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Mechanisms of DNA damage by chromium(V) carcinogens

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ABSTRACT

Reactions of bis(2-ethyl-2-hydroxy-butanato)oxochromate(V) with pUC19 DNA, single-stranded calf thymus DNA (ss-ctDNA), a synthetic oligonucleotide, 5′-GATCT-ATGGACTTACTTCAAGGCCGGGTAATGCTA-3′ (35mer), deoxyguanosine and guanine were carried out in Bis–Tris buffer at pH 7.0. The plasmid DNA was only nicked, whereas the single-stranded DNA suffered extensive damage due to oxidation of the ribose moiety. The primary oxidation product was characterized as 5-methylene-2-furanone. Although all four bases (A, C, G and T) were released during the oxidation process, the concentration of guanine exceeds the other three. Orthophosphate and 3′-phosphates were also detected in this reaction. Likewise, the synthetic oligomer exhibits cleavage at all bases with a higher frequecncy at G sites. This increased cleavage at G sites was more apparent after treating the primary oxidation products with piperidine, which may indicate base oxidation as well. DNA oxidation is shown to proceed through a Cr(V)–DNA intermediate in which chromium(V) is coordinated through the phosphodiester moiety. Two alternative mechanisms for DNA oxidation by oxochromate(V) are proposed to account for formation of 5-methylene-2-furanone, based on hydrogen abstraction or hydride transfer from the C1′ site of the ribose followed by hydration and two successive β-eliminations. It appears that phosphate coordination is a prerequisite for DNA oxidation, since no reactions between chromium(V) and deoxyguanosine or guanine were observed. Two other additional pathways, hydrogen abstraction from C4′ and guanine base oxidation, are also discussed.

INTRODUCTION

A number of metal ions cleave nucleic acids in the presence of oxygen or hydrogen peroxide. High valency oxometal complexes are often suspected to be the primary reactive intermediates in these reactions (1–5), although direct participation of hydroxyl and peroxy radicals has not yet been ruled out (6,7). The role of oxometallates in these oxidative damage processes can therefore best be elucidated by examining direct reactions of nucleic acids with authentic oxometal complexes. Unfortunately, many of these putative DNA damaging agents, such as Fe(IV/V) and Mn(IV/V) species, are not stable enough at physiological pH to allow unambiguous characterization of these species (1–7). Therefore, other stable (or metastable) oxometal centers must be used to determine the redox chemistry of metal ions and nucleic acids. Oxoruthenium(IV) and oxoosmium(IV) complexes have thus been utilized in oxidation of calf thymus DNA and synthetic oligonucleotides by Thorp and co-workers (8–10). These authors reported that ruthenium(IV) complexes cleave DNA primarily through oxidation of the ribose and guanine base. Sugar oxidation proceeds mainly at the C1′ position. However, results obtained from one metal ion may not apply to others, since tremendous diversity exists in coordination and the redox chemistry of transition metal ions.

In a recent communication we reported that oxochromium(V) complexes oxidize single-stranded calf thymus DNA (ss-ctDNA) mainly through the C1′ site of the ribose (11). An understanding of redox reactions of oxochromium(V/IV) complexes with DNA is important, since these oxidation states are implicated in Cr(VI)-induced carcinogenesis (12–18). Earlier, Lay and co-workers used an authentic Cr(V) compound, bis(2-ethyl-2-hydroxy butanoato)oxochromate(V) (**I**) in reaction with pUC9 DNA in acidic solution (19). These workers observed that the plasmid was only nicked. Subsequently, the same laboratory employed our method of *in situ* preparation (20) of oxochromium(IV) and examined its reactions with the same plasmid DNA (21). Like its parent oxochromium(V) complex, chromium(IV) nicked the plasmid. All these reactions were carried out in acidic solution, since these hypervalent oxochromium complexes are not stable at neutral pH (19–21). Recently we have observed that the polyalcoholic multidentate ligand bis(hydroxyethyl)amino tris(hydroxymethyl)-methane (BT) stabilizes Cr(V) in aqueous solution at neutral pH by retarding its disproportionation (22). This new stable Cr(V) compound (**II**) provides us with an opportunity to examine the DNA reaction in detail. Furthermore, this oxochromium Cr(V) compound, unlike the oxoruthenium bipyridyl complexes, carries an overall negative charge. Formation of a DNA–metal outer sphere complex, held together largely by electrostatic forces, is unlikely. Therefore,

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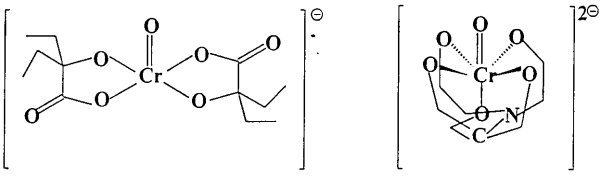
these anionic chromium(V) complexes might add to the mechanistic diversity associated with oxidative damage of DNA.

In this article we describe the reactions of plasmid pUC19 DNA, ss-ctDNA and a synthetic 35mer oligonucleotide with oxochromium(V) complexes (**I** and **II**; Scheme 1). To our knowledge this is the first report that characterizes DNA oxidation products, identifies intermediates and pinpoints the specific sites of reaction with oligonucleotides.



I II

Scheme 1.

MATERIALS AND METHODS

Reagents

Sodium bis(2-ethyl-2-hydroxy butanoato)oxochromate(V) was prepared following the method of Krumpolc and Rocek (23). A chromium(V)–Bis–Tris (Cr^{V}–BT) complex was generated *in situ* by direct ligand exchange reaction of BT ligand with **I** (22). More than 95% of the parent Cr(V) compound was converted to the Cr^{V}–BT complex. 5-Methylene-2-furanone (5-MF) was synthesized following a published method (24). The oligonucleotide (35mer) 5′-GATCTATGGACTTAGTTCAAGGCCGGGTAATGCTA-3′ was synthesized by Gibco BRL (Gaithersburg, MD). The radioactive isotope [α-^{32}P]dCTP (3000 Ci/mol) was obtained from DuPont/NEN (Boston, MA). Terminal deoxynucleotidyl transferase was purchased from Promega (Madison, WI). Molecular weight markers (a *Hae*III digest of pUC18 and a 1 kb DNA ladder) were obtained from Sigma. Unless otherwise stated all other common reagents were of highest purity (Sigma or Fisher Scientific).

