

# Unusual reactivity in a commercial chromium supplement compared to baseline DNA cleavage with synthetic chromium complexes

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## Abstract

Commercially available chromium supplements were tested for their DNA cleavage ability compared with synthetic chromium(III) complexes, including chromium(III) *tris*-picolinate [Cr(pic)<sub>3</sub>], basic chromium acetate [Cr<sub>3</sub>O(OAc)<sub>6</sub>]<sup>+</sup>, model complexes, and recently patented Cr-complexes for use in supplements or therapy. Four different supplements (**P1–P4**) were tested for their DNA cleaving activity in the presence and the absence of H<sub>2</sub>O<sub>2</sub>, dithiothreitol (DTT) or ascorbate. One supplement, **P1**, showed nicking of DNA in the *absence* of oxidant or reductant at 120 μM metal concentration. Different lot numbers of **P1** were also tested for DNA cleavage activity with similar results. Commercial supplements containing Cr(pic)<sub>3</sub> nicked DNA at ≥ 120 μM metal concentrations in the presence of 5 mM ascorbate or with excess hydrogen peroxide, analogous to reactions with synthetic Cr(pic)<sub>3</sub> reported elsewhere. Another chromium (non-Cr(pic)<sub>3</sub>) supplement, **P2**, behaves in a comparable manner to simple Cr(III) salts in the DNA nicking assay. Chromium(III) malonate [Cr(mal)<sub>2</sub>] and chromium(III) acetate [Cr(OAc)] can nick DNA in the presence of ascorbate or hydrogen peroxide, respectively, only at higher metal concentrations. The Cr(III) complexes of histidine, succinate or *N*-acetyl-L-glutamate do not nick DNA to a significant degree.

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## 1. Introduction

DNA cleavage assays in the presence of various metals are a simple, rapid and sensitive probe of metal mediated processes, such as hydrolysis or metal–DNA redox reactions, that may have biological significance [1,2]. These assays may use plasmid, single-stranded DNA or double-stranded DNA fragments or other nucleic acids to determine initial modes of reactivity between

metal ions and these biological materials. Plasmid DNA cleavage reactions giving rise to uncut (supercoiled), nicked (relaxed supercoil) or cut (linear) forms of the plasmid may be used for concentration and time dependent assays of redox, hydrolytic or other reactivity with the nucleic acids.

While much is known about the toxicology of chromium (especially the oxidation states 4, 5, and 6) [3–6], literature on the necessity of Cr(III) as an essential trace element and its biochemical speciation, even with extensive reviews [7–11], is not definitive [11–13]. There are good examples of work exploring amino acid–Cr(III) chemistry [14–20], especially redox processes related to biological systems [21], but a definitive answer as to

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the nature of a Cr(III) “glucose tolerance factor”, the biological form of chromium(III) and its mechanism of action has not been determined [11,22,23].

The work of John Vincent and coworkers in characterizing a “low molecular weight” chromium binding peptide (LMWCr) has sought to clarify the role and mechanism of chromium usage in the body [23]; this complex may turn out to be the elusive “glucose tolerance factor”. LMWCr has recently been shown to possibly play a role in the insulin-dependent glucose uptake pathway. The apparent action of the chromium complex is to stimulate and prolong glucose metabolism inside insulin sensitive cells after external binding of insulin. The role of Cr(III) and of LMWCr are of interest to researchers in the areas of adult-onset diabetes, carbohydrate and lipid metabolism, toxicology, and bioinorganic chemistry.

Chromium(III) picolinate ( $\text{Cr}(\text{pic})_3$ ) (Fig. 1) has some advantage over simple chromium salts in dietary uptake [11] and has become a popular nutritional supplement over the past decade.  $\text{Cr}(\text{pic})_3$  is available over the counter in the form of tablets, sports drinks, etc., however, there is concern regarding deleterious effects of  $\text{Cr}(\text{pic})_3$  in the recent literature [2,24,25]. In addition to supplements for human consumption, chromium supplementa-

tion of animal feeds is practiced [26], and there are recent patents for the use of other Cr(III) complexes as supplements of both types [27,28].

Here, we are using DNA reactivity as a sensitive probe of interaction of Cr(III) complexes with DNA. The present study focuses on the testing of store brand chromium supplements,  $\text{Cr}(\text{pic})_3$  and various models of the “low molecular weight chromium” substance for their DNA cleavage activity and making general comparisons among the compounds tested. We tested reactivity of four store brands of nutritional chromium supplements with pUC 19 DNA. Also, we repeated previously reported DNA reactions with synthetic  $\text{Cr}(\text{pic})_3$  [2] and with ‘basic’ chromium acetate ( $\text{Cr}(\text{OAc})$ ) (Fig. 1) [29]. The purpose for repeating those reactions is to confirm the expected results in our hands and to compare these with supplements and other Cr(III) compounds. Also completed was the testing of various new model complexes of low molecular weight chromium binding peptide. The models synthesized and tested were *trans*-chromium malonate ( $\text{Cr}(\text{mal})_2$ ), a chromium *bis*-histidine complex, and chromium diacid complexes: chromium succinate and chromium(*N*-acetyl-L-glutamate) (Fig. 1).

