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The nutritional supplement chromium picolinate generates oxidative DNA damage and peroxidized lipids in vivo

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Abstract

The nutritional supplement chromium picolinate, $[Cr(pic)_3]$, has been proposed to damage DNA, presumably through the catalytic formation of reactive oxygen species. For this mechanism to be important in vivo, the compound needs to bind to or be in close proximity of DNA. Recent studies have shown that $[Cr(pic)_3]$ does not accumulate in the nucleus and is not stable for prolonged periods of time in cells; this work also demonstrates that the supplement has little if any propensity to bind to DNA. However, as the supplement enters cells intact and is potentially capable of generating oxidative damage where it does occur, experiments were performed to probe for several forms of oxidative damage in rats. $[Cr(pic)_3]$ was found to raise urinary and cellular 8-OHdG levels in a time dependent fashion, and the compound was found to significantly increase lipid peroxidation in vivo. Thus, oxidative DNA damage (and lipid damage) from $[Cr(pic)_3]$ in whole animals has been observed for the first time.

Keywords: Chromium picolinate; 8-OHdG; Lipid peroxidation; Rats; DNA

1. Introduction

In 1995 questions first arose about the safety of chromium picolinate, $[Cr(pic)_3]$, as a dietary supplement when Wetterhahn and coworkers reported that the compound caused clasotogenic damage in a Chinese hamster ovary (CHO) cell model [1]. When intracellular chromium levels generated using $CrCl_3$ or chromium nicotinate were comparable to those generated using $[Cr(pic)_3]$, no chromosome aberrations were found. Unfortunately, these studies used high, non-physiological levels of chromium in the culture media that cast doubt on the interpretation and significance of these findings. Stohs and coworkers have subsequently observed DNA fragmentation in cultured macrophages treated with $[Cr(pic)_3]$, although the Cr concentations were also non-physiological [2]. Wetterhahn and coworkers have suggested that taking $[Cr(pic)_3]$ supplements for five years could result in liver tissue concentrations of 13 μM [3].

In a recent in vitro investigation [4], potentially physiologically relevant concentrations of chromium as $[Cr(pic)_3]$ (as low as 120 nM, greater than 100-fold less than the estimate above) and of biological reductants, such as ascorbic acid and thiols, resulted in catalytic production of reactive oxygen species, which could cleave DNA and could possibly cause other oxidative damage. This ability apparently stems from the combination of chromium and picolinate; the picolinate ligands shift the redox potential of the chromic center such that it is susceptible to reduction. The reduced chromous species then could interact with dioxygen to produce reduced oxygen species including hydroxyl radical [4,5]. These findings are consistent with earlier results that showed mutagenic forms of chromium(III) possessed chelating ligands containing pyridine-type nitrogens coordinated to the metal [6].

Recent studies have also shown that $[Cr(pic)_3]$ is remarkably stable in buffered aqueous solution [4,7] and in synthetic gastric fluid, and it passes unhindered through the jejunum [8]. Consequently $[Cr(pic)_3]$ probably enters cells intact, i.e. in the potentially harmful

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form; a recent in vivo study with rats showed that the complex enters hepatocytes intact [9]. $[Cr(pic)_3]$ does not release its chromium efficiently to biological chromium-binding species such as apotransferrin or apochromodulin unless the metal is reduced to the chromous level [5]—the type of chemistry demonstrated to lead to the catalytic generation of reactive oxygen species—or the organic ligand is enzymatically modified [10].

Only a few studies have examined potential deleterious effects of $[Cr(pic)_3]$ supplementation of humans or animal models. No effect on 5-hydroxymethyl uracil, a product of oxidative DNA damage, levels was observed in 10 obese women given 400 μg $[Cr(pic)_3]$ per day for 8 weeks [11]. A preliminary report of a study on potential chromosome damage in which rats were given a single oral dose of $[Cr(pic)_3]$ up to 2000 mg complex per kg body mass appeared recently; chromosomes from bone marrow cells of femurs removed 18 or 42 h after the dose showed no increase in damage versus controls [12]. However, the significance of studies utilizing a single dose of $[Cr(pic)_3]$ in tissues not known to accumulate chromium is difficult to assess.

Isolated incidents of deleterious effects of $[Cr(pic)_3]$ supplementation have been reported: weight loss, anemia, thrombocytopenia, liver dysfunction, and renal failure [13]; renal failure [14]; rhaddomyolysis [15]; dermatitis [16]; acute, short-lasting cognitive, perceptial and motor changes [17]; and exanthematous pustulosis [18]. The significance of these incidents is difficult to ascertain.