Preparation and purification of plasmid pUC19 DNA

Escherichia coli DH5α (r^{-}_{k} m^{+}_{k}) harboring pUC19 was grown overnight in LB medium containing 100 µg/ml ampicillin at 37°C with shaking (25). The plasmid was purified using a Qiagen Plasmid Kit (Qiagen Inc., Studio City, CA). After further purification through a 1% agarose gel the purity and concentration of the DNA was determined spectrophotometrically at 260 nm. The ratio of absorbances measured at 260 and 280 nm was 1.77.

Preparation and purification of end-labeled oligomer

The synthetic oligomer was end-labeled by addition of [α-^{32}P]dCTP with terminal deoxynucleotidyl transferase and gel purified by excising the labeled oligomer from a 15% polyacrylamide gel. The oligomer was sequenced following the method of Maxam and Gilbert (26).

Purification of calf thymus DNA

ss-ctDNA (Fluka) was dissolved (3.2 mg/ml) in a mixture of Bis–Tris buffer and NaCl solution. This was passed through a Chelex 100 ion exchange column (5 ml bed volume, 1 cm inner diameter). The DNA was eluted with 10 ml aliquots of Bis–Tris buffer (100 mM). The concentration of this DNA was estimated from its molar absorptivity, 6.6×10^{3}/cm/M at 260 nm.

Physical measurements

Electron spin resonance measurements. Electron paramagnetic resonance experiments were performed on an X-band (9.5 GHz) IBM instrument (200D-SCR) in a flat quartz cell in aqueous solution. A Hewlett Packard microwave frequency counter (5351A) was used to measure the frequency. The spectrometer was calibrated at $g = 2.0023$ using an NMR gaussmeter. Typical data acquisition parameters were: data points, 2K; frequency window, 3450 ± 250 G; acquisition time, 100 s; modulation frequency, 100 KHz; modulation amplitude, 0.5–1.5 G; attenuation, 10 dB. In a typical ESR experiment the Cr(V) compound (1.0–5.0 mM **I** or **II**) was mixed with ctDNA (5.0–10.0 mM) or pyrophosphate (2.0–10.0 mM) in Bis–Tris buffer (10–50 mM, pH 6.6–7.6). The solution was immediately transferred to a flat quartz cell. The ESR spectra were measured at regular time intervals. In order to relate the ESR intensity to [Cr^{V}] the signal intensities (calculated through double integration) were compared with standard concentrations of **I** at pH 3.3. **I** is most stable at pH 3.3.

Nuclear magnetic resonance measurements. NMR experiments were performed on a GE 300 MHz (GN 300) instrument. The proton chemical shifts are with respect to the H-O-D resonance at 4.67 p.p.m. ^{31}P resonances are reported with respect to 85% phosphoric acid at 0.0 p.p.m. For ^{31}P a 25 µs pulse with a repetition time of 2.0 s was used. Typically 8–16K data points were collected within a frequency window of 10 000 Hz. A line-broadening factor of 3 Hz was introduced before Fourier transformation.

In a typical NMR experiment 1–2 mM Cr(V) complex was mixed with ctDNA (5–10 mM) in Bis–Tris buffer, pH 7.0, in D_2O. After 2 h reaction at room temperature EDTA (30 mM) was added to the reaction mixture. ^{31}P NMR spectra were recorded before and after adding EDTA. The spectra were also recorded at various time intervals after EDTA was added. For proton NMR spectra fractions from HPLC separation were collected and concentrated on a rotary evaporator under reduced pressure (~10 torr) and then subjected to NMR measurements.

Mass spectrometric measurements. Mass spectra were recorded on a VG Autospec tandem mass spectrometer with an EBE geometry. In this geometry the first two sectors function as MS-1 and the third sector serves as MS-2. An electron impact ionization source (70 eV) was utilized.

High performance liquid chromatographic measurements. HPLC measurements were performed either on an ISCO ternary gradient or on a Waters gradient instrument using a µ-bonded reverse phase C18 (3.9 × 300 mm) column. The Waters instrument is equipped with a diode array detector. Isocratic separations were done using either 50 mM phosphate buffer, pH 6.8, or ammonium formate, pH 6.8–7.0, in 80% water, 20% acetonitrile as the mobile phase with a flow rate of 1 ml/min. The retention times of analytes were compared with those of standard samples, which were measured under identical conditions. The spectra of the analytes were also compared with standard samples recorded by the diode array detector during separation. Fractions collected from columns after several injections were

concentrated on a rotary evaporator and were then subjected to NMR measurements for further characterization.

In order to compare the concentrations of A, C, G and T bases released during oxidation of DNA by Cr(V) complexes the area under the peak for a given nucleotide was divided by its molar absorptivity. Chromatographic detection was at 260 nm. Since bases (A, C, G and T) exhibit absorption maxima at different wavelengths which are pH dependent, molar absorptivities for three bases were determined at 260 nm at pH 7.0 by dissolving a known quantity of the base in Bis–Tris buffer. These molar absorptivity values expressed as per M/cm at 260 nm are: A, 13.29×10^3; C, 5.55×10^3; T, 7.52×10^3. The molar absorptivity of the guanine base was taken from the literature.

Disproportionation of chromium(V) complexes

Chromium(V) disproportionates to Cr(VI) and Cr(III). The extent of disproportionation was estimated by measuring the Cr(VI) concentration spectrophotometrically at 372 nm at neutral pH using a molar absorptivity of 4.8×10^3/M/cm. Nucleotides do not absorb at this wavelength. Furthermore, interference by Cr(III) is minimal, since the trivalent oxidation state has a very low molar absorptivity (<50) at this wavelength.