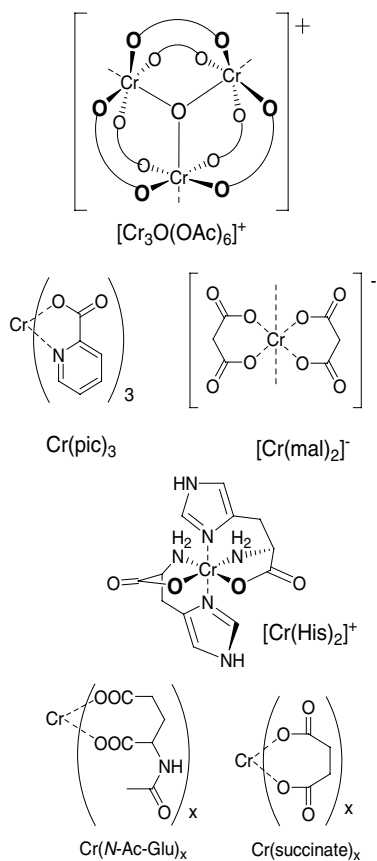


Fig. 1. Cr complexes used in this study. (coordinated water and counter ions omitted for clarity.)

## 2. Materials and methods

### 2.1. Caution

Ethidium bromide is a suspected mutagen. Chromium in the oxidation states, II, IV, V, and VI, are known or potentially hazardous species. Chromium(III) may also present hazard under certain conditions. Care should be taken while handling these materials.

### 2.2. General

Reagents were used as supplied unless otherwise noted. pUC 19 DNA was obtained from New England BioLabs Inc. and used as received. L-Ascorbic acid was obtained from Sigma–Aldrich Chemical, Inc.  $\text{Cr}(\text{pic})_3$  and *trans*-chromium(III) malonate [ $\text{Cr}(\text{mal})_2$ ] were synthesized by known literature procedures [30,31]. Basic chromium(III) acetate was purchased from Strem Chemicals (Product # 93-2401). It should be noted that while the composition and stoichiometry of this product generally corresponds to  $\text{Cr}_3(\text{O-H})_2(\text{O}_2\text{CCH}_3)_7$ , the commercial material may contain impurities (Merck 12:2275) and various forms of ‘chromium acetate’; these may include chromium(III) hexaquo acetate and other multinuclear Cr clusters [32]. Chromium diacid complexes were synthesized by modifying the procedure for the synthesis of chromium propionate [33]; the full characterization of these complexes will be reported elsewhere. *Trans*-(imidaz-

ole)chromium(III)bis-histidine nitrate was synthesized by modification of procedures found in the literature for chromium(III) histidine salts [28,34–36]. A solution of NaOH (0.04 mol) in water (25 mL) was added to L-Histidine (6.2 g, 0.04 mol) and  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  (8 g, 0.02 mol). The solution was covered with reflux tube and gently heated on a steam bath 12 h. The solution was further refluxed and stirred 24 h and air evaporated to reduce the volume. The above solution was filtered and 5.00 g (0.0125 mol, 62.5%) of crude salt was obtained. The crude sample was recrystallized by dissolving in a minimum water and refrigerating for several days to get 1.85 g (0.00462 mol, 23.1%) of the crystalline material. Single-crystal X-ray analysis confirmed the identity of this compound [34].

Chelex 100 resin was obtained from Bio-Rad Laboratories. Phosphate buffer (pH 7) was treated with Chelex 100 resin to remove trace metals. Commercially available chromium supplements were purchased locally and are listed in Table 1 (subscript letters indicate different lot numbers). The percent plasmid relaxation [1] relative to supercoiled DNA was quantified using the UN-SCAN-IT gel software version (Silk Scientific Corp., Orem, UT). Quantified reactions were done in duplicate or triplicate ( $P < 0.05$ , ANOVA). Standard deviations are indicated on figures of quantified gel results. Metal analysis (ICP; EPA 6010B) for **P1** and **P4** (Table S1) were performed at MD Chemical and Test, Inc., Topeka, KS.

### 2.3. Cr supplement extraction procedure

**P1**, **P3** and **P4** are reported to contain 200  $\mu\text{g}$  chromium as chromium picolinate while **P2** contains 200  $\mu\text{g}$  chromium as “GTF chromium yeast”. **P1** and **P4** were in the form of a powder in capsules. The powder was carefully removed from the capsule and dissolved in DNA-grade water (FisherSci) to make solutions at  $1.8 \times 10^{-4}$  M final metal concentration. **P2** and **P3** were in tablet form and dissolved in water in a similar way. All solutions were filtered (syringe, 0.45  $\mu\text{m}$  disc) and the presence of Cr(pic) was confirmed in **P1**, **P3** and **P4** by UV–Vis spectroscopy ( $\lambda_{\text{max}} = 265 \text{ nm}$ ;  $\epsilon = 15,546$ ; Merck 12:2291). The con-

centrations used were based on the assumption that the reported amounts of chromium in the samples was correct. Metals analysis indicated that **P1** and **P4** contained 405 and 420  $\mu\text{g/g}$  chromium, respectively, in good agreement (<10%) with the listed amounts. The filtered solutions were used in the DNA reactions with appropriate dilution.

