Identification of the Oxidized Products Formed upon Reaction of Chromium(V) with Thymidine Nucleotides

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Received July 15, 1996⊗

Abstract: Two pathways have been observed for Cr(V)-mediated nucleotide oxidation in reactions of bis(2-ethyl-2-hydroxybutyrato)oxochromate(V) $[CrO(ehba)_2]^-$ with thymidine nucleotides dTMP, dTDP, and dTTP. The extent of Cr(V)-induced nucleotide oxidation was greatest for thymidine diphosphate (dTDP), as measured by the production of thiobarbituric acid reactive species (TBARS; indicative of pathway 1) and thymine release (indicative of pathway 2). The nucleoside thymidine showed no reaction, suggesting a phosphate-dependent oxidation. Amounts of TBARS and thymine were maximal at a pH range of 6.0−6.5, and both TBARS formation and thymine release correlated with decay of the Cr(V) EPR signal. Formation of TBARS was maximal in 100% O_2 but decreased markedly under argon, whereas thymine release was maximal under argon, but remained the major product observed under aerobic conditions. Pathway 1 for the reaction of Cr(V) with dTDP led to formation of glycolic acid and *trans*-thymine propenal at approximately equimolar amounts, consistent with a mechanism involving oxygen-dependent sugar oxidation following hydrogen atom abstraction at the C-4′ carbon of the deoxyribose sugar. Pathway 2 led to release of free thymine, but much less (barely detectable) 2-deoxy-D-pentitol was formed from postreduction of the reactive aldehydic sugar fragment. Thus, the oxygen-independent release of thymine does not appear to result from reaction at the C-4′ hydrogen unless decomposition of the aldehydic intermediate occurred. Determination of the oxidation state of chromium responsible for the observed oxidative damage was carried out using Mn(II), a Cr(IV)-specific reductant. Mn(II) essentially abolished all activity for both TBARS formation (pathway 1) and thymine release (pathway 2). These results suggest that Cr(IV), formed upon disproportionation of Cr(V), oxidizes the nucleotide deoxyribose sugar moiety *via* a phosphate-bound intermediate. Pathway 1 involves oxygen-dependent oxidation at the C-4′ position; however, the mechanism for oxygen-independent thymine release (pathway 2) is still unclear.

Introduction

The carcinogenicity of chromium(VI), is well established but this oxidation state is not considered to be responsible for the DNA lesions associated with the observed carcinogenic activity. Instead, the role of the Cr(VI) is in membrane transport where its tetrahedral conformation is isostructural with phosphate and sulfate and allows active transport into the cell *via* nonspecific anion transport channels. Once inside the cell, intracellular reduction occurs and generates the "ultimate" carcinogenic chromium species.[1] Chromium(V) is considered to be a candidate for one of these ultimate carcinogenic species since it has been observed both *in vivo* and *in vitro* during the biological reduction of Cr(VI) to the final intracellular stable oxidation state Cr(III).[2−4] This biological reduction of Cr(VI) to lower oxidation states has been observed with a wide variety of naturally occurring cellular reductants including ascorbate, glutathione, cysteine, NADH, and cytochrome P450 reductase.[5−8] A consequence of this intracellular reduction is the production of a wide array of adverse biological effects including DNA lesions induced by oxidative stress (DNA strand breaks, 8-oxo-2′-deoxyguanosine, abasic sites) and chromium directly metalated to the DNA strands (Cr−DNA adducts, both inter- and intrastrand cross-links, and Cr−DNA−protein cross-links).[9−12] At the genetic level, chromium has been shown to affect the expression of some inducible genes and to interact with oxidative stress inducible transcription factors in the signal transduction pathway.[13,14] In general, the chromium oxidation state which is responsible for producing a particular DNA lesion and the mechanism by which the lesion is produced are currently unknown. However, there is a general belief that the higher valent states of chromium, acting either directly or through reactive oxygen species produced from molecular oxygen or hydrogen peroxide, are responsible for much of the oxidative stress and DNA damage observed in biological systems.

Bis(2-ethyl-2-hydroxybutyrato)oxochromate(V) $[CrO(ehba)_2]^-$ has properties that make it a favorable Cr(V) model for substrate oxidation studies. The pentacoordinate complex with oxygen ligands resembles the postulated ligand environment, and has a

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1996.

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similar EPR *g* value, as the Cr(V)−ascorbate complex.[5,15] The α-hydroxy ligands of the model Cr(V) complex are not as prone to intramolecular oxidation as the corresponding ascorbate ligands, thus limiting the reactivity observed to the metal center. Also, the decay of this Cr(V) complex has been well studied and is known to proceed by disproportionation through a Cr(IV) intermediate at neutral pH.[16] At progressively more acidic pH values, disproportionation is slower, with the Cr(V) complex being quite stable at pH 3−4. Importantly, the Cr(V) complex does not show formation of carbon-based or oxygen radicals;[17] thus, any oxidative damage should be due to a direct metal−substrate oxidative mechanism.

The mode of interaction of Cr(V) with DNA is currently unknown, although Farrell *et al.* initially postulated that direct binding of Cr(V) to DNA led to oxidation and DNA strand breakage.[18] Recently, the ability for $[CrO(ehba)_2]^-$ to undergo ligand exchange with free phosphate and pyrophosphate has been shown,[19] suggesting phosphate as a tethering point with potential relevance to binding of Cr(V) to DNA and deoxyribonucleotides. Upon reduction of the high valent chromium(V) species to the more stable +3 state, the ensuing oxidation of nucleotides can be envisioned. Previously, *in situ* generated Cr(V)−glutathione and Cr(V)−ascorbate species had been suggested to undergo an oxidative mechanism with DNA at the sugar moiety.[11,20,21] However, under the reaction conditions employed, carbon-based radicals[5] and thiyl radicals[22] are formed which could account for the observed reactivity and no product analysis was performed to show formation of the specific sugar and base degradation products expected.

In this paper, we show two oxidative pathways for the reaction of a high valent chromium(V) species with thymidine nucleotides by the identification and quantitation of specific degradation products. We have found that Cr(V)-induced oxidative damage of the thymidine series of nucleot(s)ides is wholly dependent on the presence of a phosphate moiety within the substrate. Of the thymidine nucleotides, dTDP was significantly more prone to oxidation than either dTMP or dTTP, while dT alone showed no reactivity. Identification of thiobarbituric acid reactive species (TBARS) arising from *trans*-thymine propenal, and formation of the remaining 3-carbon sugar unit, glycolic acid, showed that pathway 1 involved an oxygen-dependent oxidation at the C-4′ hydrogen atom of the deoxyribose sugar. In addition, the release of the free base thymine in this reaction showed the occurrence of an oxygen-independent mechanism (pathway 2). Finally, we demonstrated using Mn(II) as a Cr(IV)-specific reductant that these two pathways involve Cr(IV) as the reactive high valent species in these sugar oxidation mechanisms and not Cr(V) itself.