DNA cleavage by chromium(V)

The 35mer (10 000 c.p.m. end-labeled probe) was treated with Cr(V) complex **I** (1.0 mM) with or without Bis–Tris buffer (30 mM, pH 7.0) in a 100 µl volume for 90 min at 37°C. At the end of the reaction, EDTA (50 mM, pH 8.0) was added to bind Cr(III) and destroy unreacted Cr(V). EDTA reduces Cr(V) to Cr(III) with formation of ethylenediamine triacetic acid and CO_2 (27). After incubation for 30 min with EDTA the samples were precipitated by addition of 25 µl dimethylsulfate stop solution (1.5 M sodium acetate, 1 M β-mercaptoethanol, 100 µg tRNA) and 300 µl ethanol. The precipitate was collected by centrifugation. The dried pellet was resuspended in 5 µl formaldehyde loading solution. The cleavage products were separated on an 8% acrylamide sequencing gel containing 6 M urea. The bands were visualized by exposure to X-ray film and were quantitated with IP Labgel software (Signal Analytics Corp., Vienna, VA) in conjunction with a Molecular Dynamics phosphorimager.

Reaction of the Cr(V)–BT complex with pUC19 DNA (0.3 µg/10 µl) was carried out for 90 min at 37°C in Bis–Tris buffer at pH 7.0. The concentration of Cr(V) was varied from 0.3 to 3.0 mM. After the reaction 10 µl 0.2% bromophenol, 50% glycerol stain was added to samples which were then loaded onto a 0.8% agarose gel. The samples were then immediately electrophoresed in buffer containing 89 mM Tris–HCl, 89 mM boric acid, 2.5 mM EDTA and 0.5 µg/ml ethidium bromide for 15 h at 250 V. Gels were photographed with 254 nm UV light using a Polaroid camera.

RESULTS

Figure 1 shows a polyacrylamide gel that exhibits the products of the 35mer cleaved by the Cr(V) complexes. The identity of the cleavage products was determined by comparison with cleavage products obtained by subjecting the same oligmer to sequence-specific chemical cleavage (lanes 1–4). Lanes 5–8 contain products of cleavage of the oligonucleotide by chromium(V) in the presence and absence EDTA and piperidine. In the absence of EDTA bands are seen to be retarded compared with the unreacted

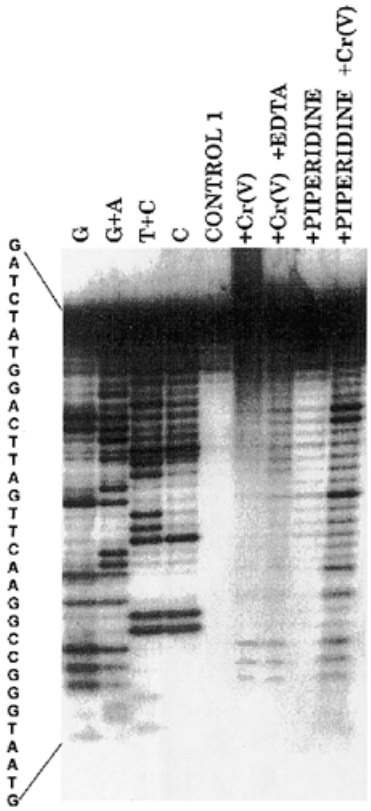


Figure 1. Autoradiagram of a polyacrylamide gel of a 35mer (3′-end-labeled with ^{32}P), 5′-GATCTATGGACTTAGTTCAAGGCCGGGTAATGCTA-3′, and the products of the reaction with Cr(V) in Bis–Tris buffer. Four lanes corresponding to Maxam–Gilbert sequences are indicated. The lanes marked as control 1 and piperidine are for the 35mer alone and with piperidine. The lane marked +Cr(V) resresents reaction of the 35mer with compound **I**. Lanes marked +Cr(V) +EDTA and +Cr(V) +piperidine were subjected to EDTA and piperidine treatment at the end of the Cr(V)–DNA reaction.

oligo probe, due perhaps to coordination of nucleotides with the tripositive chromium center, slowing migration towards the anode due to the change in charge or structure. Upon treatment with EDTA several distinct bands appear, corresponding to cleavage at G and to a lesser extent at A, C and T bases. After treating with piperidine these bands were further intensified. Intense bands were observed corresponding to G9, G10, G11, G14, G15, G27 and G28 (counted from the 3′-labeled-end). Weaker bands due to cleavage at other bases were also distinctly visible. Cleavage of pUC19 DNA by Cr(V) was monitored on an agarose gel. The gel revealed a slower/relaxed moving band as compared with the original supercoiled plasmid, indicative of nicked DNA. The intensity of the nicked DNA band was increased as a function of Cr(V) concentration (data not shown). No other cleavage was observed for this plasmid DNA up to 5 mM Cr(V).

Figure 2 shows a ^{31}P NMR spectrum of the reaction mixture containing **I** (1.0 mM) and ctDNA (10 mM) at pH 7.6 in Bis–Tris buffer (20 mM). The spectrum was recorded at the end of the reaction (2 h) and after adding 30 mM EDTA. This sequestering agent binds Cr(III) relatively quickly ($t_{½}$ ~ 5 min) and releases phosphate species which are coordinated to the metal center. The inset in Figure 2 displays the spectrum of DNA alone under identical

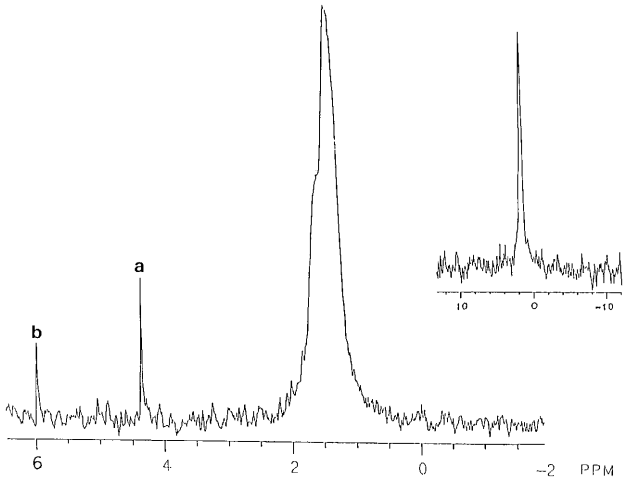


Figure 2. ^{31}P NMR spectrum acquired after reaction between Cr(V) (2.0 mM) and DNA (10 mM) at pH 7.0. Disodium EDTA was added to the mixture before recording the spectra. The peaks at 4.34 and 5.97 p.p.m. are for 3′-dGMP and inorganic phosphate. The peak at 1.47 p.p.m. is for the ss-ctDNA. The inset displays the ^{31}P NMR spectrum for ss-ctDNA alone under identical experimental condition.