### 2.4. Gel electrophoresis

Literature methods [1,2] were followed with some modifications. DNA cleavage assays were carried out by mixing aliquots of 39  $\mu\text{M}$  (in base pairs) pUC 19 DNA in phosphate buffer (pH 7.0, the final phosphate buffer concentration was 50 mM), reductant or oxidant in water and chromium complexes were added in water to a final volume of 15  $\mu\text{L}$ . Reactions were allowed to proceed for 2 h. The addition of 5  $\mu\text{L}$  loading buffer was used to quench all reactions (24% glycerol and 0.1% bromophenol blue). Nicking of supercoiled plasmid DNA was observed by gel electrophoresis on 1% agarose gel with  $1 \times \text{TBE}$  (tris/boric acid/EDTA) as running buffer at 140 V for 75 min. The gel was stained by addition of 0.5  $\mu\text{g/ml}$  ethidium bromide for 45 min and de-stained by soaking with DNA-grade water for 30 min. The gel was photographed on a UV transilluminator using Polaroid® 667 film. Timed assay experiments were also performed with the reactions quenched at various times (the addition of DNA to the reaction mixture was set as time<sub>0</sub>) by adding loading buffer.

## 3. Results

Reactions of pUC 19 DNA with chromium(III) complexes were analyzed by gel electrophoresis. The reactions were evaluated by comparing the relative amounts of nicked (relaxed supercoil) or cut (linear) forms of the plasmid to the amount of uncut (supercoiled) plasmid present in control reactions. The reactivity of DNA was compared with commercial chromium supplements,  $\text{Cr}(\text{pic})_3$ , and other synthetic chromium(III) complexes.

Table 1  
Commercially available chromium supplements used in this study

Sample	Name, Cr(III) ingredient	Other ingredients	Lot#
<b>P1<sub>a</sub></b>	Chromium picolinate, $\text{Cr}(\text{pic})_3$	Millet, cellulose, maltodextrin, gelatin(capsule)	225323
<b>P1<sub>b</sub></b>	Chromium picolinate, $\text{Cr}(\text{pic})_3$	Millet, cellulose, maltodextrin, gelatin(capsule)	235200
<b>P1<sub>c</sub></b>	Chromium picolinate, $\text{Cr}(\text{pic})_3$	Millet, cellulose, maltodextrin, gelatin(capsule)	310226
<b>P1<sub>d</sub></b>	Chromium picolinate, $\text{Cr}(\text{pic})_3$	Millet, cellulose, maltodextrin, gelatin(capsule)	330692
<b>P2</b>	GTF chromium, unknown	Cellulose, Stearic acid, silica, magnesium stearate, cellulose, glycerin coating	N2A522B
<b>P3</b>	Chromium picolinate 200, $\text{Cr}(\text{pic})_3$	Dicalcium phosphate, cellulose	4476KC0098
<b>P4</b>	Chromium picolinate, $\text{Cr}(\text{pic})_3$	White rice flour, gelatin(capsule)	520311

### 3.1. Comparison of commercially available supplements with synthetic $\text{Cr}(\text{pic})_3$ and $\text{Cr}(\text{OAc})$

The commercial Cr supplement, **P1<sub>a</sub>**, can cleave DNA at 120  $\mu\text{M}$  concentrations alone in the absence of oxidant/reductant in 1 and 2 h reactions (Fig. 2, lanes 3–6 top). Supplement **P2**, which is a non- $\text{Cr}(\text{pic})_3$  product, showed some nicking at 120  $\mu\text{M}$  concentration (Fig. 2, lane 10 top). Other store brands containing  $\text{Cr}(\text{pic})_3$  did not show much reactivity alone or with added oxidant (Fig. 2, lanes 11–18 top; Figures S1a, S2). A timed assay was also carried out for **P1<sub>a</sub>** at 120  $\mu\text{M}$  metal concentration without an oxidant. Nicking was observed even at 0 min (Figure S1b, lane 3), however this may be due to inadequate quenching of the individual reactions. Different lot numbers of **P1** (**P1<sub>b</sub>**, **P1<sub>c</sub>**, **P1<sub>d</sub>**) were then tested with DNA to confirm the unusual behavior of **P1<sub>a</sub>** (Fig. 2, bottom). Each lot tested exhibited similar reactivity to the original supplement. Supplement **P1** was tested with hydrogen peroxide, ascorbate and *D,L*-1,4-dithiothreitol (DTT) also. There is little change in nicking of DNA in the presence of oxidant or reductant when compared to reactions without these added reagents (Figure S1c and d).