Recently $[Cr(pic)_3]$ has been shown to possess a lifetime of less than 24 h in vivo in rats [9], although low steady state concentrations of $[Cr(pic)_3]$, just at or below the detection level of the study, may have been present. Thus, the form of chromium which accumulates in tissues from $[Cr(pic)_3]$ supplementation is not $[Cr(pic)_3]$. The results are consistent with those of Kelley and coworkers who demonstrated that microsomal enzyme preparations and hepatocytes could rapidly degrade $[Cr(pic)_3]$ in vitro [10]. In this work, the ability of chromium picolinate to produce oxidative damage in vivo was examined and compared to the results of in vitro studies to estimate the potential of the supplement to oxidatively damage DNA and other biomolecules.

2. Experimental

2.1. Materials

^{51}Cr-labeled chromium picolinate was prepared as previously described [19] except that a tracer amount of $^{51}CrCl_3$ was mixed with the initial aqueous solution of $CrCl_3$. $^{51}CrCl_3$ and $^{51}CrO_4^{2-}$ were obtained from ICN. Calf thymus DNA was obtained from Life Technologies. All chemicals were used as received. Doubly deionized water was used throughout. All operations were performed in plasticware whenever possible.

2.2. Thermal denaturation studies

Thermal denaturation studies were performed on control solutions containing 20 μM calf thymus DNA and 10 mM phosphate buffer, pH 7.0; for $[Cr(pic)_3]$ studies, 200 μM $[Cr(pic)_3]$ was added to the buffer and DNA. The melting points (T_m's) were determined from smoothed first derivative plots generated from absorbance data. At the maximum of the derivative plot, the fraction of strands in the double helical state, α, is 0.414. To determine the T_m's ($\alpha = 0.5$), each T_{max} value was multiplied by [0.167/0.172] [20]. Ultraviolet absorbance data were recorded at 260 nm with a Pharmacia Ultrospec 2000 spectrophotometer fitted with a Peltier-heated cell holder and a computer-driven temperature controller. Samples were heated at a rate of 0.5 °C min^{-1}.

2.3. Modified equilibrium dialysis studies

Solutions were prepared with a fixed calf thymus DNA concentration (8.2 μM) and varying $[Cr(pic)_3]$ concentrations (50, 27.5, 5, 2.75, and 0.5 μM) in pH 7.0 phosphate buffer (10 mM) in the presence and absence of 50 mM ascorbic acid. The $[Cr(pic)_3]$ contained a tracer amount of $[^{51}Cr(pic)_3]$. The solutions were allowed to incubate approximately 16 h, after which 400 μl of each solution were pipetted into microcentrifuge tubes fitted with Microcon filters (molecular weight cutoff 10 000). Samples were spun at room temperature at circa 1000 rpm on a Jouan MR 1812 refrigerated tabletop centrifuge. After centrifuging until roughly half of the solution had passed through the membrane, equal aliquots of the solutions above the filters (DNA-containing) and the filtrates were collected. Gamma counting was performed on a Packard Cobra II auto-gamma counter.

2.4. Rat studies

The University of Alabama Institutional Animal Use and Care Committee approved all studies involving rats. Nine male Sprague–Dawley rats ($\sim$600 g) were randomly divided into three groups. One group of four rats received an injection in the tail vein every morning for 60 days of 150 μl of doubly deionized water. The second group, also containing four rats, received an injection of 150 μl of a saturated aqueous solution of $[Cr(pic)_3]$ ($\sim 6 \times 10^2$ μM) daily over the same period of time. The last rat was given 150 μl of doubly deionized water containing 0.2 mCi of ^{51}Cr as $Na_2^{51}Cr(VI)O_4$ every morning for 5 days; this rat, which received hexavalent rather than trivalent chromium, served as a positive

control for the detection of urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG). Urinary 8-OHdG levels of this rat increased during the first 8 days of the study and then decreased, indicating that changes in 8-OHdG levels could be monitored readily. All rats were kept in metabolic cages, and urine was collected daily. The rats were weighed daily; no statistical differences in body mass among the groups were found throughout the study. Rats were allowed to feed ad libitum on a commercial rat chow and tap water. The rat food contained 2.04 mg Cr/kg diet; the Cr concentration of the water was 0.073 μg g^{-1} [21]. After 60 days the rats were sacrificed by CO_2 asphyxiation, and the liver and kidneys were harvested. Livers and kidneys were washed with 0.25 M sucrose solution and weighed and were homogenized in the sucrose solution using a hand-held tissue grinder. Hepatocyte DNA was extracted using a DNAzol reagent kit from Life Technologies (Gaithersburg, MD). Phosphodiester bonds of the DNA were hydrolyzed using *Crotalus adamanteus* Type VI phosphodiesterase (Sigma). The isolated DNA was maintained at 37 °C in 50 mM Tris buffer (pH 8.8) overnight in the presence of ~0.7 units of phosphodiesterase. (One unit is defined as the ability to hydrolyze one μmole of bis(*p*-nitrophenyl)phosphate per min at pH 8.8 and 37 °C). 8-OHdG (and 8-OHdG 5′-monophosphate) content was determined using kits from Genox Corporation (Baltimore, MD), following the manufacturer's instructions. Lipid peroxidation was assayed by two methods: thiobarbituric acid assay [22,23] and diene conjugation assay [22]. UV–Vis measurements were made on a Hewlett-Packard 8453 spectrophotometer.