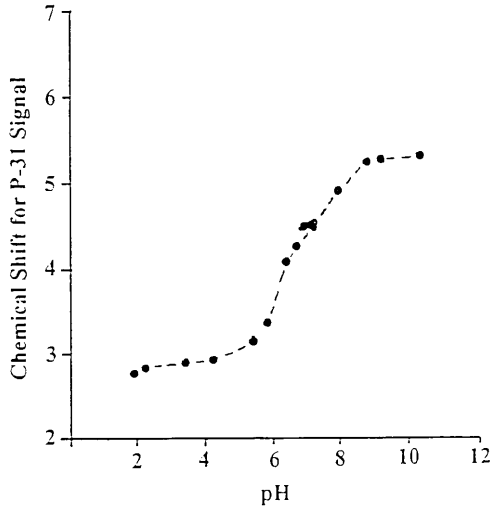


Figure 3. Variation in ^{31}P chemical shifts with pH for peak b in Figure 2. This chemical shift–pH profile matches that of 3′-dGMP but not with other nucleotides, including 5′-analogs.

conditions. Two distinct peaks, at 4.34 (a) and 5.97 p.p.m. (b), down field from the DNA resonance (1.48 p.p.m.) appear in the spectrum. Usually orthophosphate and nucleoside 5′- and 3′-phosphates appear in this chemical shift region. Both inorganic and nucleoside phosphates exhibit significant changes in chemical shift as a function of pH. The pH–chemical shift profile for peak b is shown in Figure 3. This profile matches that observed for deoxyguanosine 3′-monophosphate. In addition to two peaks (a and b), several shoulders also appeared in the signal for DNA which were absent in pure DNA. These shoulders may indicate fragmented oligonucleotides generated from cleavage of ctDNA. This fragmentation, leading to generation of several oligonucleotides, was also observed in the HPLC experiments discussed below.

HPLC experiments were performed to detect and identify small molecules produced in the oxidation of DNA. In some experiments

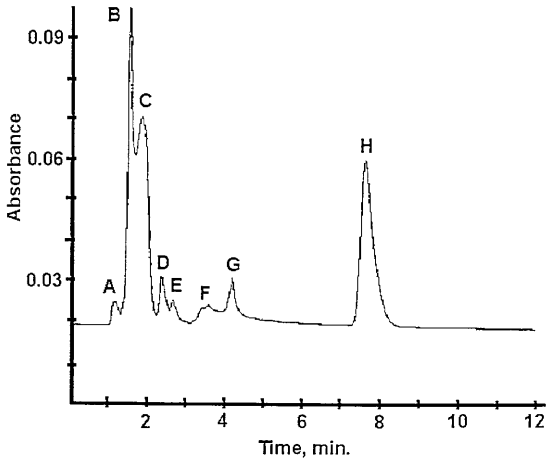


Figure 4. HPLC chromatogram of the Cr(V)/DNA reaction mixture after acid precipitation recorded at 260 nm using a μ-bond C-18 reversed phase column exhibiting DNA cleavage products. These products were formed by reaction between compound **II** (1.0 mM) and ss-ctDNA (5.0 mM) in Bis–Tris buffer (30 mM) at pH 3.0. The peaks resresent: A, Cr(III); B, cytosine plus chromate; C, guanine; D, thymine; E and F, unknown products; G, adenine; H, 5-MF. The mobile phase and flow rate were 50 mM ammonium formate, pH 7.0, in 10% acetonitrile and 1 ml/min.

unreacted DNA and larger oligonucleotides were removed by precipitation with trifluroacetic acid or perchloric acid. Figure 4 shows an HPLC chromatogram of acid-soluble oxidized DNA products. The peaks are labeled A–H. The peak width of H changes markedly over time. For example, chromatograms recorded after several days reaction exhibited a much wider peak. The spectrum of each of the fragments was also recorded. The relative intensities of these peaks varied with the nature of the DNA and with time. However, the number of peaks and their retention times were reproducible for a given column with a given flow rate. The best resolution was achieved using 0.05 M ammonium formate in 80% water and 10% acetonitrile (v/v) at pH 7.0. These retention times were compared with the bases (A, C, G and T), Cr(III)–EDTA, CrO_4^{2-} and some expected oxidized products of deoxyribose, e.g. 5-MF, furfural and base propenal.

HPLC chromatograms were also recorded without acid precipitation using methanol/acetonitrile/water mixtures in a gradient experiment. One such chromatogram is displayed in Figure 5. These gradient separations, utilizing methanol as a component of the mobile phase, were necessary to elute oligonucleotides, as well as small molecules. As expected, unreacted DNA was retained in the column for a longer time and was eluted as a group of peaks centered at ~30 min. In the time domain 1–7 min chromatograms of the reaction mixture displayed peaks similar to those observed with acid precipitation except that they were not well resolved. Some peaks overlap. Furthermore, several other peaks were observed, due to formation of oligonucleotides resulting from cleavage in the time domain 18–25 min.

Some of the fractions from the chromatograms were collected and subjected to proton and ^{31}P NMR measurements. Unfortunately, for the proton NMR spectra the aromatic region was partly obscured by the broad ammonium formate peaks. No signals were observed in the ^{31}P and proton NMR spectra of fraction A. Based on the matching retention time of the Cr(III)–EDTA complex and the absence of NMR signals, peak A is assigned to the Cr(III)–EDTA complex. Fraction D exhibited a singlet at 1.2 p.p.m.