Experimental Section

Materials. Thymine, thymidine, dTMP, dTDP, dTTP, $MgCl_2 \cdot 6H_2O$, glycolic acid, 2-deoxy-D-ribose, $Na_2Cr_2O_7 \cdot 2H_2O$, alkaline phosphatase (type VII-L from bovine intestinal mucosa), and Sephadex DEAE A-25 anion exchange resin were purchased from the Sigma Chemical Co. The deoxyribonucleotides, as the sodium salts, were used as supplied. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and dry acetonitrile as the silylation cosolvent was purchased from the Pierce Chemical Co. Propargyl alcohol, $NaBH_4$, 2-ethyl-2-hydroxybutyric acid, 1,1,3,3-tetramethoxypropane, and 2-thiobarbituric acid were obtained from Aldrich Chemical Co. Dowex AG 50W-X8 cation exchange resin (H^+ form), Dowex AG 50W-X2 cation exchange resin (H^+ form), Dowex AG 1-X2 anion exchange resin (Cl^- form), and Chelex 100 were obtained from Bio-Rad Co. Whatman silica gel TLC plates (both analytical and preparative), trichloroacetic acid, $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, and HPLC grade methanol were purchased from Fisher Scientific.

HPLC Conditions. HPLC separations and quantitations were carried out on a HP-1090 HPLC system with diode array detection at wavelengths of 254 nm for thymine, 275 nm for *cis*-thymine propenal, and 305 nm for *trans*-thymine propenal. Analyses were performed on a Rainen Microsorb-MV 100 Å C18 column, 3 μm particle size (4.6 mm i.d. × 10 cm length), as described[23] and afforded the following retention times and detection limits (in parentheses where applicable) for the compounds of interest: thymine (1.65−1.70 min, 0.1 μM), thymidine (1.75−1.80 min, 0.1 μM), dTMP (1.10−1.20 min), dTDP (0.90−1.00 min), *trans*-thymine propenal (3.05−3.15 min, 0.1 μM), *cis*-thyminepropenal (2.10−2.20 min).

EPR Conditions. EPR spectra were recorded at RT using a Bruker ESP-300 spectrometer with spectral parameters of 100 kHz field modulation, 1.0 G modulation amplitude, 5.12 ms time constant, 9.769−9.773 GHz microwave frequency, 1×10^5 receiver gain, 2 mW microwave power attenuated at 20 dB, 3380−3580 G sweep width, and a 21 s scan time. All signals were averaged over nine scans. Measurements were done on *ca.* 100 μL volume samples, drawn into a capillary tube, and sealed on one end with Dow-Corning high vacuum grease. The *g* values were determined with respect to 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), $g = 2.0036$. Concentrations were determined from a standard curve of $[CrO(ehba)_2]^-$ in 100 mM aqueous 2-ethyl-2-hydroxybutyric acid.

GC/MS Conditions. GC/MS analyses were carried out on the trimethylsilylated derivatives of the compounds by using a HP-5890 GC with a HP-5971 mass selective detector. Silylations were accomplished by addition of a 1:1 solution of 1% TMCS in BSTFA/acetonitrile followed by heating at 60 °C for 1 h. GC conditions were 100 mL/min He flow rate with 2.5 mL/min split flow, injector temperature of 250 °C, detector temperature of 312 °C, and a column temperature gradient of 100−300 °C at 16 °C/min with a Supelco SPB-5 column (30 m × 0.2 mm).

Synthesis of Cr(V). The sodium salt of bis(2-ethyl-2-hydroxybutyrato)oxochromate(V) was prepared in a crystalline form using the method of Krumpolc and Rocek.[15] IR and UV−vis analysis yielded identical results as that shown previously for this complex. Purity was determined to be >98% by oxidation to Cr(VI) using alkaline H_2O_2 and analysis by UV−vis using $\epsilon = 4830\ cm^{-1}\ M^{-1}$.[24] *Caution! Cr(VI) is a known human carcinogen, and Cr(V) complexes are potentially carcinogenic. Appropriate precautions should be taken in handling these materials.*

Synthesis of *cis*- and *trans*-Thymine Propenals. Preparation of *cis*- and *trans*-thymine propenals was carried out by the method of Ajmera *et al.*[23] by reacting bis(trimethylsilyl)thymine[25] in a neat solution of propargylaldehyde.[26] The product of interest, *trans*-thymine propenal, was purified by preparative TLC,[27] and was determined by HPLC to be ∼95% pure. The contaminants were a small amount of free thymine and the corresponding *cis* isomer. UV−vis spectra of *trans*-thymine propenal demonstrated a wavelength maximum of 306 nm and a calculated extinction coefficient of 11 270 $M^{-1}\ cm^{-1}$. ^{1}H-NMR (300 MHz, D_2O, δ (HDO) = 4.67 ppm) δ 9.63 (1H, d, $J = 7.8$ Hz), δ 8.09 (1H, d, $J = 14.4$ Hz), δ 7.67 (1H, s), δ 6.25 (1H, q, $J = 6.6$ and 14.7 Hz), δ 1.82 (3H, s).

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Synthesis of 2-Deoxy-D-*erythro*-pentitol. The synthesis of 2-deoxy-D-*erythro*-pentitol was accomplished using the method of Tymiak and Rinehart,[28] as modified by Rabow *et al.*[29] Derivatization of the oil with 1% TMCS in BSTFA and subsequent GC/MS afforded a mass spectrum identical with that reported by Rabow *et al.*,[29] *m/z* 321, 307, 231, 219, 205, and the 103 base peak. ^{1}H-NMR (300 MHz, D_2O, 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid internal standard) δ 3.74 (4 H, m), δ 3.60 (2 H, m), δ 1.86 (1 H, dtd, J = 14.4, 7.5, 3 Hz), δ 1.65 (1 H, tdd, J = 12.3, 5.3, 6.6 Hz). ^{13}C NMR (75.4 MHz, D_2O, 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid internal standard) δ 74.9 (C-4), 69.1 (C-3), 62.6 (C-5), 58.7 (C-1), 34.3 (C-2). The carbon numbering scheme is in reference to the sugar group on a nucleotide and does not conform to IUPAC conventions for this complex.