To assess the extraordinary reactivity of the **P1** sample, a number of experiments were conducted in an effort to determine the reactive species present in the supplement. An EPR (electron paramagnetic resonance) measurement of **P1** indicated no organic radicals present, and iodometry indicated no oxidizers were present (data not shown). Trace metals analysis (Table S1) indicated that Fenton-type chemistry may not give rise to the observed activity in **P1**. Namely, trace copper was not detected, and trace iron (22.9 and 12.1 ppm in **P1** and **P4**, respectively) appears to be insignificant in comparison to the observed reactivity. In addition, the concentrations of mono- and divalent cations in the samples appear to be similar, and may rule out a metal-hydrolysis mediated reactivity of supplement **P1**. Finally, titration against potassium permanganate indicated that extra reducing equivalents may be present in **P1** when compared to **P4** or similar masses of rice flour

(Figure S3), but an exact species is unknown and composition of the other ingredients in **P1** differs from **P4**.

### 3.2. Comparison to synthetic $\text{Cr}(\text{pic})_3$

Noting the disparity in results with the samples above, published reactions [2] of  $[\text{Cr}(\text{pic})_3]$  were repeated with DNA at concentrations 0.12–120  $\mu\text{M}$  for comparison purposes. Additionally, these reactions were carried out at higher concentrations (3 mM and saturated solutions) with  $\text{H}_2\text{O}_2$ , DTT and ascorbate. DNA was nicked at 120  $\mu\text{M}$  and 3 mM  $[\text{Cr}(\text{pic})_3]$  in the presence of 5 mM ascorbate. At saturated concentrations,  $[\text{Cr}(\text{pic})_3]$  cuts the DNA to form linear species (which migrates in between intact and nicked DNA). The reaction of DNA with  $\text{H}_2\text{O}_2$  nicked DNA only at the highest concentration of  $[\text{Cr}(\text{pic})_3]$ . As expected, DNA cleavage reactions with  $\text{Cr}(\text{pic})_3$  increase with concentration and time. Also, DTT showed cleavage of DNA with  $[\text{Cr}(\text{pic})_3]$  at concentrations  $\geq 3$  mM. These results are consistent with published work [2] except at the 120  $\mu\text{M}$  concentration, where nicking was not observed with added hydrogen peroxide or DTT (Figure S4).

Basic chromium acetate,  $[\text{Cr}(\text{OAc})]$ , requires higher concentrations (1–9 mM) to nick pUC 19 DNA with  $\text{H}_2\text{O}_2$  [29]. Also, timed assays of DNA with 9 mM  $[\text{Cr}(\text{OAc})]$  showed increasing amount of nicked pUC 19 with increasing time from 1 to 60 min (Figure S5).  $[\text{Cr}(\text{OAc})]$  did not show any nicking with ascorbate and DTT at these concentrations.

### 3.3. Models for low molecular weight chromium

Potassium *trans*-chromium(III) malonate  $[\text{Cr}(\text{mal})_2]$  did not show nicking of DNA with  $\text{H}_2\text{O}_2$  at 0.12, 1.2 and 12  $\mu\text{M}$  concentrations. Nicking was observed upon increasing metal concentration to  $\geq 120$   $\mu\text{M}$ . Further, increasing the concentration to 9.0 mM led to significant increases in nicking that may be seen in concentration dependent manner (Fig. 3, lanes 16–19).  $\text{Cr}(\text{mal})_2$  at 3, 6 and 9 mM concentrations in the presence of ascorbate nicked the plasmid DNA extensively (Fig. 3, lanes 7–9).

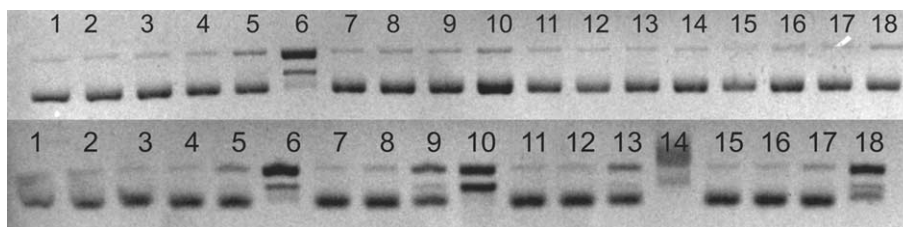


Fig. 2. Unusual reactivity in commercial chromium supplement, **P1**, without added oxidant or reductant (top). DNA (lane 1); DNA + 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (lane 2); DNA + 0.12, 1.2, 12, 120  $\mu\text{M}$  **P1**, **P2**, **P3**, **P4** (lanes 3–6, 7–10, 11–14, 15–18, respectively). Comparison of different lot numbers of **P1** in DNA cleavage assay (bottom). pUC19 DNA (lane 1); DNA + 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (lane 2); DNA + 120  $\mu\text{M}$  **P1<sub>a</sub>**, **P1<sub>b</sub>**, **P1<sub>c</sub>**, and **P1<sub>d</sub>** (lanes 3–6, 7–10, 11–14, 15–18, respectively).

