Chapter 3
Chromium: Sources, Speciation, Toxicity, and Chemistry

John B. Vincent

Abstract In the environment, chromium is found almost exclusively in the trivalent, 3^+, and hexavalent, 6^+, oxidation states. Chromium^{3+} is stable in the presence of water and air; however, chromium^{6+} as chromate is kinetically stable, but thermodynamically unstable. Chromium has a range of effects in living organisms. In plants, as will be the focus of the following chapters of this work, chromium is a toxin as either Cr^{3+} or Cr^{6+}. In animals, the status has been highly debated since the element as the trivalent ion was first proposed as an essential element about seventy years ago; however, the element is no longer considered to be essential but may have beneficial pharmacological effects. The modes of Cr^{3+} transport in animals has recently been elucidated and may provide insight on how the metal ion can be transported in plants. Chromate is toxic and carcinogenic. A focus of the chapter will be on techniques to characterize chromium in mammals in terms of structure and potential function to suggest how the methods cold be extended to plants.

Keywords Chromium · Insulin sensitivity · Trace element

3.1 Introduction

Chromium is known in eleven oxidation states from 4 to 6^+, although essentially only two are found in the environment, Cr^{3+} and Cr^{6+}. Chromium is the 7th most abundant element on the earth but only the 21st most abundant element in the earth's crust. In the presence of air and moisture, Cr^{3+} is the most stable oxidation state. Cr^{3+} in the environment occurs as coordination complexes with a wide variety of primarily oxygen- and nitrogen-based ligands. As Cr^{6+}, chromium is normally found in the environment as chromate or dichromate. Chromate and dichromate are thermodynamically unstable but kinetically stable. In other words, in the absence of an appropriate reductant, these forms of Cr^{6+} can sit in the environment, despite their oxidizing potential. However, they are readily reduced releasing their oxidizing power when an

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appropriate reductant is encountered. The natural occurrence of chromium is heterogeneous in rock, and its occurrence in the environment is made more heterogeneous by anthropogenic activity. The major applications of chromium are in stainless steel (>11% Cr), where introduction of chromium increases corrosion resistance, chrome plating, and pigments. Chromium is also important in the tanning industry, where Cr^{3+} crosslinks collagen fibers in tanned leather stabilizing the leather, and in the preservation of wood, where added chromate resists infiltration by insects and fungi. These applications have led and continue to lead to the introduction of significant amounts of toxic Cr^{6+} into the environment.

The toxicology and biochemistry of both Cr^{3+} and Cr^{6+} have extremely controversial histories. The public is perhaps more familiar with toxicology, chemistry, and biochemistry of Cr^{6+} because of its carcinogenicity and toxicity, which was the focus of the Hollywood movie *Erin Brockovich* in 2000, starring Julia Roberts. The d^0 Cr^{6+} ion is most commonly encountered as the intensely colored chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) anions, which are interconvertible as a function of pH in water. Chromate occurs at basic pH's where the dianion has a distinctive yellow color, which led to its use as the insoluble salt $PbCrO_4$ as the yellow pigment in the paint used for highway lines. Below pH 6, chromate and orange dichromate are in equilibrium; acidic solutions of dichromate are potent oxidants. The Cr^{6+} center in both chromate and dichromate possesses tetrahedral geometry (Fig. 3.1). Their intense colors arise from charge transfer bands. Mixed ligands complexes of Cr^{6+} with oxides and halides or oxides and amines are also well known, as are peroxo complexes. As the Cr^{6+} center is diamagnetic, it does not give rise to electron spin resonance (ESR) spectra, while nuclear magnetic resonance (NMR) studies of complexes with oxo, peroxo, and halo ligands are of limited utility (Fig. 3.2).