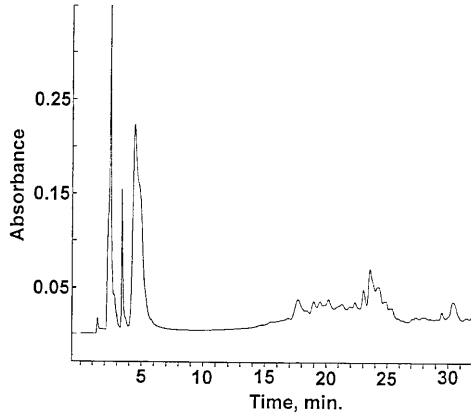


Figure 5. HPLC chromatogram of the Cr(V) (1.0 mM)/ss-ctDNA (5.0 mM) reaction mixture recorded at 260 nm using a μ-bonded C-18 reversed phase column without acid precipitation. A linear gradient of 0–100% 10% acetonitle in water containing 70 mM ammonium formate, pH 6.8, and methanol as the mobile phase over 40 min was used. Peaks appearing up to 7 min are due to released bases and oxidized products which are not resolved. Peaks between 17 and 24 min are due to formation of oligonucleotides. Unreacted ctDNA appeared after 24 min.

in the proton NMR spectrum. The retention time of this fraction matched that of thymine and therefore peak D is assigned to thymine. No other fractions exhibit any signals below 4 p.p.m. The retention times of peaks B, C and G match those of cytosine, guanine and adenine. The retention time of CrO_4^{2-}, a product formed due to the parallel disproportionation reaction of Cr(V), also matched that of cytosine. The percent contribution of Cr(VI) in peak B was calculated based on independent chromatographic detection at 372 nm, the absorption maximum for Cr(VI). Note that cytosine does not absorb at this wavelength. Taking the chromatographic peak areas for the four bases and adjusting for the differences in extinction coefficients, we determine that 15% A, 20% C, 50% G and 15% T were released during DNA oxidation.

Two smaller peaks, E and F, remained unidentified. However, the retention times for these peaks are close to those observed for base propenals. Reactions with guanine, guanosine and guanine 5′-monophosphate were also carried out. No oxidation products were observed in the HPLC chromatograms. In particular, we looked for formation of 8-hydroxyguanine, an expected base oxidation product of guanine. The chromatograms of these reactions exhibited three peaks for Cr(III), Cr(VI) and unreacted substrate. The chromium species were generated through disproportionation. The latter reaction was greatly accelerated by 5′-GMP. This enhancement was also observed with ortho- and pyrophosphate ions, discussed below.

The retention time of peak H matches that of 5-MF. Furthermore, its absorption maximum at 259 nm matches that of the authentic sample. The mass spectrum of fraction H reveals a molecular ion peak at m/z = 96, consistent with formation of 5-MF. The other peaks for 5-MF appear at 68, 54, 50, 42 and 26, due to fragmentation. The peaks can be explained as due to formation of the following ions: $CH_2COCHCH$ (m/z = 68, due to loss of CO), CHCHCO (m/z = 54), CHCCCH (m/z = 50), H_2CCO (m/z = 42) and C_2H_2 (m/z = 26). The retention time for this peak matches that of 5-MF.

The ESR spectrum of the Cr(V)/ctDNA reaction mixture, recorded immediately after mixing, is shown in Figure 6A. The peaks at g = 1.976 and 1.996 are for the parent Cr(V) and

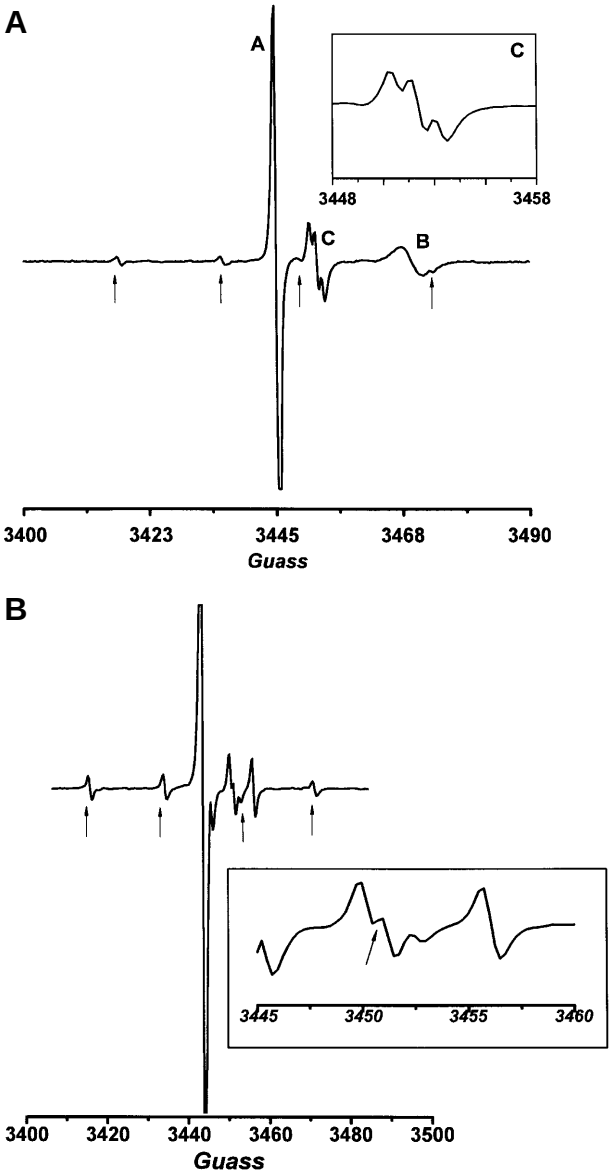


Figure 6. (**A**) ESR spectrum of the reaction mixture described in Figure 2. Peaks A and B are for Cr(V) complexes **I** and **II** and C is for a Cr(V)–DNA complex coordinated through the phosphodiester moiety indicated in Scheme 2. The inset is an expansion of C. The two outer-lines in C are due to hyperfine coupling with ^{31}P nuclei. The center line is due to a Cr(V)–BT–EBA intermediate. The hyperfine lines due to coupling with ^{53}Cr (I = 3/2, 9.8%) are indicated by arrows. (**B**) ESR spectrum of the reaction mixture containing compound I (1.0 mM) and pyrophosphate ion (10 mM) at pH 6.0. The intense signal is for compound **I** and the three lines appearing between 3445 and 3460 G are due to formation of a Cr(V)–pyrophosphate complex. The inset is an expansion of the region exhibiting signals for the Cr(V)–pyrophosphate complex. Hyperfine lines due to coupling with ^{53}Cr for compound **I** are indicated by arrows.

Cr(V)–BT complexes. An intermediate formed during the reaction was detected as a pair of signals centered at g = 1.974. The two peaks show equal intensity with a separation of 3.2 G. In order to address the origin of these peaks, a reaction between Cr(V) complex **I** and sodium pyrophosphate was carried out. This reaction afforded a Cr(V)–pyrophosphate complex which rapidly disproportionates to Cr(VI) and Cr(III). This pyrophosphate complex exhibits three lines in the ESR signals with a hyperfine coupling constant of 3.4 G, which appear at g = 1.974 (Fig. 6B).