2.5. Statistical analyses

The control and $[Cr(pic)_3]$-treated group of rats each consisted of four rats, and each sample analysis was performed in triplicate. In vitro studies were performed in triplicate. Comparisons among groups were evaluated for statistical significance by analysis of variance, followed by pair-wise comparisons to derive probability values for the differences between specific groups. Differences were considered significant at $P < 0.05$. Values are means ± SD.

3. Results

3.1. In vivo effects of $[Cr(pic)_3]$

Treating rats intravenously with $Cr(pic)_3$ (0.090 μmol) daily for 60 days had no effect on the visible appearance or behavior of the rats, on the appearance of their organs, or on their body mass. However, the treatment had a significant effect on urinary 8-OHdG output. Urinary 8-OHdG is a marker for oxidative DNA damage; 8-OHdG is produced by the modification of the base guanine by hydroxyl radical and other reactive oxygen species. The formation of 8-OHdG causes misincorporation during DNA replication and subsequently leads to G→T transversions [24,25]. Repair enzymes can recognize and excise the damaged nucleotide, resulting in its appearance in the urine. Over the 60-day period, the daily output of 8-OHdg of rats not receiving the supplement was generally lower than that of $[Cr(pic)_3]$-treated rats, although the difference was significant only on day 8 and after day 31 (Fig. 1). After day 31, the increase in levels of urinary 8-OHdG for $[Cr(pic)_3]$-treated rats relative to that of the control rats appears to become greater. Thus, $[Cr(pic)_3]$ appears to result in increased levels of oxidative DNA damage. This needs to be put into the context of the large quantities of $[Cr(pic)_3]$ utilized. The amount of the complex injected daily corresponds to 5 μg of chromium. Human nutritional supplements generally provide 200–600 μg of Cr per day. Assuming an average human body mass of 75 kg, this is equivalent to 1.3–3.9 μg of Cr per day for a 0.5 kg rat. Thus, 5 μg of Cr is slightly more than rats would receive if given a dose comparable to a typical human dose. Because 5% or less of Cr from oral $[Cr(pic)_3]$ supplements is actually absorbed, the injection represents an approximately ≥ 25-fold excess of Cr compared to humans taking the supplement orally. This quantity of $[Cr(pic)_3]$ was used to guarantee that any deleterious effects would be observed. Injection of the supplement rather than oral administration assured that any differences or problems associated with absorption were overcome and that the amount of $[Cr(pic)_3]$ entering the blood stream was known. Anderson et al. have fed rats diets containing up to 15 mg Cr per kg body mass as $[Cr(pic)_3]$ for 24 weeks without observing any acute toxic effects [26]. Assuming 5% absorption [27], this would correspond to 375 μg of Cr entering the bloodstream of a 0.5 kg rat; thus, the dosage of Cr in the current work was expected to have no acute toxic effects.

The urine results are mirrored by studies of the concentration of 8-OHdG in the DNA of the rats' livers and kidneys. The 8-OHdG content of DNA extracted from the livers and kidneys is significantly increased by $[Cr(pic)_3]$ treatment (Fig. 2). Thus, $[Cr(pic)_3]$ treatment leads to oxidative damage of DNA and the production of 8-OHdG.

In addition to potential oxidative effects on DNA, the ability of $[Cr(pic)_3]$ to generate another form of oxidative damage, lipid peroxidation, was probed. One of the products of the breakdown of peroxidized lipids is malondialdehyde; malondialdehyde can readily be assayed by reaction with thiobarbituric acid to generate a species absorbing at 535 nm [22]. While the assay is