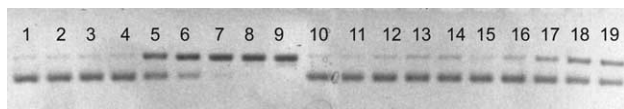


Fig. 3. Cleavage assay with  $\text{Cr}(\text{mal})_2$ . DNA + 1, 3, 6, 9 mM  $\text{Cr}(\text{mal})_2$  (lanes 1–4); 5 mM ascorbate (lane 5); 5 mM ascorbate + 1, 3, 6, 9 mM  $\text{Cr}(\text{mal})_2$  (lanes 6–9); 5 mM DTT (lane 10); 5 mM DTT + 1, 3, 6, 9 mM  $\text{Cr}(\text{mal})_2$  (lanes 11–14); 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (lane 15); 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$  + 1, 3, 6, 9 mM  $\text{Cr}(\text{mal})_2$  (lanes 16–19).

DTT was found to marginally increase nicking with  $\text{Cr}(\text{mal})_2$  (Fig. 3, lanes 11–14). The nicking cannot be seen in control reactions with DNA and  $\text{H}_2\text{O}_2$  or with  $\text{Cr}(\text{mal})_2$  alone. Timed assays were also performed with  $\text{Cr}(\text{mal})_2$  (9 mM) quenched at different times, 1, 5, 10, 30, 45, 60 to 120 min, respectively. The results showed intensified nicking with increasing time (Fig. 4, lanes 2–8).

Although buffers were passed through Chelex-100 to remove trace metals, nicking can be seen in the control reaction with ascorbate (Fig. 3, lane 5). Since plasmid DNA is slowly nicked the presence of ascorbate and atmospheric oxygen [37–39], timed assays were performed for 5 mM ascorbate with  $\text{Cr}(\text{pic})_3$  or without metal complex; these reactions showed slight nicking after only 5 or 30 min, respectively (Figures S6–S7). All ascorbate reactions were evaluated by comparing

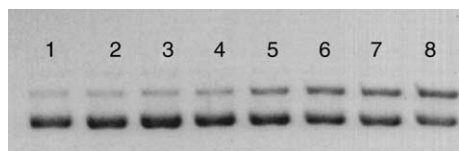


Fig. 4. Timed assay of pUC19 with  $[\text{Cr}(\text{mal})_2]$  in presence of 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . DNA + 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (lane 1); 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$  + 9 mM  $[\text{Cr}(\text{mal})_2]$  at 0, 5, 10, 30, 45, 60, 120 min, respectively (lanes 2–8).

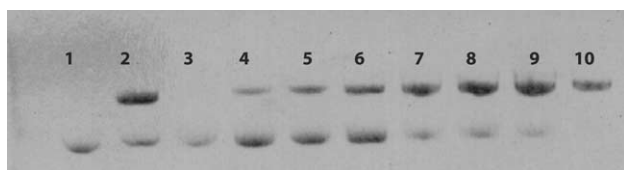


Fig. 5. Timed assay of pUC19 DNA with 3 mM  $[\text{Cr}(\text{mal})_2]$  in the presence of 5 mM ascorbate. DNA (lane 1); 5 mM ascorbate (lane 2); 3 mM  $[\text{Cr}(\text{mal})_2]$  (lane 3); 3 mM  $[\text{Cr}(\text{mal})_2]$  + 5 mM ascorbate for 0, 5, 10, 30, 45, 60, 120 min, respectively (lanes 4–10).

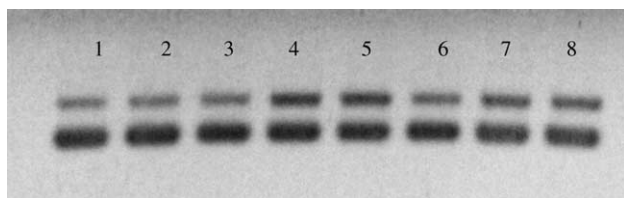


Fig. 6. Cleavage assay with  $\text{Cr}(\text{His})_2$  and ascorbate. DNA + 5 mM Ascorbate (lane 1); 0.12, 1.2, 12, 120  $\mu\text{M}$   $\text{Cr}(\text{His})_2$  (lanes 2–5); 3, 6, 9 mM  $\text{Cr}(\text{His})_2$  (lanes 6–8).

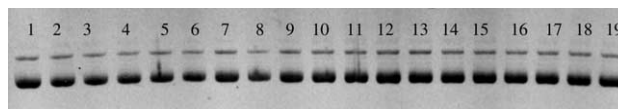


Fig. 7. Cleavage assay with  $\text{Cr}(\text{His})_2$ . DNA (lane 1); 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (lane 2); +120  $\mu\text{M}$   $\text{Cr}(\text{His})_2$  (lane 3); 9 mM  $\text{Cr}(\text{His})_2$  (lane 4); 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$  + 0.12, 1.2, 12, 120  $\mu\text{M}$   $\text{Cr}(\text{His})_2$  (lanes 5–8); 3, 6, 9 mM  $\text{Cr}(\text{His})_2$  (lanes 9–11); 5 mM DTT (lane 12); 5 mM DTT + 0.12, 1.2, 12, 120  $\mu\text{M}$   $\text{Cr}(\text{His})_2$  (lanes 13–16); 3, 6, 9 mM  $\text{Cr}(\text{His})_2$  (lanes 17–19).

the timed assay of ascorbate alone and the Cr complexes. Timed assays in the presence of 5 mM ascorbate were also performed with  $\text{Cr}(\text{mal})_2$  at 3 mM concentration for 2 h (Fig. 5, lanes 4–10).  $[\text{Cr}(\text{mal})_2]$  with 5 mM ascorbate showed increasing nicking with increase in time and complete nicking at 2 h (Fig. 5).