Coordination complexes of Cr^{3+}, the chromic ion, are almost always octahedral (Fig. 3.1). As a result, the chromic center has a d^3 electron configuration with three unpaired electrons giving an electron spin of 3/2. This is responsible for the kinetic inertness of complexes of Cr^{3+}, where at room temperature the ligand exchange half-times are often in the range of hours. While the hexaaquo ion of Cr^{3+}. $[Cr(H_2O)_6]^{3+}$, is purple in color in aqueous solution, aqueous solutions of the cation are acidic. At neutral or basic pH's, the ion is susceptible to oligomerization generating hydroxo-or-oxo bridged species. The most commonly used source of Cr^{3+} in the laboratory is green crystalline $CrCl_3 \cdot 6H_2O$, which is actually *trans*-$[Cr(H_2O)_4Cl_2]Cl \cdot 2H_2O$ (Fig. 3.1). Dissolving this salt in water initially generates the green cation $[Cr(H_2O)_4Cl_2]^+$, which hydrolyzers with the replacement of one (and with time both chlorides depending on concentration) with water. The chromic ion has a large charge to size ratio, so that it is a hard Lewis acid and prefers oxygen-based ligands and nitrogen-based ligands.

The electronic configuration of Cr^{3+} makes obtaining information about its environment at biologically relevant concentrations difficult using spectroscopic and magnetic techniques. No charge transfer bands normally occur in the visible region of the electronic spectrum, while two spin-forbidden d-d transitions in the visible region have extinction coefficients well under 100 M^{-1} cm^{-1}. A third d-d transition occurs in the ultraviolet region, but it often obscured by ligand-based transitions. Chromic

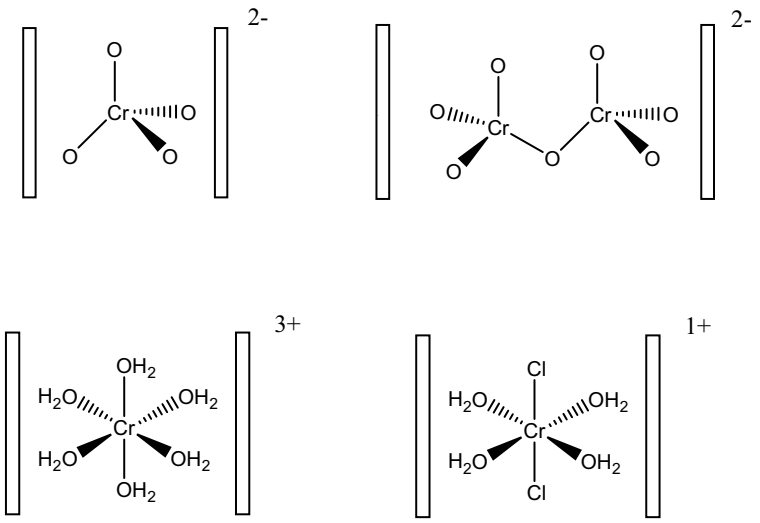

Fig. 3.1 Structures of common chromium compounds: (Top left) chromate anion, (Top right) dichromate anion, (Bottom left) hexaaquochromium(III) cation, and (Bottom right), *trans*-tetraaquadichlorochromium(III) cation

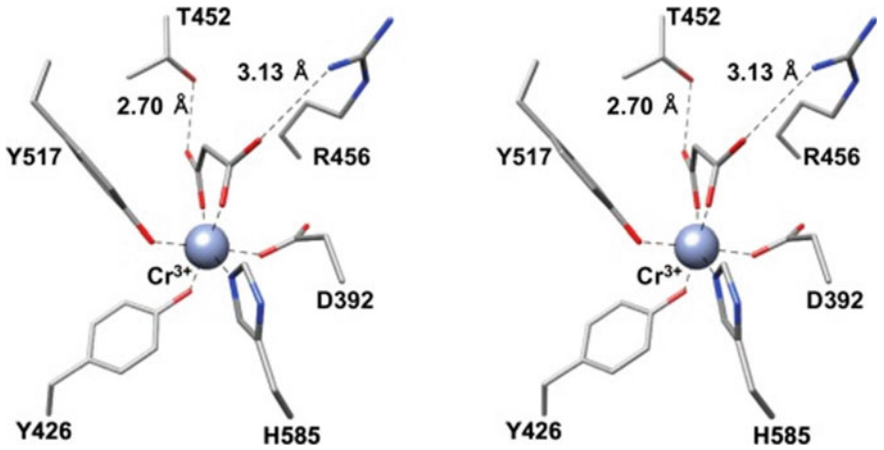

Fig. 3.2 Stereo view of the Cr^{3+}-binding site at the C-lobe of Cr^{3+}-transferrin (PDB entry 6UJ6). Adopted from Petersen et al. (2020). The ligand not provided by the protein is malonate from the crystalloization buffer