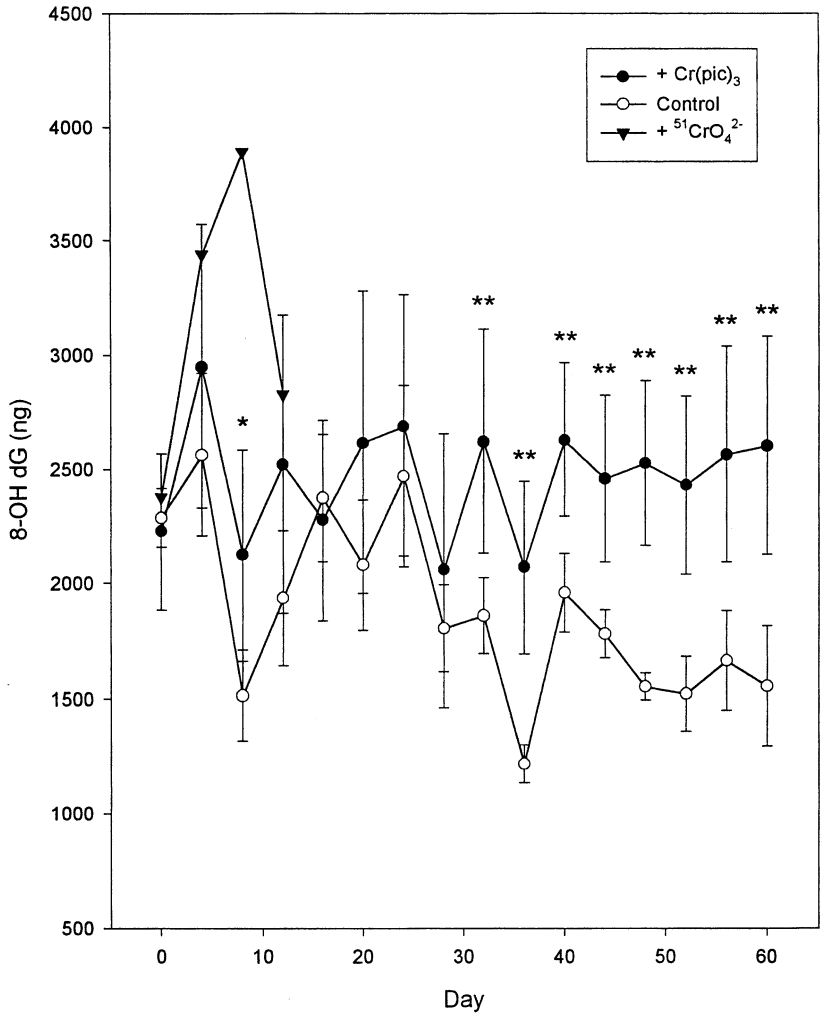

Fig. 1. Daily 8-OHdG content (ng) of rat urine from day 20 to day 60. Dark circles correspond to urine 8-OHdG content of rats receiving a daily injection of an aqueous solution of $[Cr(pic)_3]$; open circles, content from control rats receiving water. *Significant difference between urinary 8-OHdG content of urine of control and $[Cr(pic)_3]$-treated rats ($P < 0.01$); **$P < 0.002$.

susceptible to small levels of interference, the concentration of thiobarbituric acid reactive species (TBARS) in tissues is an excellent estimate of the levels of peroxidized lipids. As shown in Fig. 3, the amount of lipid peroxidation in liver and kidney cells as estimated by measuring TBARS is significantly increased by $[Cr(pic)_3]$ treatment. As confirmation, the levels of lipid peroxidation were also examined by determining the amount of conjugated dienes, which absorb at 233 nm. Conjugated dienes are formed by rearrangement of polyunsaturated fatty acid double bonds that accompanies lipid peroxidation [22]. The levels of conjugated dienes (Fig. 4) follows those of TBARS as the levels are appreciably greater in the liver and kidney tissues of rats treated with $[Cr(pic)_3]$. The similar results of two independent assays of products of lipid peroxidation combined with the urinary and cellular 8-OHdG results conclusively demonstrates that $[Cr(pic)_3]$ treatment results in oxidative damage in cells.

3.2. Interaction between $[Cr(pic)_3]$ and DNA

For reactive oxygen species such as hydroxyl radical generated by $[Cr(pic)_3]$ to be able to damage DNA, the diffusing species must be produced in close proximity to DNA, preventing the reactive species from first coming in contact with other molecules susceptible to reaction. This raises the question as to whether $[Cr(pic)_3]$ actually binds to DNA or only needs to be in close proximity to DNA. The potential of the nutritional supplement to bind to DNA was probed first by thermal denaturation experiments and then by attempts to measure a binding constant by utilizing a variation of equilibrium dialysis. The poor solubility of $[Cr(pic)_3]$ in water (~ 0.6 mM) limits the range of concentrations available for these experiments.