Conditions for Reaction of Cr(V) with Thymidine and Thymidine Deoxyribonucleotides. (A) Identification and Quantification of TBARS, Base Release, and *trans*-Thymine Propenals. Typical reactions for determination of TBARS, base release and *trans*-thymine propenals were carried out simultaneously using 0.8 mL reaction volumes containing 10 mM nucleotide or nucleoside and 0.618 mM Cr(V) (from a 50 mM stock solution of $Na[CrO(ehba)_2]$ in H_2O). The pH was maintained at 6.0 unless otherwise specified with 100 mM NaOAc buffer rigorously demetalated with Chelex-100 ion exchange resin. The reactions were carried out under ambient oxygen pressure at RT for 1 h, except for reactions aimed at determination of oxygen dependence which were carried out under an atmosphere of 100% oxygen or after rigorously degassing with argon (note: only the reaction itself was conducted under the argon or oxygen atmosphere but not the subsequent TBARS, thymine release, and *trans*-thymine propenal analyses which were carried out in air).

Half of the reaction solution was assayed for TBARS using the method of Greenwald *et al.*[30] and quantified from the absorbance at 532 nm using a standard of 1,1,3,3-tetramethoxypropane ($\epsilon = 1.6 \times 10^5\ M^{-1}\ cm^{-1}$).[23] Thymine release and *trans*-thymine propenal formation were determined by passing the remaining 0.4 mL reaction volume through a column (0.5 × 5 cm) of DEAE Sephadex A-25 anion exchange resin followed by elution with 0.6 mL of H_2O. Thymine was quantified by HPLC at 254 nm *versus* a standard curve of authentic thymine, and *trans*-thymine propenal was quantified by HPLC at 305 nm using synthetic *trans*-thymine propenal as a standard.

(B) Identification and Quantification of Glycolic Acid and 2-Deoxy-D-*erythro*-pentitol. Determination of glycolic acid and 2-deoxy-D-*erythro*-pentitol were hampered by the lack of a chromophore thus entailing the use of larger volume reactions and subsequent analysis by GC/MS. Glycolic acid formed in the reaction between dTDP and Cr(V) was determined essentially by the method of McGall *et al.*[31] and 2-deoxy-D-*erythro*-pentitol determined by the method of Rabow *et al.*[29] The glycolic acid and 2-deoxy-D-*erythro*-pentitol were derivatized with TMCS in BSTFA as described above and analyzed by GC/MS. Quantitations were achieved using authentic glycolic acid and synthetic 2-deoxy-D-*erythro*-pentitol as standards. Essentially quantitative recovery was achieved using these methods as demonstrated with reactions using these standards. The detection limit was *ca.* 1.0 μM for both compounds.

(C) Trapping of Cr(IV) by Mn(II). The role of Cr(IV) in the oxidative mechanism was determined using Mn(II) as a Cr(IV) trap and Mg(II) as a cationic control under the conditions described above for the formation of TBARS, thymine release, and *trans*-thymine-propenal. Mg(II) or Mn(II) (final concentration of 1.25 mM) was added to the deoxyribonucleotide solution prior to addition of Cr(V).

Results

Role of Phosphate in Cr(V) Reactions with Nucleotides. Thymidine nucleotides were chosen as a model for Cr(V)-mediated nucleotide oxidation because of the inherent stability of the pyrimidine base and the known oxidation products derived from these substrates' reaction with ionizing radiation and bleomycin.[32,33] These studies have shown oxygen-dependent formation of base propenal (a TBA reactive substance) as well as oxygen-independent base release as a result of two separate pathways for sugar degradation. The role of the phosphate moiety in Cr(V)-mediated nucleotide oxidation was probed by reacting Cr(V) with the dTMP, dTDP, and dTTP nucleotide series, and the dT nucleoside at pH 6.0 under ambient oxygen pressure for 1 h. Formation of thiobarbituric acid reactive species (TBARS) as well as release of free thymine was observed for all three nucleotides, while thymidine showed no reaction (Figure 1A). TBARS formation was greatest for dTDP (2.15 ± 0.08 μM) followed by dTMP (0.71 ± 0.05 μM) and dTTP (0.70 ± 0.01 μM). An identical trend was observed for thymine release: dTDP (20.95 ± 0.62 μM) > dTMP (7.5 ± 0.10 μM) > dTTP (6.79 ± 0.76 μM).

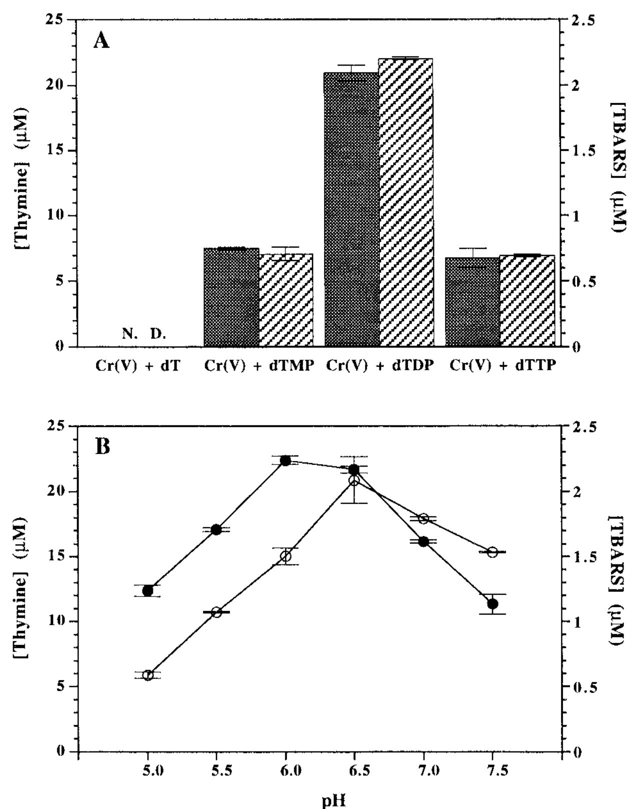

Figure 1. (A) Comparison of the reactivity of the thymine-containing nucleoside and nucleotides (10 mM) with Cr(V) (0.618 mM) at pH 6.0. Solid bars represent thymine release, and cross-hatched bars represent TBARS formation. (B) pH dependence for the formation of TBARS (open circles) and thymine release (closed circles) in the reaction of 0.618 mM Cr(V) with 10 mM dTDP. Reactions were carried out in 100 mM NaOAc for 1 h at RT in air. Data represent the mean ± SD (n = 3).