complexes, particularly with biological ligands, are generally stable against oxidation or reduction under biological conditions. The S = 3/2 center results in greatly broadened (if even observable) and shifted resonances in NMR spectra. As a general rule, species that give rise to sharp features in NMR spectra do not give rise to EPR

signals or to very broad EPR signal, and vice versa. Chromic complexes can give rise to sharp features in ESR spectra. Unfortunately, for biomolecules containing bound Cr^{3+}, the features in ESR spectra have been found to be rather broad, limiting interpretation. Thus, Cr^{3+} is one of the exceptions to the general rule where features in both NMR and EPR are broad, if observable, and difficult to interpret. Still, ESR is an underutilized technique in probing Cr^{3+}-containing biomolecules and has recently shown significant value in following the transport of Cr^{3+} in vitro (Vincent 2013a). Applying spectroscopic and magnetic techniques to characterize the environment of Cr^{3+} in a biologically relevant system will be a focus of this chapter.

Chromium has four stable isotopes, with chromium-52 being the most abundant comprising about 84%. Chromium-50 and chromium-53 have been used as stable isotope tracers. Synthetic chromium-51 is stylized experimentally as a radiotracer; the isotope decays via electron capture with associated loss of gamma rays, which can readily be detected.

The objective of the chapter is to summarize the fate of chromium in organisms other than plants for comparison with those of plants in the subsequent chapters.

A note of nomenclature is warranted. The free trivalent chromic ion is symbolized by Cr^{3+}. This is also used to refer in a general sense to the chromic ion. When this ion is part of a coordination complex, then it is properly referred to as Cr(III). In aqueous solution, the free chromic ion does not occur as it is bound by coordinate covalent bonds to water molecules. To avoid going back and forth between Cr(III) and Cr^{3+} in the text depending on context, which is often confusing to readers (particularly those who are not inorganic chemists), Cr^{3+} will be used throughout this chapter to refer to the Cr^{3+} ion regardless of context.

3.2 Yeast

The fields of the biochemistry and nutrition of chromium started with studies utilizing yeast, although the focus was orginally not on chromium nor on the yeasts themselves, but rather on the effects of various yeasts on human nutritional health. The effects of chromium on yeast are still poorly understood, while human and rodent studies examining the effects of the chromium content of yeast are a roadmap for how nutritional and biochemical studies should not be performed.

In the late 1950s and early 1960s, a Torula yeast-based diet fed to rats was found to led to a liver disorder (resulting from the low selenium content of the diet) and an apparent inability to efficiently restore blood glucose levels after an intravenous glucose challenge. The existence of a dietary requirement that was absent from the diet leading to the glucose intolerance, Glucose Tolerance Factor (GTF), was postulated. The addition of inorganic compounds of over three dozen different elements to the diet (at 200–500 μg/kg body mass) failed to restore glucose tolerance while several Cr^{3+} complexes (200 μg Cr/kg body mass) apparently did, leading to the postulate that Cr^{3+} was GTF (Schwarz and Mertz 1959).

Unfortuantely, the studies were flawed, which in hindsight limits ntreperation and value of these studies. For example, the chromium content of the diet was not examined. The rats were kept in cages with metal components (potentially including stainless steel with its large chromium content). As a result, the chromium intake of the rats cannot be ascertained. In fact, at least one study has found that Torula yeast diets are not low in chromium compared to other common rat diets (Shepherd et al. 2002) Additional questions about data handling and the significance of the effect observed have been raised (Woolliscroft and Barbosa 1977). More recently studies with rats generally fail to observe effects on glucose levels in glucose tolerance tests (Vincent 2013a). Also, the amount of chromium used to supplement the diet was about 10^3 times the typical content of commercial rat chows, far above nutritionally relevant levels. This raises the possibility that the effect, if real, was pharmacological in origin.