$\text{Cr}(\text{III})$  complexes with diacids,  $\text{Cr}(\text{III})$ (succinate) and  $\text{Cr}(\text{III})$ (*N*-acetyl-L-glutamate), and a  $\text{Cr}(\text{III})$ (histidine)<sub>2</sub> complex were also tested for their reactivity with DNA at metal concentrations of 0.12, 1.2, 12 and 120  $\mu\text{M}$ .  $\text{Cr}(\text{succinate})$  did not show any cleavage with ascorbate and DTT, but a small amount of nicking was observed at 120  $\mu\text{M}$  concentrations with  $\text{H}_2\text{O}_2$  (Figure S8).  $\text{Cr}(\text{N-acetyl-L-glutamate})$  showed no cleavage at any concentration or with any of the oxidants or reductants (Figure S9). Recently, compositions of chromium(III)–histidine complexes have been patented as a chromium supplement [28]. This compound may exhibit lower toxicity than  $\text{Cr}(\text{pic})_3$  and has improved absorption compared to other commercially available supplements [28]. *Trans*-imidazole-chromium(III)-bis-histidinato nitrate  $[\text{Cr}(\text{His})_2]$  was synthesized by the modification of published literature procedures [34–36] and tested for its reactivity with DNA. The chromium–histidine complex was easily soluble in water even at higher concentrations. This complex has little or no reactivity in the presence of oxidants or reductants even at the concentrations as high as 9 mM (Figs. 6 and 7).

#### 4. Discussion

In this project, commercially available Cr supplements were tested for their ability to nick DNA. By coincidence, the first  $\text{Cr}(\text{pic})_3$  containing supplement tested, **P1<sub>a</sub>**, exhibited unusual ability to cleave DNA compared to other manufacturers' chromium supplements containing various forms of Cr. Two other supplements containing  $\text{Cr}(\text{pic})_3$  (**P3**, **P4**) showed lower levels of DNA nicking activity consistent with  $\text{Cr}(\text{pic})_3$  studied by Vincent and coworkers [2]. A third Cr supplement containing another form of Cr (**P2**) showed similar low-level activity. While initial reactions contained no added oxidant or reactant, subsequent reactions with the Cr supplements exhibited similar patterns of reactivity. Namely, **P1** had excessive reactivity and the others,

various low-level activities. Quantified data for reactions with supplement **P1** and **P4** are compared to controls and to reactions with  $\text{Cr}(\text{pic})_3$  in Fig. 8. To test whether sample **P1<sub>a</sub>** was a unique case, different lot numbers of **P1** were tested to determine variability over the same manufacturer's product. This series of experiments yielded the same results. Since subsequent study showed (see below) that Cr nicking activity should not be this substantial *in vitro*, i.e.,  $\text{Cr}(\text{pic})_3$  containing supplements should be similar to synthetic  $\text{Cr}(\text{pic})_3$ , we surmise that extra reducing equivalents are present in **P1** (Fig. 8, S3). The unexpected and significant activity in this supplement may possibly be in the form of Cr(II) or other reducing species, and may arise as a result of some manufacturing process.

For comparison, the ability of synthetic  $\text{Cr}(\text{pic})_3$  to cleave DNA was reexamined for a direct comparison to the activities of the Cr supplements. Repetition of published reactions with synthetic  $\text{Cr}(\text{pic})_3$  confirm this previous work and indicate that commercially available Cr supplements should behave like synthetic  $\text{Cr}(\text{pic})_3$  or other Cr complexes except in unusual cases, (e.g., **P1**). In further comparison, the Cr salts of organic acids,  $\text{Cr}(\text{mal})_2$  and  $\text{Cr}(\text{OAc})$ , nicked DNA in the presence of  $\text{H}_2\text{O}_2$  or ascorbate at higher concentrations. The results