The *g* value of this pyrophosphate complex is identical to that observed for a Cr(V)–DNA intermediate. Attempts to detect ESR signals for a Cr(V)–orthophosphate complex were also made. The reaction between orthophosphate and Cr(V) quickly leads to disproportionation products and no ESR signals were detected within 1.5 min of mixing.

Chromium(V) compounds **I** and **II** undergo disproportionation reactions to form Cr(VI) and Cr(III). **I** completely decomposes within 5 min at neutral pH using 1.0–2.0 mM solutions, whereas **II** undergoes slow decomposition, for which the estimated half-life lies between 45 and 90 min in 30 mM Bis–Tris buffer. The extent of Cr(V) decomposition through disproportionation during DNA oxidation was monitored by measuring the amount of Cr(VI) produced. The amount of Cr(VI) thus generated largely depends on the nature of the DNA and the Cr(V) concentration employed. For double-stranded DNA reactions >95% Cr(V) (both **I** and **II**) suffered disproportionation, whereas for the ss-DNA counterpart such decomposition lay between 30 and 75%.

DISCUSSION

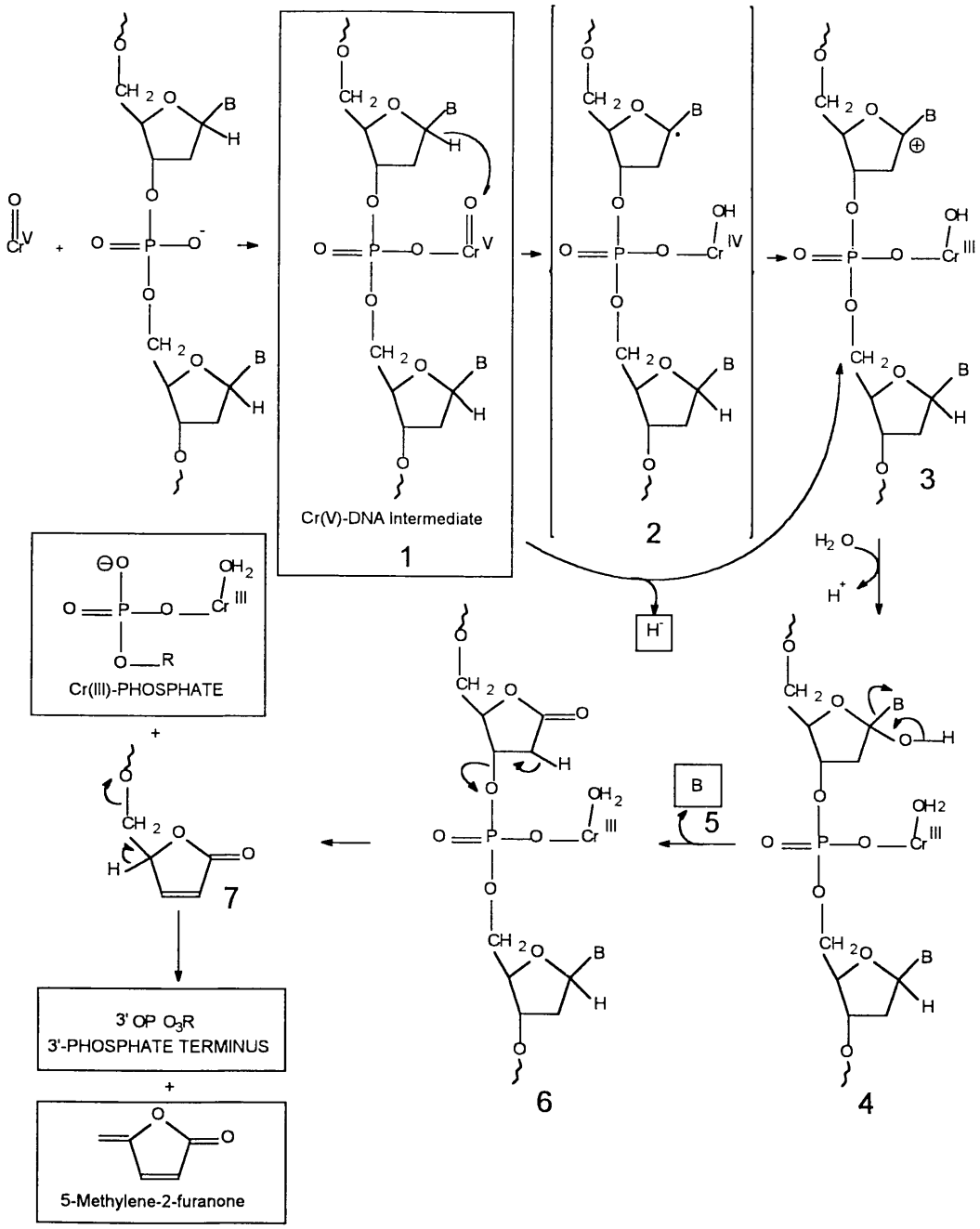
Oxochromium(V) complexes efficiently cleave ss-DNA predominantly through oxidation of deoxyribose, since 5-MF, an oxidation product of the sugar, was formed in this reaction (8–10,28–38). Oxidation of DNA is accompanied by release of the four bases. Formation of orthophosphate and guanosine 3′-monophosphate were also evident, based on the ^{31}P NMR characterization. Furthermore, several oligonucleotides formed by cleavage of highly polymerized ctDNA were detected in the HPLC chromatograms. The Cr(V) species was reduced to Cr(III). Some Cr(VI) was also detected in the products. This hexavalent chromium compound was formed through a parallel disproportionation reaction. The exact ligand environment of Cr(III) is not clear. However, the presence of orthophosphate in the ^{31}P NMR spectrum was detected only after treating with EDTA, implying that inorganic phosphate was initially coordinated to the tripositive chromium center.