In thermal denaturation studies, double-stranded DNA is slowly heated, ultimately inducing strand separation. The loss of pi-stacking between the DNA

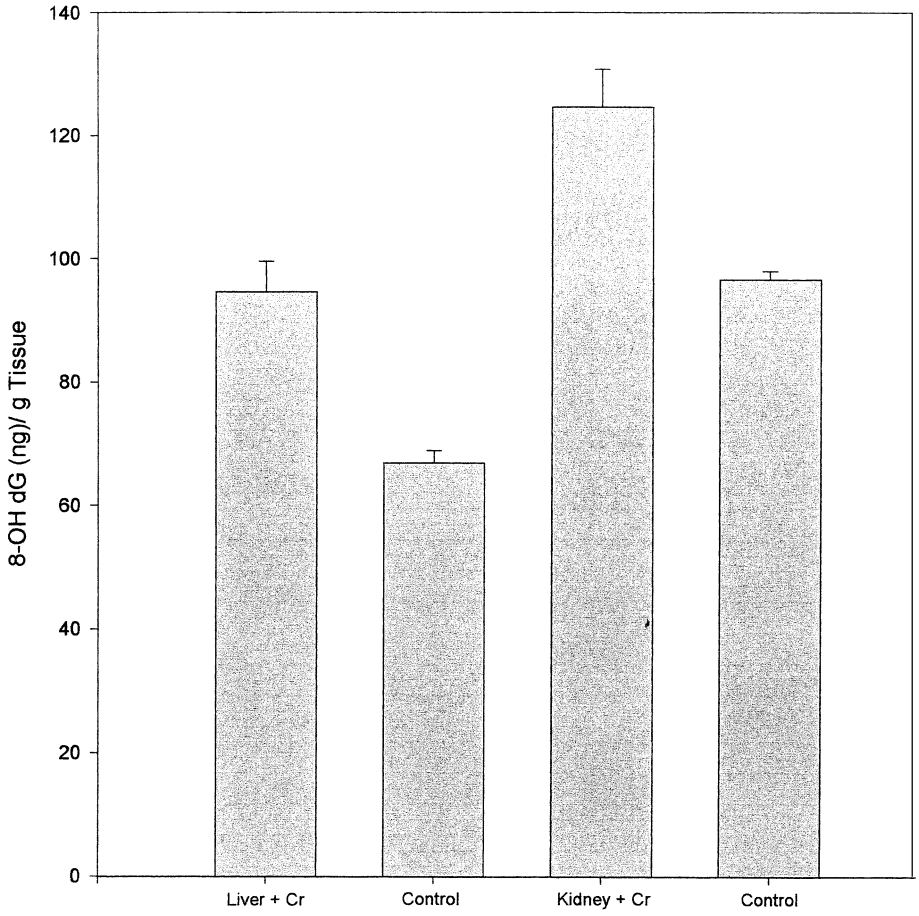

Fig. 2. 8-OHdG content of kidney and liver tissue of rats receiving [Cr(pic)$_3$] and control rats. Differences between 8-OHdG content of tissues of control rats and [Cr(pic)$_3$]-treated rats are significant ($P < 0.002$).

bases results in absorption changes (a hyperchromic shift) in the ultraviolet region which can readily be monitored by ultraviolet spectroscopy. The temperature at which half of the DNA exists in the double-strand form, T_m, is a measure of the stability of the duplex. Many DNA-binding molecules can enhance duplex stability by binding selectively to the double-stranded form. Molecules that bind to DNA through a number of modes including minor groove binding and intercalation between the bases exhibit this property. In this work, thermal denaturation studies of 20 μmol l^{-1} calf thymus DNA in the presence and absence of 0.200 mmol l^{-1} [Cr(pic)$_3$] revealed that the chromium species had no discernable effect on the T_m (of ~ 66 °C). The lack of an appreciable change suggests the complex interacts only weakly if at all.

To attempt to determine the strength of any interaction between the supplement and DNA, the extent of association of [Cr(pic)$_3$] and DNA was assayed by an ultrafiltration method. ^{51}Cr-labelled complex was incubated overnight with DNA to allow equilibrium to be achieved. Rapid ultrafiltration of part of this solution using a Microcon apparatus equipped with a 10 000 molecular weight cutoff membrane allowed DNA to be retained while [^{51}Cr(pic)$_3$] was free to pass through the membrane. Association of [^{51}Cr(pic)$_3$] with DNA would result in the ^{51}Cr content of the DNA-containing solution being greater than that of the ultrafiltrate. The experiments were performed in the presence and absence of 50 mmol l^{-1} ascorbate. Ascorbate has previously been shown to be capable of reducing the chromic center of [Cr(pic)$_3$] to Cr(II), entering the complex into the cycle by which it can generate reactive oxygen species such as hydroxyl radical [4]. If significant levels of intermediates in the production of hydroxyl radical are generated and bind to DNA, this should be reflected in the ascorbate-containing experiments. As shown in Fig. 5, over the [^{51}Cr(pic)$_3$] concentration range of 0.5–50 μM in the presence of ascorbate, the concentration of [^{51}Cr(pic)$_3$] is the same within error in the DNA-containing solution and the ultrafiltrate, demonstrating that the species or species derived from it do not associate significantly with DNA. Similar results are obtained in the absence of ascorbate. (A control experiment in which DNA was not added to the buffer indicates that [^{51}Cr(pic)$_3$] passes freely through