Disproportionation of Cr(V) in aqueous neutral solutions, 3Cr(V) → 2Cr(VI) + Cr(III), yields a stoichiometry of 67% Cr(VI) and 33% Cr(III).[16] The presence of an oxidizable substrate, such as a nucleotide, should shift the redox percentages toward the more reduced form Cr(III), resulting in a decrease in the final Cr(VI) concentration. Measurement of the final Cr(VI) concentration formed during the reactions described

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Table 1. Oxidation Products and Cr(VI) Loss Observed in the Reaction between Cr(V) and dTDP[a]

oxidized product	concn (μM)
glycolic acid, **III**	6.5 ± 0.7[b]
trans-thymine propenal, **I**	1.1 ± 0.1,[b] 5.6 ± 0.5[c]
thymine	20.95 ± 0.62[b]
2-deoxy-D-pentitol, **V**	<1.0
loss of Cr(VI)	19.8 ± 4.6[b]

[a] All reactions were carried out at 100 mM NaOAc, pH 6.0, in air at RT for 1 h with 10 mM dTDP and 0.618 mM Cr(V). [b] Mean ± SD (n = 3). [c] Corrected concentration for anion exchange column loss.

above showed that disproportionation was stoichiometric, yielding 67% Cr(VI), for the control Cr(V) reaction as well as the reaction with dT. A small yet significant decrease in Cr(VI) concentration, between 3 and 4%, was observed in reactions of Cr(V) in the presence of dTDP (Table 1) and dTTP (23.5 ± 4.5 μM), indicating that substrate oxidation had occurred. This number was considered to correspond roughly to the "oxidative reactivity range" for Cr(V) with these nucleotides. However, the low overall reaction with dTMP could not be detected using this method. This "oxidative reactivity range" was consistent with the total oxidized products measured by TBARS and thymine release for the Cr(V) reaction with dTDP (Table 1). The fact that dT showed no reaction in any of these assay systems demonstrates the absolute requirement for phosphate in the redox reaction between Cr(V) and nucleotides.

pH and Time Dependence of TBARS Formation and Thymine Release. Maximum formation of both TBARS and free thymine for the reaction of Cr(V) with dTDP was observed at pH 6.0−6.5 when carried out at RT for 1 h under ambient oxygen pressure (Figure 1B). However, some reaction could be detected in the full pH range measured, 5.0−7.5. The range of greatest reactivity was considered to be a consequence of balancing the ligand exchange lability of Cr(V) leading to rapid disproportionation at higher pH values with ligand exchange stability and thus lower reactivity at more acidic pH values.

The 1 h reaction time used throughout this study corresponded to complete decay of the Cr(V) EPR signal in the pH ranges of 7.5−6.5 at RT, and approximately 95% Cr(V) decay at pH 6.0 (Figure 2A). The Cr(V) EPR signal showed significantly greater stability at pH < 6.0, which would account for the lower reactivity observed under these conditions. The presence of dTDP in the reaction solution with Cr(V) did not significantly alter the normal decay pathway for Cr(V) as measured by EPR (Figure 2A). The appearance of oxidative damage, as measured by thymine release and TBARS formation (Figure 2B), was inversely related to the disappearance of Cr(V) (Figure 2A). Neither Cr(VI) itself nor an aged solution of Cr(V) (which had been allowed to completely disproportionate to Cr(VI) and Cr(III)) showed significant TBARS formation or thymine release (data not shown). Reaction of Cr(V) with dTMP and dTTP at pH 6.0 demonstrated similar conditions for maximal formation of TBARS and thymine release (data not shown). These data support the hypothesis that oxidative damage arises from either Cr(V) itself or an *in situ* generated Cr(IV) species under conditions which are within a physiologically relevant range.

Oxygen Dependence for TBARS Formation and Thymine Release. The requirement for oxygen was determined for the reaction of Cr(V) with dTDP by measuring the formation of TBARS and thymine release under an oxygen, air, or argon atmosphere. Under a pure oxygen atmosphere, TBARS formation was enhanced only slightly over air but was decreased by 42% in the absence of oxygen (Figure 3A). The opposite was true for thymine release where production of free thymine was maximal under argon, but decreased by 35% under oxygen with

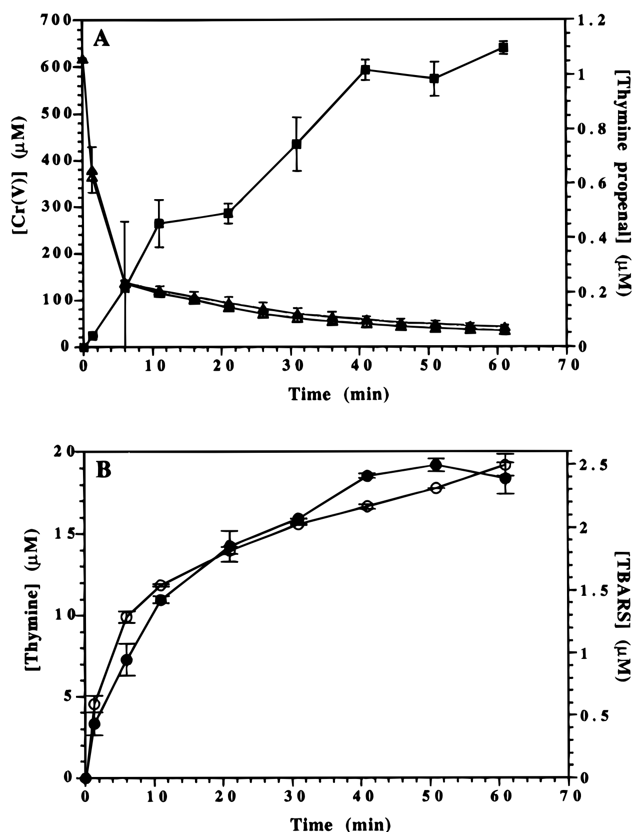

Figure 2. (A) Loss of Cr(V) EPR signal over time for the disproportionation reaction of Cr(V) in the presence (open triangles) or absence (closed triangles) of dTDP and time-dependent formation of *trans*-thymine propenal (closed squares) in the reaction of 0.618 mM Cr(V) with 10 mM dTDP in 100 mM NaOAc, pH 6.0. Data represent the mean ± SD (n = 3). (B) Time-dependent formation of TBARS (open circles) and thymine release (closed circles) in the reaction of 0.618 mM Cr(V) with 10 mM dTDP in 100 mM NaOAc, pH 6.0, at RT.

air being intermediate in reactivity (Figure 3A). Since only the direct 1 h reaction of Cr(V) with dTDP was carried out under a pure argon or oxygen atmosphere, it is possible that the subsequent addition of thiobarbituric acid to form the TBARS chromophore (carried out in air) may have been measuring a long-lived O_2-dependent species. This long-lived O_2-dependent species may account for the residual TBARS which were observed under anoxic conditions. The oxygen-dependent formation of TBARS is a classical marker of C-4′ H-atom abstraction and suggests an oxygen-dependent mechanism for Cr(V)-induced oxidation of dTDP (pathway 1) outlined in Scheme 1.[34] However, the formation of free thymine in an oxygen-independent fashion suggests a separate pathway of sugar oxidation (pathway 2).