ESR spectroscopy helped us to detect and characterize a Cr(V) intermediate in this reaction. The intermediate exhibits ESR signals at g = 1.974, comparable with that of a chromium(V)–pyrophosphate complex (g = 1.974). Furthermore, the hyperfine coupling constant for this intermediate is very close to that of the pyrophosphate complex. Splitting of the signals resulted from coupling between the Cr(V) electron spin and 100% ^{31}P nuclear spin (**I** = 1/2). The g value, hyperfine coupling constant and splitting pattern of the signals indicate that the intermediate is a phosphate-bound Cr(V) species.

A mechanism based on characterization of the intermediate and products is shown in Scheme 2. In this mechanism a Cr(V)–phosphate intermediate is proposed. This is formed by co-ordination between Cr(V) and the phosphodiester backbone of the DNA. This activated phosphate complex is perhaps a better oxidizing agent than the parent compound. Hydrogen abstraction from the C1′ position of the ribose to the oxochromium(V) center would lead to formation of 5-MF, as observed in our reactions. This hydrogen abstraction by the oxocenter followed by an electron transfer step generates a carbocation at the C1′ position and a chromium(III) species. Attack by a water molecule at this carbocation followed by two β-elimination reactions would result in formation of 5-MF, 3′-phosphate and a Cr(III)–orthophosphate complex. However, our data cannot eliminate the possibility of hydride transfer, which would give rise to the same products. The latter reaction followed by the same hydration and β-elimination steps would afford identical products. Examples of hydride transfer reactions for oxochromium(V) and (IV) complexes have been reported (39–41).

The possibility of hydride transfer as an alternative mechanism needs to be addressed. Hydride transfer reactions involving Cr(V)/Cr(IV) complexes in acidic solutions are documented in the oxidation of a series of alcohols by Espenson and co-workers (39,40) and others (41). In order to understand the importance of hydride transfer over hydrogen abstraction the reduction potential related to 2e transactions, i.e. the Cr(V)/Cr(III) couple, should be compared with the Cr(V)/Cr(IV) potential. Hydride transfer would produce Cr(III) products directly without intervention by the intermediate oxidation state Cr(IV), whereas the latter mechanism would involve Cr(IV) following a sequential 1e transfer. The hydride transfer pathway is also shown in Scheme 2. A comparison between hydride and hydrogen transfer must be made at physiological pH, since we are performing DNA cleavage at this pH. We have recently measured the reduction potentials of the Cr(V)/Cr(IV) and Cr(V)/Cr(III) couples of compound **I** (42). The Cr(V)/(IV) couple ($E°$ = 0.44 V versus NHE) did not show any pH dependency above pH 3.4 due to retention of the oxo configuration in both oxidation states. However, since the Cr(III) compound is predominantly $Cr(EBA)_2(H_2O)_2$, the potential for the Cr(IV)/(III) couple varied above that pH. The estimated Cr(V)/(IV) potential at pH 7.0 lies between 0.44 and 0.65 V. The lower estimate is based on retention of the Cr(III) configuration, as observed at pH 4.0, while the upper limit is based on formation of a hydroxochromium(III) species, $Cr(EBA)_2(OH)(H_2O)$. Since formation of a hydroxochromium(III) complex is not unreasonable, a Cr(V)/(III) path may be more favorable. The above analysis assumes that the redox potentials for the phosphate-coordinated Cr(V) complex will follow a similar trend to that observed for compound **I**.

We have also considered pathways of DNA damage including oxidation by hydrogen abstraction from other carbon centers in the ribose moiety and by base oxidation, in particular oxidation of guanine to produce 8-hydroxyguanine. First, we did not observe 8-hydroxyguanine formation. Generally 8-hydroxyguanine is observed in reactions between the base and hydrogen peroxide in the presence of intense UV radiation (43,44). The extent of 8-hydroxyguanine formation correlated with the concentration of hydroxyl radical. In fact, formation of 8-hydroxyguanine is found to be minimal in Bis–Tris buffer, since BT works as a radical scavenger. The possibility of hydroxyl radical formation can be ruled out in our system since the oxidation products were observed in the absence of oxygen in a N_2 atmosphere. However, direct guanine oxidation by the oxochromate species to produce 8-hydroxy- or 8-oxyguanine without involving the hydroxyl radical cannot be completely ruled out. Formation of these base oxidation products does not necessarily release the modified base and therefore might escape our HPLC detection. Furthermore, we cannot rule out an oxidation mechanism based on hydrogen abstraction from C4′, since two HPLC peaks, E and F, appear to be due to base propenal. Oxidation initiated through C4′ cannot be the predominant mechanism of DNA damage, because of the very small amount of propenal formed. This small amount of propenal formed is consistent with a mechanism of oxidation of the thymine mononucleotides TDP and TTP by Cr(IV), as proposed by Wetterhahn and co-workers (45).



Scheme 2. Proposed mechanism of DNA cleavage by Cr(V) complexes. The species in the boxes have been identified. Ligands to Cr(V) have been omitted for simplicity. The species within the braces is putative intermediate in the hydrogen abstraction pathway.

Formation of orthophosphate and 3′-phosphate can also be related to metal-assisted hydrolysis reactions (46–49). Coordination through the phosphodiester bond followed by transfer of the coordinated hydroxide or water from chromium(V) to phosphorus could give rise to such hydrolysis. However, this hydrolysis is not a redox reaction and therefore no oxidation products would be expected to form. We conclude that formation of phosphate moieties by metal-promoted hydrolysis must be minimal.

The postulated mechanism does not address the base or sequence specificity of DNA oxidation. However, autoradiograms of polyacrylamide gels indicate cleavage at all bases, with some preference at G (65% compared with the statistically expected 25%) over other bases. Likewise, HPLC chromatograms revealed release of all four bases, however, the relative concentration of G was double that expected based on random non-preferential oxidation. Cleavage at all bases, including G, was further intensified by treatment with piperidine. This enhancement of cleavage at G sites reminds us of similarities between our system and those observed by Thorp and co-workers (8–10) for oxoruthenium(IV)-mediated DNA damage. The existence of

base labile sites in polyacrylamide gels is usually taken to be an indication of base oxidation. In order to understand the relative contributions from sugar versus base oxidation processes to DNA damage, 8-oxyguanine and 8-hydroxyguanine must be measured independently. Unfortunately, these oxidized guanine products readily decompose in piperidine (50). Piperidine also accelerates decomposition of the α,β-unsaturated lactone (8–10,28–30), an intermediate formed by hydrogen abstraction at the C1′ position, followed by the first β-elimination reaction. Currently we are using a variety of enzymatic digestion methods to assess the relative contribution of guanine base oxidation (Moghaddas and Bose, work in progress).