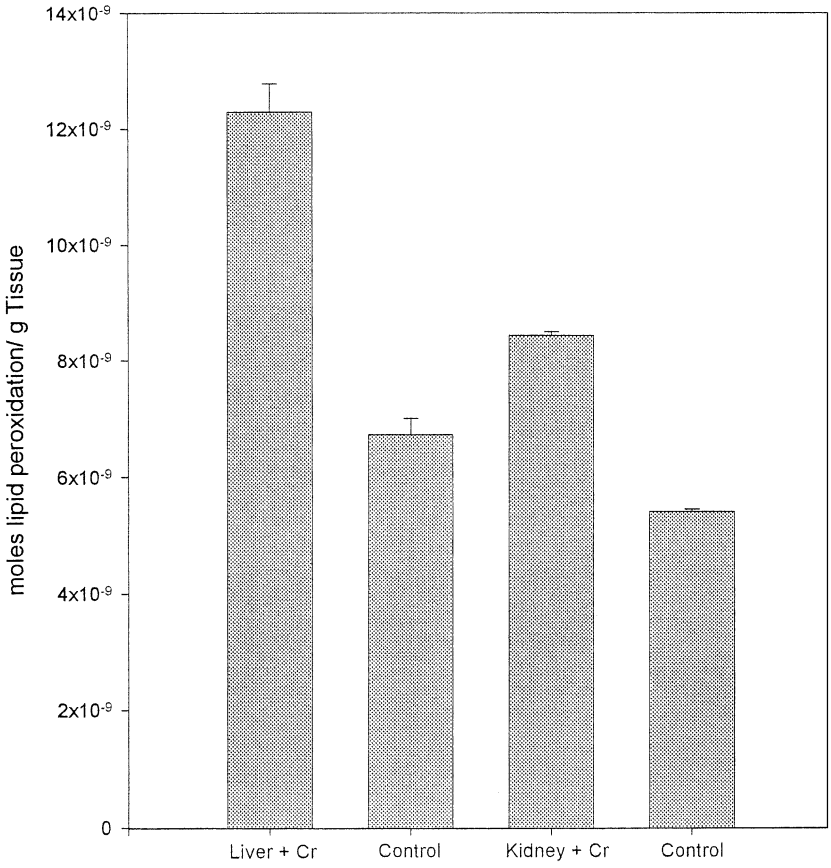

Fig. 3. Lipid peroxidation levels (TBARS) (moles $\times 10^{10}$ lipid peroxidation/g tissue) of kidney and liver tissue of rats receiving $[Cr(pic)_3]$ and control rats. Differences between levels of TBARS in tissues of control rats and $[Cr(pic)_3]$-treated rats are significant ($P < 0.002$).

the membrane and does not adhere to components of the Microcon.)

4. Discussion

$[Cr(pic)_3]$ is absorbed more efficiently than dietary Cr (2–5% vs. 0.5–2%) [27], presumably because the nutritional supplement remains intact in vivo until entering cells. Once inside cells, the complex appears to be degraded rapidly [9,10]. Within liver cells, Cr(III) from $[Cr(pic)_3]$ (given intravenously daily for 2 weeks) resides mainly in the cytosol [9]); little of the metal ion is found in the nucleus. Thus, while Cr(III) from the supplement accumulates in hepatocytes, the actual concentration of the intact supplement is very low in the cytosol (< 1 μmol l^{-1}) and undetectably small in the nucleus [9]. However, a recent study of the transport of $[Cr(pic)_3]$ in rats during the first 24 h after intravenous injection demonstrated that Cr enters hepatocyte nuclei rapidly, reaching a maximum concentration one hour after injection; Cr levels decrease rapidly thereafter [28]. Thus, $[Cr(pic)_3]$ may reside in the nuclei of cells for brief periods of time.

The results of this study which indicate that $[Cr(pic)_3]$ leads to oxidative damage of DNA in vivo are consistent with the earlier in vitro studies using isolated DNA or cultured cells. Hence, supplementation of the diet with $[Cr(pic)_3]$ could present a hazard in terms of oxidative DNA damage as previously suggested. However, this does not rule out other mechanisms of DNA damage such as crosslinks formed from Cr^{3+} released from $[Cr(pic)_3]$ that may also contribute to the previously observed clastogenic damage [1,2]. Indeed, lipid peroxides can also decompose to generate genotoxic products that form DNA adducts [29] and could readily explain the previous results. Thus, both direct oxidative DNA damage and indirect damage arising from lipid peroxidation could potentially lead to mutations following $[Cr(pic)_3]$ treatment. Recently, Stearns et al. have shown that the supplement is mutagenic at the hypoxanthine phosphoribosyltransferase locus in CHO cells [30], although the concentrations of $[Cr(pic)_3]$ utilized (0.2–1.0 mM) were not physiologically significant. Recently, this laboratory has observed mutational effects from the supplement in *Drosophila* at levels in food comparable to supplemented human diets (D.D.D. Hepburn, J. O'Donnell, and J.B. Vincent, unpublished results).