Pathway 1: Identification of Products from the Oxygen-Dependent Pathway in the Cr(V)−dTDP Reaction. (A) *trans*-Thymine Propenal. The specific species being measured in the TBARS assay is considered to be *trans*-thymine propenal (for this substrate), **I**, formed following abstraction of the C-4′ hydrogen of deoxyribose, addition of molecular oxygen to the reactive C-4′ radical intermediate, and anti-elimination of the 2′-*pro-R* hydrogen of the oxidized sugar (Scheme 1).[34] Concurrent with the formation of **I** would be a phosphorylated glycolic acid species, **II**, in a 1:1 ratio. Specifically, this

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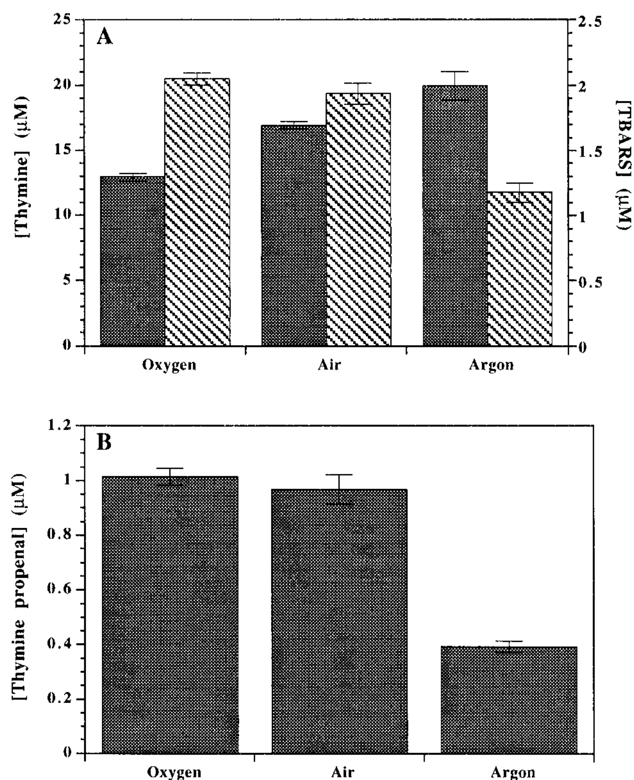


Figure 3. Oxygen dependence of (A) the formation of TBARS (cross-hatched bars) and thymine release (solid bars) and (B) the formation of *trans*-thymine propenal in the reaction of 0.618 mM Cr(V) with 10 mM dTDP in 100 mM NaOAc, pH 6.0, for 1 h at RT. Data represent the mean ± SD ($n = 3$).

would be a diphosphorylated species from the dTDP substrate, but a monophosphorylated species if this reaction occurred in DNA.

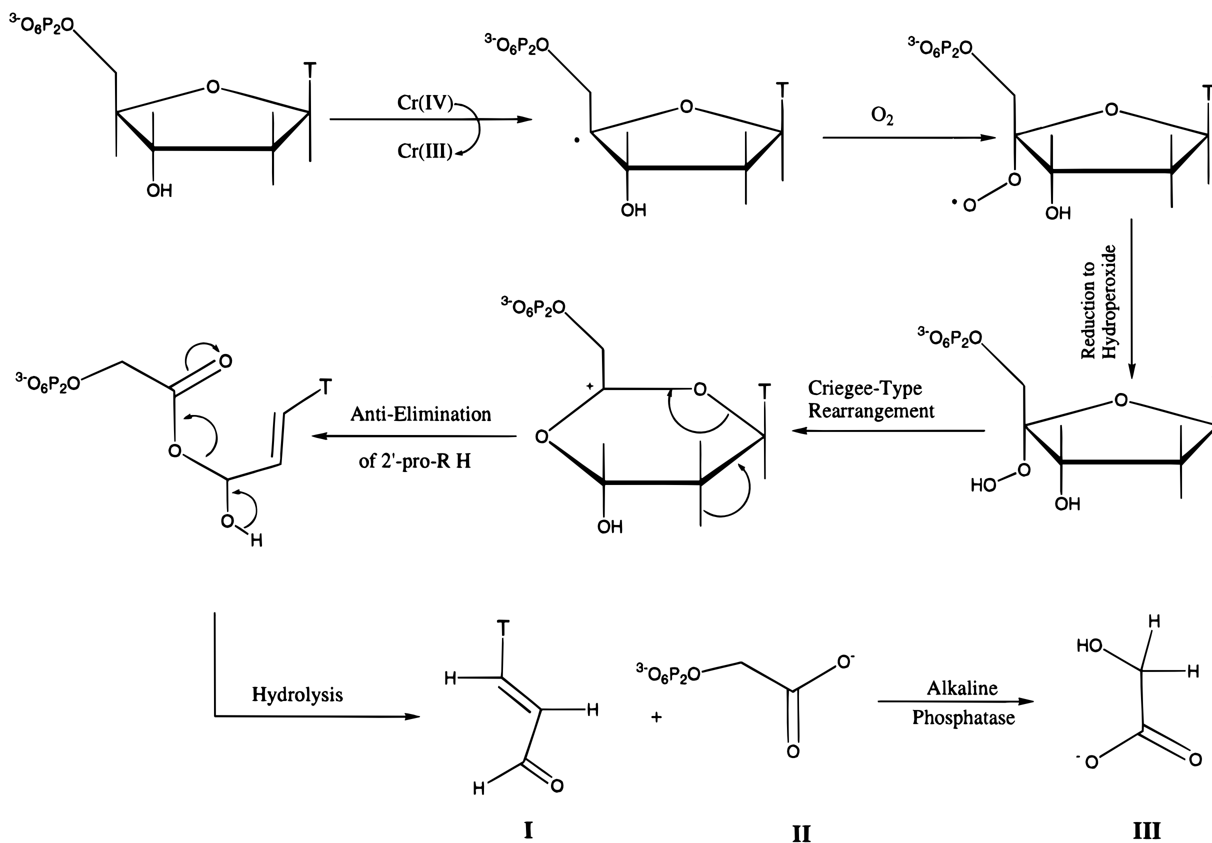
Formation of **I** was demonstrated in the reaction of Cr(V) with dTDP at pH 6.0 using HPLC (Figure 4). The HPLC trace showed that, in this reaction, a peak eluting at ~3.1 min had an identical retention time as synthetic *trans*-thymine propenal (Figure 4). Diode array detection of the HPLC eluent showed that the 3.1 min peak had an identical absorption spectra as authentic **I** with a wavelength maximum at 306 nm. Collection of the 3.1 min peak and subsequent thin layer chromatography *versus* authentic **I** demonstrated an identical R_f value (0.73), and when sprayed with a 1% solution of 2-thiobarbituric acid in 0.1 N NaOH developed the red-pink color associated with TBARS. No *cis* isomer of **I** was detected in any of the reactions, which is consistent with the mechanism of anti-elimination of the 2′-*pro-R* hydrogen.[34]