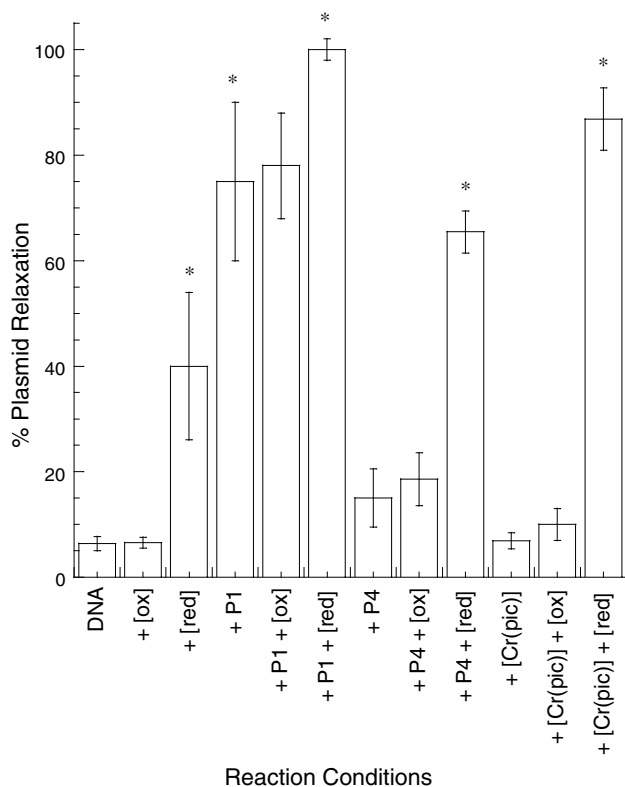


Fig. 8. Quantification of plasmid relaxation relative to super coiled DNA in the presence of commercial  $\text{Cr}(\text{pic})_3$  supplements and synthetic  $\text{Cr}(\text{pic})_3$ . [ox] = 215  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ; [Cr(pic)] = synthetic chromium picolinate; Pn = chromium supplement; [Cr] = 120  $\mu\text{M}$ ; [red] = 5 mM ascorbate.

with  $\text{Cr}(\text{OAc})$  are presumably through the action of the trinuclear Cr cluster (Fig. 1), with the qualification that the commercially obtained composition is probably inhomogeneous [32], and other Cr species may be the active entities in these reactions. Finally, reactions were conducted with  $\text{Cr}(\text{succinate})$  and  $\text{Cr}(\text{N-acetyl-L-glutamate})$ , and the amino acid complex,  $[\text{Cr}(\text{His})_2]$ . The diacid complexes of malonate, succinate and *N-acetyl-L-glutamate* are used here are carboxylate-rich models for the “low molecular weight binding substance” described by Vincent and coworkers [23,40]. The last three complexes showed only low levels of activity and only at the highest metal concentrations. Reactions with ascorbate are compared in Fig. 9. When the background ascorbate reactions is accounted for, only  $\text{Cr}(\text{pic})_3$  and  $\text{Cr}(\text{mal})_2$  exhibit a statistically significant amount of DNA nicking in this assay. These results confirm earlier work by others, provides data on new complexes and demonstrates the lower reactivity of acidic or amino acid complexes of chromium. In the oxidation reactions, the trichromium cluster complex, basic chromium acetate is the only complex that yields appreciable amounts of nicking at submillimolar concentrations. This result is in accord with a recent paper that proposes a redox action for Cr(III) *in vivo* and notes the lower oxidation potential of such Cr(III) clusters [41]. The mechanistic details and DNA cleavage products of this reaction require further examination. It is difficult to generalize about potential binding interactions between DNA and Cr(III) complexes from this study [18–20]. Neutral ( $\text{Cr}(\text{pic})_3$ ), cationic ( $\text{Cr}(\text{OAc})$ ) and anionic ( $\text{Cr}(\text{mal})_2$ ) complexes are represented in reactions that can affect DNA, and cationic ( $\text{Cr}(\text{His})_2$ ) and anionic ( $\text{Cr}(\text{succinate})$ ) complexes are represented in reactions with very little nicking of DNA under the same conditions.

A summary of the cleavage reactions of Cr complexes with DNA from this work and others' is condensed in Table 2. This comparison of reactions may establish a baseline view of DNA cleavage caused by chromium(III) complexes in this type of assay. While the reducing conditions (5 mM reductant) in this report are similar to what may be found in biological systems, it is important to note that metal concentrations and the oxidizing conditions are extreme. Table 2 presents the data in a relative ranking of activity from highest to lowest, and groups together supplements and complexes found in this report and others. Of note is the high reactivity found in one commercial supplement (**P1**) compared to other supplements.  $\text{Cr}(\text{pic})_3$  with added oxidant exhibits low activity (presumably through a mechanism involving higher oxidation states of Cr) [41] which is comparable with the less reactive supplements tested (**P2**, **P3**, **P4**), but exhibits high reactivity under reducing conditions, presumably through a mechanism involving Cr(II) as described elsewhere [2]. This trend is reversed slightly with  $\text{Cr}(\text{mal})_2$  and  $\text{Cr}(\text{OAc})$