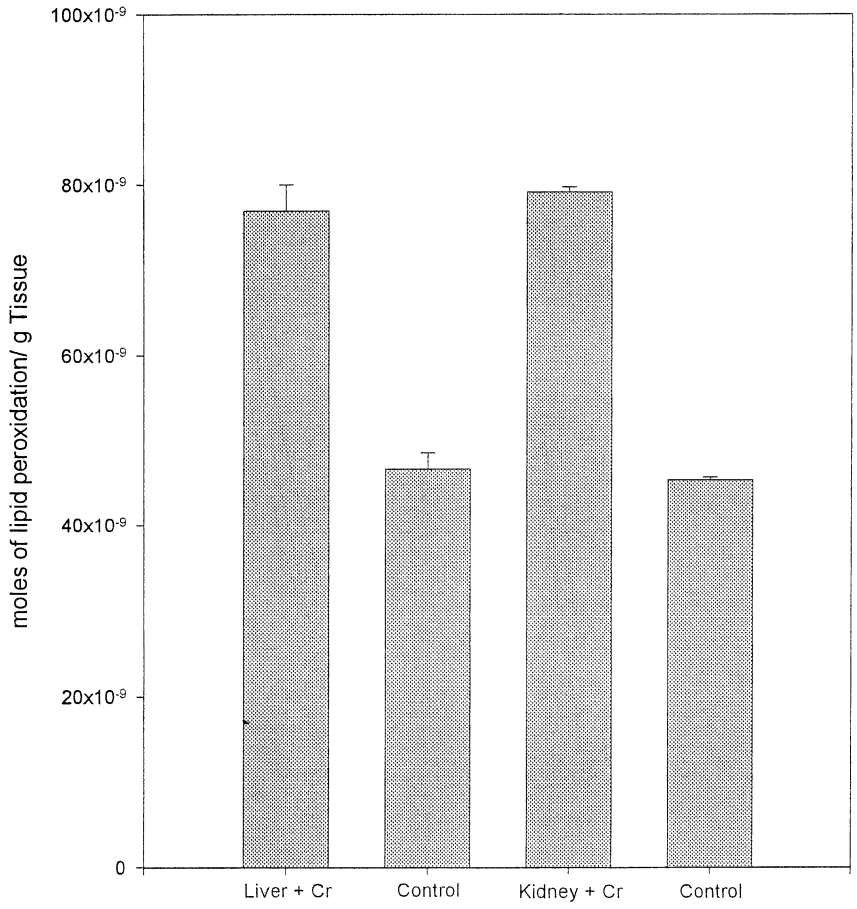

Fig. 4. Levels of conjugated dienes (as absorbance at 233 nm) in lipid extracts from kidney and liver tissue of rats receiving [Cr(pic)$_3$] and control rats. Differences between levels of conjugated dienes in tissues of control rats and [Cr(pic)$_3$]-treated rats are significant ($P < 0.002$).

Preuss et al. have also examined the effect of Cr(pic)$_3$ on TBARS in rats [23]. Spontaneously hypertensive rats were fed a diet containing 5 mg kg^{-1} Cr as [Cr(pic)$_3$] for 30 days. Subsequently the diet was changed to laboratory rat chow for 30 days during which time the drinking water was replaced with 5% w/w sucrose solution [23]. For another 14 days, the diet was maintained while the concentration of sucrose in the water was doubled. Hence the rats received [Cr(pic)$_3$] for 30 days, but they were then off the supplement for 44 days prior to sacrifice and determination of lipid peroxidation levels. The researchers found that hepatic levels of TBARS in rats receiving [Cr(pic)$_3$] were significantly lower than those of the controls (although no numbers are presented) while renal levels of TBARS were not significantly affected. These results are not difficult to interpret given the short lifetime of [Cr(pic)$_3$] in the cells and the instability of peroxidized lipids. Overall, these results point to an initial increase in nuclear and cytosolic oxidative damage upon treatment with [Cr(pic)$_3$]. Termination of the supplementation probably allows for the repair of damaged cellular components. Evaluation of the significance of this initial damage will require further study.

It should also be noted that Witmer et al. have examined the ability of $CrCl_3$ and [Cr(pic)$_3$] to promote the formation of reactive oxygen species capable of generating oxidative damage in cultured human lung cells [31]. Using a dye that fluoresces when oxidized, $CrCl_3$ (200 μmol l^{-1}) was found to have no effect. [Cr(pic)$_3$] at the same concentration partially quenched the fluorescence [31]. However, the [Cr(pic)$_3$] was added as a methanol solution to increase the solubility of the complex; alcohols are traps for reactive oxygen species such as hydroxyl radicals, preventing any conclusions from being drawn from the study.