Production of TBARS should directly correlate with formation of **I**, since the malondialdehyde-like species measured at 532 nm is derived from acid hydrolysis of **I**.[27] Formation of **I** in the reaction of Cr(V) with dTDP (Figure 2A) followed the same time dependence as the formation of TBARS (Figure 2B), suggesting that **I** is the ultimate species measured in this assay. Formation of **I** was also observed to follow the same trend as TBARS formation for oxygen dependence (Figure 3B). However, when quantified versus a standard curve of authentic *trans*-thymine propenal, the concentration of **I** was determined to be 1.1 ± 0.1 μM, much less than expected on the basis of the concentration of TBARS. This result suggested either decomposition of **I** was occurring during the reaction, or there was an incomplete release of **I** from the phosphate moiety. Incomplete release would result in binding to the anion exchange resin used during workup of the product and thus an underestimation of total **I** produced during the reaction. Precedence for this incomplete release of **I** from phosphate was demonstrated in the reaction of bleomycin with DNA.[34a] In control reactions, authentic **I** was inert to decomposition by either chromium(V) or chromium(VI) under the normal reaction conditions. However, a control reaction for TBARS production either with or without passing through an anion exchange resin demonstrated that only 19% of the total TBARS formed in the one hour reaction had undergone hydrolysis resulting in release of the phosphate. This demonstrated that the actual concentration of **I** formed in the reaction of Cr(V) with dTDP was ~5-fold higher (5.6 ± 0.5 μM). Formation of **I** was also observed by HPLC in reactions between Cr(V) and dTMP or dTTP at pH 6.0, but at much lower levels as suggested by the ~3-fold lower level of TBARS observed.

(B) Glycolic Acid. The second sugar fragment formed in pathway 1 (see Scheme 1) is expected to be the phosphorylated glycolic acid species **II**. Treatment with alkaline phosphatase releases glycolic acid, **III**, which is readily measured by GC/MS upon derivatization to the bis(trimethylsilyl)glycolate species. Formation of **III** in the reaction of Cr(V) with dTDP at pH 6.0 was confirmed by GC/MS analysis (Table 1). The glycolic acid formed in the reaction had an identical retention time on the GC as authentic **III**, 4.6 min, as well as identical molecular ion, $M - CH_3$ of $m/z = 205$, and identical fragmentation pattern. The concentration of **III** formed was consistent with that obtained for fragment **I** (Table 1), as expected from the 1:1 ratio predicted in Scheme 1. The fact that the amounts of **I** and **III** are consistent with each other but higher than the TBARS suggests that a reaction between chromium and TBARS may occur and lead to an underestimation of the actual reaction. Formation of **III** in the reaction between Cr(V) and dTMP and dTTP was not attempted due to the low level of reaction for these two substrates and the fact that levels of **III** were near the detection limits for the much higher yielding Cr(V)−dTDP reaction.

Pathway 2: Identification of Products from an Oxygen-Independent Pathway in the Cr(V)−dTDP Reaction. (A) 2-Deoxy-D-*erythro*-pentitol. A two-electron, O_2-independent pathway (Scheme 2)[29] or a one-electron pathway where OH^- addition is favored over O_2 addition could account for the release of the free base thymine. The mechanism invoked involves formation of a carbocation intermediate at the C-4′ position of deoxyribose by a two electron Cr(IV)/Cr(II) redox mechanism, followed by addition of OH^- from water and concomitant thymine release. In tandem with thymine release is the production of the reactive 4′-keto-1′-aldehyde species **IV** (Scheme 2).[29] This reactive aldehydic species is not isolable itself[29] but instead is reduced by $NaBH_4$ to the polyalcoholic species 2-deoxy-D-pentitol, **V**, with both *erythro* and *threo* isomers expected.[29] With dTDP as the substrate, any **V** formed would be the diphosphorylated species that upon dephosphorylation with alkaline phosphatase would give free **V** for GC/MS analysis. Reaction of Cr(V) with dTDP at pH 6 resulted in production of **V** that was below the detection limits afforded by this assay (data not shown). A small, unquantifiable peak eluting at a retention time of 10.6 min, identical to that for authentic **V**, was observed but the concentration was less than lowest standard (<1.0 μM, Table 1). It cannot be ruled out that the reactive aldehydic intermediate **IV** was formed but decomposed in the presence of oxidizing chromium species in solution, although the reduced species **V** was observed to be stable to the presence of both Cr(V) and Cr(VI). Repeating the experiment under anaerobic conditions to maximize formation of **V** (suggested by the anaerobic results for thymine release, Figure 3A) resulted in no detectable **V**. This result strongly

Scheme 1



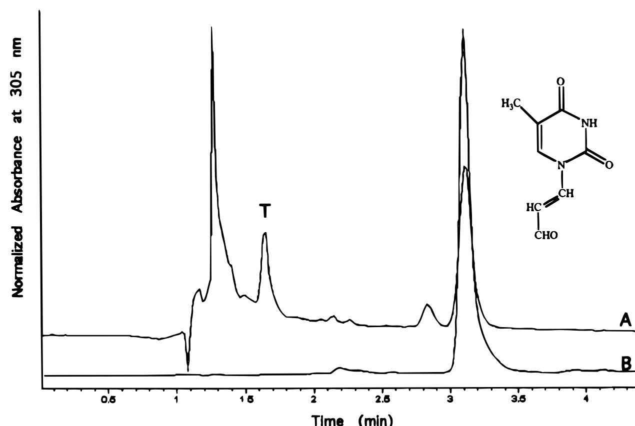


Figure 4. HPLC chromatogram of the products formed from the reaction of Cr(V) (0.618 mM) and dTDP (10 mM) in 100 mM NaOAc, pH 6.0, at ambient oxygen pressure and RT for 1 h. Trace A: the reaction monitored at 305 nm for detection of *trans*-thymine propenal at 3.1 min, also showing thymine, **T**, at 1.6 min. Trace B shows the elution profile of authentic *trans*-thymine propenal, synthesized as described in the Experimental Section.

suggests that production of **V**, and the mechanism that it represents, was a minor pathway and reaction at different sugar sites such as C-3′, C-5′, or C-1′ must be considered to explain the excess production of free thymine.