Brewer's yeast, which accumulates Cr^{3+} for an unknown reason, was also shown to apparently restore glucose tolerance in rats when added to the Torula yeast-based diet. Subsequently, efforts were made to attempt to isolate the Cr^{3+}-containing species in Brewer's yeast with the assupmption that this was the species in Brewer's yeast capable of reversing the purported effects of the Torula yeast diet. The product of this endeavor unfortunately was also termed GTF. This led to confusion as to whether the term GTF refers to the inorganic Cr^{3+} ion or to a complex or Cr^{3+} with a biomolecule(s) or to such a complex specifically isolated from yeast. The latter two became the common usage of the term GTF. GTF was reported to be a complex of Cr^{3+} with nicotinate and the amino acids aspartate, glutamate, and cysteine (Toepfer et al. 1977). Later, a three dimensional structure has even proposed for GTF based largely on this data. This resulted in chromium as the trivalent ion being considered to be an essential trace element as part of the biological molecule GTF for several decades. Unfortunately, the research probing the Brewer's yeast GTF complex was extremely flawed. The isolation procedure required refluxing an extract of Brewer's yeast for 18 h in 5 M HCl, conditions that would have destroyed any protein or peptides or many other biomolecules that could have potentially bound Cr^{3+}. The quantities of chromium, nicotinate, and the amino acids in the material were not reported so that the ratios of the components to one other, if they were stoichiometric, cannot be determined. Finally, the characterization was performed on material that was impure, so that none of the components can definitely be said to be associated the component of the mixture that was active in bioassays (for the potentiation of insulin-dependent glucose metabolism by isolated rat adipocytes); in fact the authors showed the spot isolated from paper chromatography that was active and contained chromium only possessed less than 10% of the chromium from the mixture used to establish the apparent presence of nicotinate and specific amino acids.

Subsequent investigations have shown that the bioactive component of Brewer's yeast in these assays could be separated from Cr^{3+}, so that chromium is not even a component of the species in the yeast responsible for the increased glucose metabolism (reviewed in Vincent 2013a). Amazingly, a recent attempt to isolate GTF has been reported but fell into the same pitfall of reporting the composition of

an impure material (Liu et al. 2013; Vincent 2013b). The use of the term GTF should be avoided outside of its historical context.

3.3 Mammals

3.3.1 Essential Element

Chromium is ubiquitous in foods and biological tissues and fluid at very low concentrations, generally parts per billion or lower. As a result, chromium concentrations cannot be determined by flame-based atomic absorption spectrometry (AAS) methods, which lack appropriate sensitivity. Thus, chromium concentrations are normally determined using graphite furnace atomic absorption spectrometry (GFAAS) or more recently also inductively coupled plasma mass spectrometry (ICP-MS). Early GFAAS studies measuring chromium concentrations were plagued with an artifact problem that resulted in concentrations being overestimated by orders of magnitude (Veillon and Patterson 1999). Contamination from contact with stainless steel is also a problem in chromium analyses. The more processed a food the greater its chromium content becomes. Thus, it is likely that humans in more developed nations intake more chromium than their ancestors.

In 1980, the Institute of Medicine of the National Research Council of the Food and Nutrition Board of the National Academies of Science (US) established its first estimated safe and adequate daily dietary intake (ESADDI) for chromium at 50–200 μg for an adult. This was done as the panel found insufficient data to set a recommended daily allowance (RDA). The next time this was reviewed in 1989 the ESADDI was left unchanged. However, in 2001, the recommendation was changed, based largely on the improved techniques for measuring chromium concentrations that indicated chromium intakes were considerably lower than previously believed (Institute of Medicine 2001). Again, insufficient data was found for establishing a RDA, so that an estimated intake (EI) was set at 35 μg per day for adult males and 25 μg per day for adult females. This essentially means that at least 98% of American adults should be chromium sufficient at a dietary intake of ~30 μg chromium daily. Hence, almost every American is basically not chromium deficient.

In Europe, chromium is not considered an essential element for animals nor humans (EFSA 2009, 2014). The European Food Safety Authority has found "no evidence of beneficial effects associated with chromium intake in healthy subjects" and the "setting of an adequate intake for chromium is also not appropriate" (EFSA 2014). The American EI set in 2001 is outdated and needs to be reevaluated, which eventually will probably result in chromium no longer being considered an essential element in the U.S.