HPLC data, as well as electrophoretograms, should provide quantitative information related to preferential cleavage of G bases over other sites. However, quantitative information from HPLC separation initiated by acid precipitation should be treated with caution. This difficulty is due to differences in the solubilities of DNA bases. The solubility of guanine appears to be less than other bases, yet the intensity of the guanine peak appears to be stronger than the others. Due to its limited solubility, some G may have been precipitated. The densitometry scans revealed that 65 ± 5% G cleavage occurred compared with all other bases. A relatively lower yield of G in HPLC compared with PAGE may be related to the solubility problems addressed above, additional cleavage at G sites due to base oxidation and differences in structural and/or base sequences in the two types of DNA, 35mer and ss-DNA.

The extent of single- versus double-stranded DNA damage needs to be addressed. Both single-stranded oligonucleotides, ss-DNA and 35mer, suffered extensive damage, whereas the double-stranded DNA was only nicked. In the latter reactions quantitative determination of Cr(VI) revealed that Cr(V) underwent disproportionation almost exclusively. The above observations are consistent with the proposal that once Cr(V) is bound to phosphate, which points towards the outside of the grooves of ds-DNA, H1′ could be inaccessible for abstraction by the oxo center. Due to these unfavorable steric constraints on oxidation, the fate of the phosphate-bound Cr(V)–ds-DNA complexes is perhaps largely controlled by parallel disproportionation.

The DNA oxidation observed here should be compared with the recently reported oxidation of thymine mononucleotides, thymine 5′-diphosphate and -triphosphate (TDP and TTP), by compound **I** by Wetterhahn and co-workers (45). These reactions almost exclusively afforded disproportionation products of Cr(V), i.e. Cr(VI) and Cr(III). However, formation of small amounts of thymine propenal and 2-deoxy-D-pentitol [<0.5% of the Cr(V) employed] was observed. The reactive metal center was thought to be a Cr(IV) species which was generated as an intermediate during disproportionation of Cr(V). The amount of thymine propenal generated in these reactions was dependent on the amount of oxygen in the reaction mixture. Formation of the propenal was attributed to hydrogen abstraction from the C4′ position followed by attack by O_2 at this site, a mechanism similar to DNA oxidation by metal-activated bleomycin (1–6,51–53). The products observed in our study were the same in N_2. However, we cannot exclude formation of a small amount (<1%) of propenal and other oxidation products due to oxidation of DNA through other sites, since these low concentrations would escape detection. In fact, we noted two small peaks in our chromatograms (Fig. 4, peaks E and F) which may correspond to base propenals. Our reactions did not generate detectable amounts of 8-hydoxy-2′-deoxyguanosine, a product observed by Shi *et al.* (54) for oxidation of guanine by a reaction mixture containing Cr(VI), ascorbic acid and hydrogen peroxide. This reaction (54) mixture generated hydroxyl radicals along with Cr(IV) and Cr(V) species. However, guanine oxidation to form oxy- or hydroxyguanine cannot be eliminated, as discussed earlier.

A comparison of DNA oxidation by three oxometallates, Cr(V), Mn(V)–prophyrins and Ru(IV)–bipyridyl complexes, is in order. The manganese center preferentially oxidizes A-T over G-C base pairs; reactivity toward the former is two orders of magnitude greater than for the latter (52). Oxidation proceeds mainly through hydrogen abstraction at the C5′ position. The higher reactivity toward the pyrimidine bases is attributed to stronger binding to the minor groove of A-T-rich regions (10^5–10^7, compared with 10^3/M for G-C base pairs) of the nucleic acid (31,32,56) prior to oxidation. This preferential binding is a reflection of a greater electrostatic attraction between the positively charged porphyrin complex and the high negative surface potential of the minor groove (57,58). On the other hand, a greater susceptibility to oxidation of DNA at G bases by ruthenium and osmium oxo centers has been documented (8–10). Like the porphyrin complex, these complexes are positively charged. However, oxidation is initiated predominantly by hydrogen abstraction at the C1′ site rather than at C5′ (8–10). This is attributed to enhanced basicity at C1′ over the other sites in the ribose. Recently Hecht and co-workers (59) have demonstrated that a Co(II)–bithiazole complex, through activation by molecular oxygen, cleaves oligonucleotides at G sites. Based on ESR, UV-vis and competition experiments with a radical scavenger, these authors concluded that DNA cleavage is initiated by an activated binuclear cobalt complex rather than isolated hydroxyl or peroxy radicals. The oxidation products were not characterized in this reaction.

DNA damage by Cr(V) should be discussed in the context of the carcinogenic and mutagenic properties of Cr(VI) (12–18). The former hypervalent oxidation state is observed as an intermediate in redox reactions between Cr(VI) and several biological reducing agents and is believed to be a putative DNA damaging agent. Attempts have been made to assess DNA damage by this metastable species in reactions containing Cr(VI) and glutathione or ascorbic acid in the presence or absence of hydrogen peroxide (16,60,61). These reactions generated hydroxyl and other organic radicals in addition to Cr(V). Therefore, an unambiguous assessment of the extent of DNA damage by Cr(V) alone is difficult. Furthermore, these reports did not address the products formed by oxidation and therefore mechanistic information was not available.

The results presented here unambiguously establish that oxochromium(V) species damage DNA predominantly through oxidation of ribose. Furthermore, oxidation proceeds through a Cr(V)–DNA intermediate in which chromium(V) is linked to the phosphodiester moiety. The requirement for phosphate coordination for DNA oxidation is unique and unprecedented. Oxidation appears to be somewhat selective for guanine bases. However, it remains to be seen whether changes in the ligand environment and charge of the complex play any role in base selectivity and oxidation sites in the DNA. Finally, our data cannot address the issue of sequence specificity, if any, in oxidation. Experiments with a variety of sequences are in progress to resolve this issue.

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This paper is dedicated to the memory of Professor Karen Wetterhahn, who made outstanding contributions in the field of chromium-induced carcinogenesis.

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