Role of the +4 Chromium Oxidation State in Nucleotide Oxidations. The role that the +4 oxidation state of chromium may play in the oxidation of nucleotides was investigated using a Cr(IV)-specific reductant, Mn(II).[35] Cr(IV) produced from the initial disproportionation of Cr(V), eq 1, can react with Mn(II) to produce Cr(III) and Mn(III), eq 2. The use of Mn(II)

$$2Cr(V) \rightarrow Cr(IV) + Cr(VI) \quad (1)$$

$$Cr(IV) + Mn(II) \rightarrow Cr(III) + Mn(III) \quad (2)$$

as a competitor for Cr(IV) can thus determine what role this oxidation state of chromium may play in nucleotide oxidations. At a 2-fold molar excess of Mn(II) to Cr(V) in the reaction with dTDP at pH 6.0, thymine release was completely inhibited and TBARS formation was reduced to essentially background levels (Figure 5). A control reaction using a nonredox-active divalent cation, Mg(II), under identical conditions showed essentially no inhibition of either thymine release or TBARS formation (Figure 5). Formation of *trans*-thymine propenal, **I**, was detected in both control and Mg(II) reactions but not in the Mn(II) reactions (data not shown). The data clearly demonstrate that Cr(IV) is the species responsible for oxidizing the nucleotides in both pathways 1 and 2 (Schemes 1 and 2).

Discussion

Role of Phosphate in Cr(V)-Mediated Nucleotide Oxidations. Previously, we have shown that Cr(V) can bind to both phosphate and pyrophosphate, with pyrophosphate being the more kinetically favorable.[19] Since dTDP can be considered a pyrophosphate analog and dTMP a phosphate analog, the fact that dTDP shows a greater oxidation reaction with Cr(V) than dTMP is consistent with a kinetic basis for oxidation of nucleotides. The reaction of Cr(V) with dTTP would be considered to be the most reactive if the trend of increasing phosphates giving increasing oxidation held true. This trend was observed in the loss of Cr(VI) concentration but not in either of the two biomarkers used in this study, thymine release and TBARS. This can be explained on a steric basis where a bidentate binding mode to the γ- and β-phosphates of dTTP would give a greater degree of flexibility to the putative Cr(IV)–nucleotide complex in contrast to binding at the α- and

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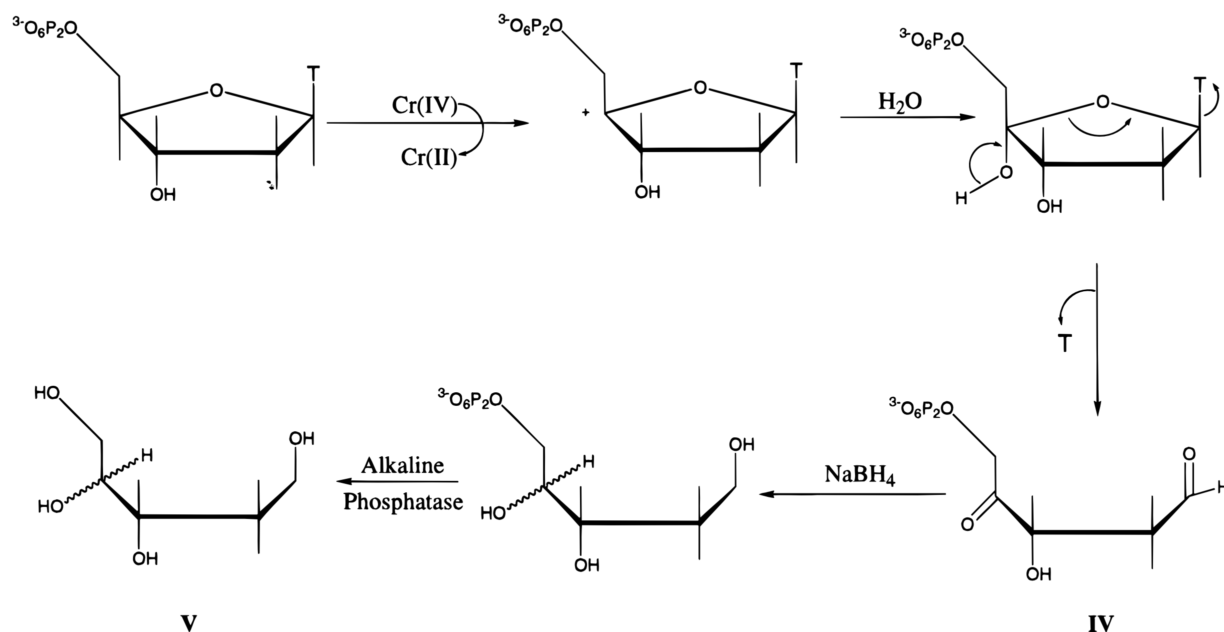
Scheme 2



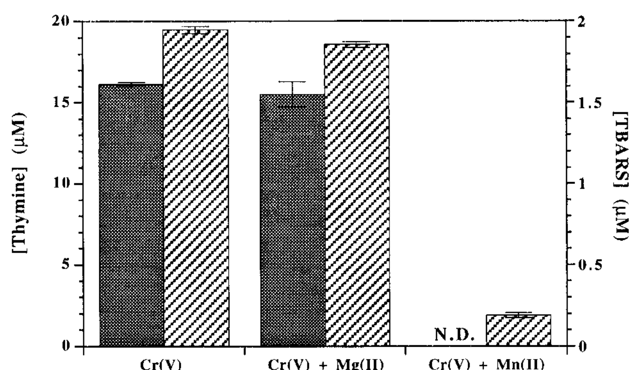


Figure 5. Effect of the Cr(IV)-specific reductant Mn(II) or the divalent cation control Mg(II) (1.2 mM) on the release of thymine (solid bars) and TBARS formation (cross-hatched bars) in the reaction of 0.618 mM Cr(V) with 10 mM dTDP in 100 mM NaOAc, pH 6.0, for 1 h at RT. Data represent the mean ± SD (n = 3). N.D. = not detectable.

β-phosphates as in dTDP. The end result of this greater flexibility would be increased oxidation at other sugar sites such as C-3′ or C-5′, yielding a higher overall oxidation as measured in Cr(VI) concentration loss but through the formation of oxidized products not measured in this study. The binding of the +3 oxidation state of chromium to nucleotides through the phosphate is well known,[36] but the ability of Cr(IV) to bind to phosphate has not been demonstrated, due to its transient nature. The importance of phosphate in chromium reactions as a potential binding site has been recently shown for the formation of ternary Cr(III)−amino acid complexes with DNA.[37] As well, reduction reactions of Cr(VI) and ascorbate leading to single strand breaks in DNA have been observed to occur primarily in phosphate buffer.[38] These results suggest a mechanism where a reactive Cr(IV) species transiently binds to a terminal or internucleotide phosphate allowing favorable steric interactions for sugar oxidation to occur.