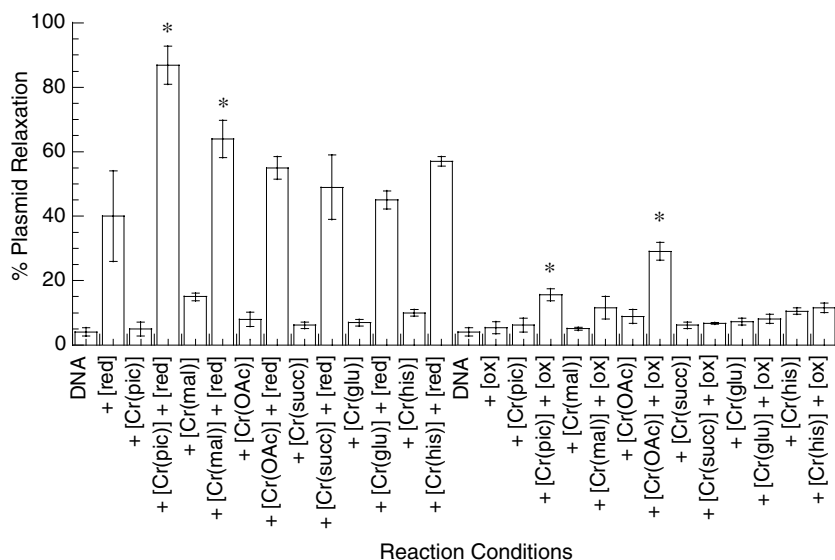


Fig. 9. Quantification of plasmid relaxation relative to supercoiled DNA in the presence of synthetic chromium(III) complexes and/or reductant or oxidant. [red] = 5 mM ascorbate; [Cr] = 120  $\mu$ M; [ox] = 215  $\mu$ M  $H_2O_2$ .

Table 2  
Relative DNA cleavage ability of Cr(III) supplements and Cr complexes<sup>a</sup>

Complex	Conditions				Figures	References
	Control	$H_2O_2$	DTT	Ascorbate		
<b>P1</b>	75 <sup>b</sup>	78	77	100 <sup>c,d</sup>	<b>2, S1</b>	
<b>P2</b>	10	15	10	40	<b>2, S1a, S2</b>	
<b>P3</b>	5	7	10	30	<b>2, S1a, S2</b>	
<b>P4</b>	10	18	12	40	<b>2, S1a, S2</b>	
[Cr(pic) <sub>3</sub> ]	5	70	15	35	<b>S4</b>	[2]
[Cr(mal) <sub>2</sub> ]	5	50	15	45	<b>3, 4, 5</b>	
[Cr(OAc)]	10	45	5	15	<b>S5</b>	[29,42]
Cr succinate	5	15	5	7	<b>S8</b>	
Cr <i>N</i> -Ac-L-Glu	5	15	5	5	<b>S9</b>	
Cr Histidine	2	4	3	10	<b>6,7</b>	

<sup>a</sup> For this table: [Cr] = 120  $\mu$ M; control reactions include only the metal complex or supplement.

<sup>b</sup> Values in the table represent the percentage of nicked DNA present after a reaction versus total DNA.

<sup>c</sup> For ascorbate, the value represents percent nicked DNA after subtracting amount of cleavage in control reactions with ascorbate alone, since these show significant activity.

<sup>d</sup> The high reactivity of **P1** alone and the control ascorbate reaction leads to essentially complete reaction in combination, including double strand breaks (Figure S1c).

showing slightly higher reactivity in oxic conditions, and lower reactivity in reducing conditions. Overall, the lowest reactivity is seen using DTT as reductant, with the diacid Cr complexes, Cr(succinate) or Cr(*N*-acetyl-L-glutamate), or with the Cr(His)<sub>2</sub> complex. Reactivity under reducing conditions apparently decreases between Cr(pic)<sub>3</sub> and simple salts of Cr and a general pattern of reactivity here is consistent with that described elsewhere in reviews of Cr toxicity [3,22]. Finally, in oxic conditions, the above in vitro experiment supports the idea that oxidation of trinuclear chromium clusters could occur under biologically relevant conditions [41].

It is evident that a significant public health concern exists in the use of chromium picolinate containing sup-

plements. The observed reactivity of Cr(pic)<sub>3</sub> suggests that alternate Cr(III) complexes are indicated for use in nutritional supplements. Suitable ligands can diminish DNA cleavage by chromium in vitro. Further studies into dietary uptake, transport, and molecular mechanisms are warranted to determine the risks and any health benefits of Cr(III).

#### Abbreviations

DTT	<i>D,L</i> -1,4-Dithiothreitol
Cr(pic) <sub>3</sub>	Chromium(III) trispicolinate
[Cr(mal) <sub>2</sub> ]	<i>trans</i> -chromium(III) malonate

ESR	electron paramagnetic resonance
[Cr(His) <sub>2</sub> ]	<i>trans</i> -imidazole-chromium(III) histidine
[Cr(OAc)]	'basic' chromium(III) acetate, [Cr <sub>3</sub> O(OAc) <sub>6</sub> ] <sup>+</sup>
LMWCr	low molecular weight chromium-binding peptide(s)
TBE	Tris/boric acid/EDTA buffer

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We thank Dr. Dipesh Ghosh (UMKC) for samples of chromium succinate and chromium *N*-acetyl-L-glutamate. This work was supported by an award from the American Heart Association, and by funds from the University of Missouri Research Board. M.M.R. was supported by an award from the UMKC SEARCH undergraduate research program.

## Appendix A. Supplementary data

Eleven pages containing Table S1 and Figures S1–S10. This material consists of metals analysis of chromium supplements P1 and P4, examples of DNA nicking reactions with all Cr complexes in this study and quantification of plasmid nicking experiments. This material is available upon request from the Corresponding Author. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinorgbio.2004.12.009.

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