The significance of the current work is that daily administration of the supplement can lead to the generation of significant levels of DNA damage in whole animals. Given the current popularity of the supplement (i.e. products containing it generating nearly one-half billion dollars (US) in sales annually, second only to calcium supplements [32]), the potential deleterious effects from this damage merit serious attention,

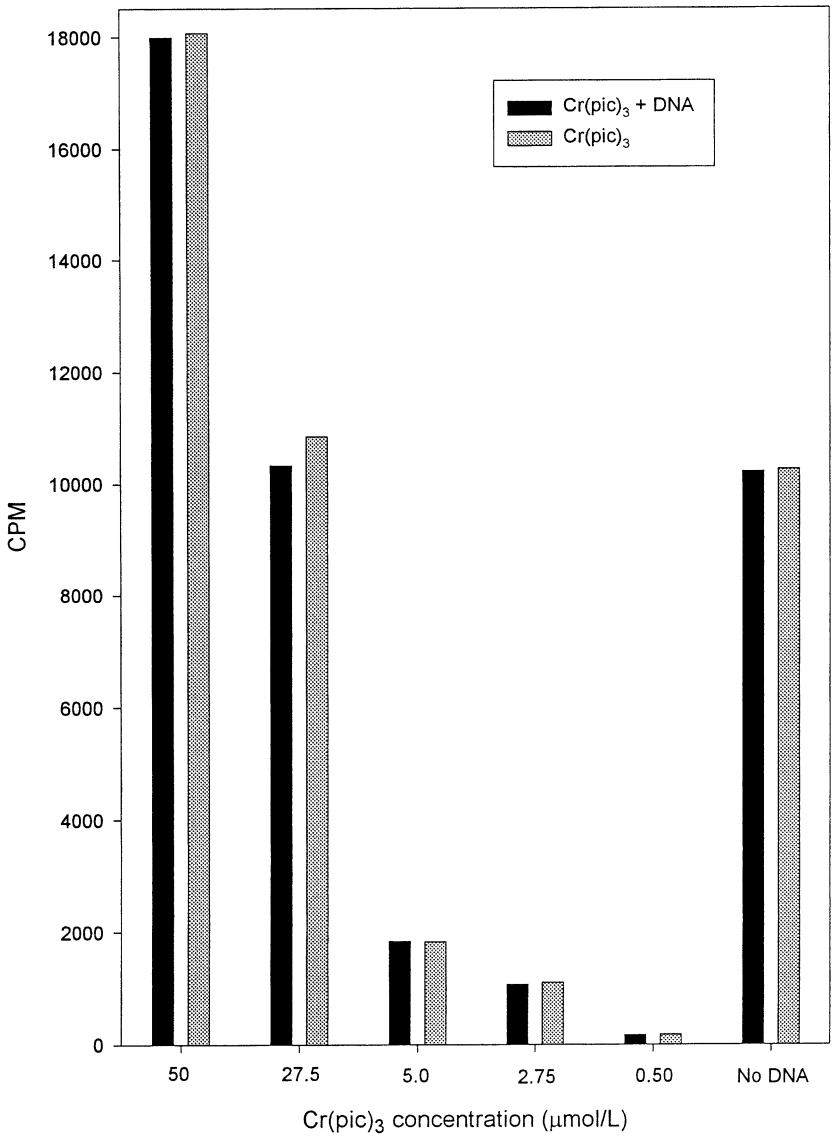

Fig. 5. ^{51}Cr content of ^{51}Cr-labelled [Cr(pic)$_3$] and DNA solutions and [Cr(pic)$_3$] solutions generated by ultrafiltration as a function of [Cr(pic)$_3$] concentration. The solutions without DNA contained 27.5 μM [Cr(pic)$_3$].

especially when reports of potential mutagenic effects are beginning to appear. A contrast exists between lack of observation of DNA oxidative damage observed in previous studies [11,12] and the current work. Problems associated with giving a single dose of the supplement to rats [12] was been noted previously. The failure to observe an effect on 5-hydroxymethyl uracil levels of women given the supplement for 56 days [11] may be the result of the dosage and time scale; rats given proportionally larger doses of [Cr(pic)$_3$] required 30 days of administration before significant levels of oxidative damage were consistently observed. Thus, an upper limit of [Cr(pic)$_3$] amount and time of administration to obtain deleterious results to DNA has been established; additional study is necessary to further evaluate the potential for the supplement to generate DNA damage and mutations; that is, the amounts of chromium picolinate which can be taken orally before significant effects are observed needs to be elucidated. Currently the Food and Drug Administration does not regulate the use of chromium picolinate.

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