Conditions for Maximum Formation of TBARS and Thymine Release. The conditions for maximal formation of TBARS and thymine release were within physiologically significant pH ranges, ionic strengths, and concentrations. The greatest reactivity appeared to be in a range which best balances stability with disproportionation. Disproportionation was necessary for reactivity, as is shown by the Mn(II) studies and by the low reactivity observed at acidic pH values, although at high pH values disproportionation appears to be favored over substrate oxidation, leading to TBARS and thymine release.

The reaction of Cr(V) with nucleotides demonstrates an oxygen-dependent mechanism (pathway 1, Scheme 1) similar to that observed with other DNA damaging agents such as bleomycin, neocarzinostatin, and ionizing radiation.[33,39] The requirement of oxygen for production of both single strand breaks (SSBs) in DNA and base release has been demonstrated for *in situ* generated Cr(V)−glutathione and Cr(V)−ascorbate complexes.[20,21] This oxygen dependence has been ascribed to a Cr(IV)− or Cr(V)−peroxo species formed from the reaction with molecular oxygen during the reduction process. In this study there was an oxygen dependence in TBARS formation, which would be manifested as SSBs in DNA, but increased oxidation as measured by thymine release was observed under anoxic conditions between Cr(V) and dTDP which is not in agreement with that seen previously.[20,21] Instead, this data is in agreement with our previous results showing that Cr(V) can directly oxidize EPR spin traps without the presence of oxygen[17] and that invoking a Cr(IV)− or Cr(V)−peroxo species with molecular oxygen is not necessary for the $[CrO(ehba)_2]^-$ species.

Identification of Oxidized Products from the Cr(V)−dTDP Reaction. Formation of base propenals in reactions with DNA or nucleotides has been shown to be oxygen-dependent, occurring through a one-electron, hydrogen atom abstraction mechanism at the C-4′ of deoxyribose.[34] In the reaction of Cr(V) with dTDP we have demonstrated the unequivocal formation of *trans*-thymine propenals. As well, we have identified the remaining sugar fragment from the C-4′ H-atom abstraction, glycolic acid. To our knowledge, this is the first time that products from a known oxidative mechanism have been isolated and characterized from the reaction of high valent chromium complexes with nucleotides. Extrapolation of this mechanism to DNA suggests that strand breakage should be

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observed. Indeed, strand breaks have been shown for this Cr(V) complex by supercoiled plasmid DNA relaxation assays.[18]

The release of the free base thymine was also observed and initially ascribed as the product of a two-electron oxidation at the C-4′ of deoxyribose (pathway 2, Scheme 2). However, this putative two-electron oxidation pathway was not verified since the sugar fragment expected in this oxidation, 2-deoxy-D-pentitol, was not observed in an equimolar amount with free thymine. A wide variety of other possible oxidation mechanisms could explain the thymine release, including C-1′, C-3′, and C-5′ oxidations. An alternative mechanism which would lead to thymine propenals, glycolic acid, and malondialdehyde formation but not to the 4′-keto-1′-aldehyde sugar fragment by hydrogen atom abstraction at the C-4′ of deoxyribose has been observed for 3′-O-acylated nucleosides.[40] This mechanism appears to be unlikely for the reaction of chromium with nucleotides since the nucleotides lack the 3′-acyl protecting group necessary for migration and polarization of the hydroperoxide intermediate formed when molecular oxygen reacts with the C-4′ radical of deoxyribose.[40] As well, equal amounts of malondialdehyde and free thymine should be detected, which was not observed.

This study shows that caution in assigning mechanisms must be used until both expected fragments from the oxidation reaction are identified. Due to the possibility of sugar oxidation intermediates reacting with high valent chromium species in solution, further studies with specifically tritiated nucleotides are needed to unequivocally establish the point of C−H bond cleavage leading to release of the free thymine.

Role of the +4 Oxidation State in Nucleotide Oxidations. During disproportionation of Cr(V), Cr(IV) is known to be formed and has been shown to be an inherently more oxidative species.[41] As well, Cr(IV) in perchloric acid solutions has been shown to readily oxidize a variety of organic complexes.[42] However, unlike Cr(V), Cr(IV) is refractory to spectroscopic observations in aqueous neutral solutions. The use of Mn(II) as a Cr(IV) trap can be used to infer the involvement of Cr(IV) in a mechanism since it does not appear to interact with the other potential oxidizing complex Cr(V).[5] The use of Mn(II) in the reaction showed that oxidation of thymidine nucleotides is dependent on the formation of Cr(IV). Neither Cr(VI) nor Cr(III) showed significant activity in any of the reactions. Previously, the use of V(IV) as a Cr(IV)-specific reductant has been observed to inhibit strand breakage by the $[Cr^{V}O(ehba)_2]^-$ complex, as measured by a supercoiled plasmid DNA relaxation assay.[43] However, this oxidation appears to be ligand specific since certain macrocyclic tetraamide Cr(V) complexes appear to relax supercoiled plasmid DNA without undergoing disproportionation/reduction to Cr(IV).[44] While it is still too early to make general conclusions on the ultimate oxidation state of chromium responsible for intracellular DNA oxidation, the +4 oxidation state must be considered a leading candidate.

Conclusions. The results reported here have established that oxidation of nucleotides by high valent chromium complexes can occur at the sugar moiety. This oxidation is dependent upon the presence of a phosphate presumably as a tethering point for the chromium complex. An oxygen-dependent mechanism (pathway 1) which involves the abstraction of the C-4′ hydrogen atom has been demonstrated by isolating and identifying the two major products which are known to be associated with this mechanism, *trans*-thymine propenal and glycolic acid. A second, as yet unidentified, mechanism is also occurring to account for the release of free thymine. As well, the oxidation state of chromium which appears to be responsible for this oxidation has been determined to be the Cr(IV) species which is formed during disproportionation of the parent Cr(V) complex. These results are the first in which a specific oxidation state of chromium has been assigned a definitive oxidative mechanism for a biologically relevant substrate unambiguously identified by product analysis. The mechanism, when coupled with phosphate dependence, has implications for oxidative DNA damage as well as disruption of intracellular nucleotide pools.

Acknowledgment. This research was funded by PHS Grant ES07167 from the National Institute of Environmental Health Sciences, DHHS (K.E.W.). The EPR spectrometer was purchased with NSF Grant CHE-8701406. K.D.S. was supported by a NRSA postdoctoral fellowship (Grant CA60434) from the National Cancer Institute, DHHS.

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