 10^{-4}$ cm^{-1}. The methylene protons typically give a triplet splitting with $a_{iso} \sim 2.5 \times 10^{-4}$ cm^{-1}, but in the case of DOPA, where they are adjacent to a chiral center, the methylene protons are inequivalent. At room temperature, the inner resonances of the 1:1:1:1 pattern are difficult to detect, since they are subjected to broadening because of restricted rotation of the methylene protons. This results in a large doublet coupling of 5.0×10^{-4} cm^{-1}, which is equal to $a_{\beta 1} + a_{\beta 2}$.[68]

The simulation of the ZnSQ signals showed that primary SQs were present during oxidation of virtually all of the catecholamines, with excess ligand (pH > 4). Additional species were observed with some substrates (Table 3), due to ring hydroxylation (TOPA-like species) or cyclized catecholamines.[54,71] The spectra show that adrenaline and noradrenaline cyclize most readily, while dopamine and DOPA did not cyclize under the reaction conditions (excess ligand), which is consistent with previous observations.[54] A surprising observation was obtained with DOPA, where the ZnSQ spectrum was most satisfactorily simulated by an approximately 50:50 mixture of dopasemiquinone, and a second SQ. The β coupling for the latter signal was much smaller than the large $a_{\beta 1} + a_{\beta 2}$ coupling reported previously[52−54,68] and is attributed to a single β proton. The results with DOPA-*ring-d*$_3$ confirmed that TOPA formation did not occur (as all the ring deuterons gave couplings), but one of the signals had again lost the coupling to one of the β protons. One possibility for this observation is that the β position has been hydroxylated to form 3,4-dihydroxyphenylserine (DOPS), but the ZnSQ spectrum obtained directly with DOPS did not give the same coupling pattern, thus the identity of the second species is not known. The favored explanation is that it is due to dopaquinone in a locked conformation, which gives only small coupling to one of the β protons.

The SQ may not result solely from direct one-electron oxidation of catecholamines by Cr(VI/V/IV) but can also form via comproportionation of a catechol and a quinone moiety (eq 4).[68] The addition of Zn(II) to the reaction may generate more SQ than the reaction of Cr(VI) with catechol(amine)s alone, as the equilibrium will be driven to the right by SQ complexation by Zn(II).[19] The appearance of strong SQ signals, coupled with the generation of fresh Cr(V) signals, is also consistent with sequestering of the SQ from EPR silent monomers, such as [Cr(SQ)(catecholate)$_2$]^0, or [Cr(SQ)$_3$]$^{2+}$.



(4)

The absence of SQ signals at lower pH values is expected, since they are readily protonated at pH < 4.[68] The lack of SQ signals with excess Cr(VI) is readily understood, as excess Cr(VI) rapidly oxidizes any SQ present, as indicated by the rapid color changes associated with formation of cyclized aminochromes[49] and polymerized products.[46,60]

Implications for Genotoxicity. The Cr(VI)/catecholamine systems generate a variety of reactive intermediates including Cr(V) complexes and organic radicals. Previous reports[36,38] suggest that H_2O_2 and reactive oxygen species (ROS) are also generated during the oxidation of catecholamines by Fe(III) and Cu(II), but the concentration of Cr(V)−peroxo species was low, as shown by the lack of effect of catalase on the EPR spectra. It is well-documented that Cr(V) complexes, such as [Cr^{V}O(ehba)$_2$]$^-$ and Cr(V)−peroxo species, are highly genotoxic,[10,41,42] and it is widely recognized that Cr(V) is likely to be one of the active components in Cr(VI)-induced carcinogenicity. The oxidation of DOPA with Cu(II) in the presence of DNA also leads to DNA base oxidation,[38] presumed to be due to ROS formed during the oxidation of DOPA. Preliminary studies[43] of the $K_2Cr_2O_7$/DOPA system in the presence of DNA have shown that extensive DNA cleavage occurs, although the mechanisms involved are not yet clear. The chemistry reported here could also be important in the formation of the Cr-induced amino acid/DNA and protein/DNA cross-links observed both in cultured cells[72,73] and in vivo.[74]

The presence of oxidized amino acids and oxidized proteins (which often contain DOPA) within cells[25] shows the potential of such systems to reduce Cr(VI) to Cr(V) and to generate ROS under metabolic conditions. Indeed, the presence of Cr within cells may promote such oxidation chemistry. Preliminary data[75] have been obtained by EPR spectroscopy for the generation of Cr(V) species on interaction of Cr(VI) with damaged proteins, and these will be reported separately.

In conclusion, the interaction of high oxidation states of Cr with catechol moieties in catecholamines is a potential pathway for the reduction of Cr(VI) in vivo. In addition to producing potentially genotoxic Cr(V) species, which have been detected by EPR spectroscopy, these systems produce organic radicals, which could participate in further radical reactions, and also ROS, such as organic peroxides, $^{\bullet}$OH and $O_2^{\bullet -}$.

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Supporting Information Available: Figure S1, UV/visible spectra obtained during the reactions of $K_2Cr_2O_7$ with DOPA, and Figure S2, EPR spectra of the Zn(II)-stabilized semiquinones generated by Cr(VI) oxidation. This material is available free of charge via the Internet at http://pubs.acs.org.

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