This leads to the need to examine the disconnect between chromium not being considered an essential element and reported beneficial effects on carbohydrate and lipid metabolism reported in rodent studies. This has recently been shown to arise

from beneficial effects of chromium resulting in increased sensitivity and lower serum cholesterol and triglycerides levels in rodents requiring supra-nutritional, pharmacologically relevant doses of chromium (Di Bona et al. 2011). Starting from a purified diet with the lowest chromium content ever used in a rodent diet and using diets ranging up to almost 100-fold higher chromium contents resulting in a chromium-intake dependent increase in insulin sensitivity in healthy rats. The increase in insulin sensitivity required chromium intakes orders of magnitude above normal dietary levels, making the effect pharmacologically relevant rather than nutritionally relevant. No human clinical trial has used doses approaching those in rodent studies, making observation of beneficial effects from chromium supplementation studies unlikely.

Despite this, claims have been made that supplemental chromium can lead to loss of body mass, loss of fat mass, increases in lean muscle mass, and beneficial effects for subjects with type 2 diabetes and related conditions. In 1997, the Federal Trade Commission (US) disallowed companies from making representations that chromium has beneficial effects on body mass, fat or muscle mass, diabetes, appetite or cravings for sugar, or serum cholesterol or glucose levels (Federal Trade Commission 1997).

Similarly, in response to a request from a nutraceutical company, the Food and Drug Administration (US) examined eight proposed qualified health claims for chromium supplementation, specifically with popular supplement chromium picolinate (Food and Drug Administration 2005). None of the claims were allowed; what was allowed was the following qualified health claim: “One small study suggests that chromium picolinate may reduce the risk of type 2 diabetes. FDA concludes that the existence of such a relationship between chromium picolinate and either insulin resistance or type 2 diabetes is highly uncertain”. The FDA did, however, establish that the use of chromium picolinate supplements appeared to be safe up to 1 mg of chromium daily.

The position of the American Diabetes Association is “there is insufficient evidence to support the routine use of herbal supplements and micronutrients, such as…chromium, to improve glycemia in people with diabetes (American Diabetes Association 2023). This position has been consistent for a number of years.

The last decade has seen a flurry of meta-analyses examining the effects of chromium supplementation on body mass or serum glucose, insulin, cholesterol, or triglycerides. The interpretation of these meta-analyses is difficult as the quality of the clinical trials utilized is generally rather poor. Most meta-analyses find no effect or statistically significant but clinically insignificant effects (Costello et al. 2019). Meta-analysis restricting utilized clinical trials to higher quality trials generally fail to observe effects. Thus, to determine if chromium supplementation has any beneficial effects in humans, clinical trials using approximately 10 mg of chromium daily (ten times the dose used in any trials to date) are required.

3.3.2 Transport

As Cr^{3+} is a d^3 transition metal ion, the metal ion is considered substitutionally inert, meaning that chemistry at the metal center that involves loss of coordinated ligands is very slow. This has been a concern about how such a substitutionally inert ion that is redox stable could readily be transported about the body efficiently if the element were essential for animals. However, recent studied have revealed how the ion is readily transported from the blood to the urine, preventing toxic accumulation of the element. The "tricks" utilized to transport Cr^{3+} in animals may have lessons to teach on how the ion might be transported in plants.

Cr^{3+} is poorly absorbed (approximately 1%) from the gastrointesinal tract by passive absorption (reviewed in Vincent 2013a). After passive absorption from the gastrointestinal tract into the blood steam, trivalent chromium initially follows the transport of iron. Transport of iron as the ferric ion from the bloodstream to the tissues of mammals is performed by the protein transferrin. Transferrin is an appropximately 80 kDa glycoprotein with two highly homologous lobes, the N-terminal and C-terminal lobe. Both lobes possesses a metal ion binding site comprised of two tyrosine residues, a histidine residue, and an aspartic acid residue. In addition, a synergistic bicarbonate anion binds concomitantly with the metal ion in the metal binding site. Transferrin is selective for Fe^{3+} in a biological environment as its two metal sites are adapted to bind ions with large charge-to-size ratios; thus, that transferrin appears to also be the transport agent for Cr^{3+}, with a similar charge to size ratio to ferric iron, in the bloodstream is not perhaps surprising (Vincent and Love 2012). A recent X-ray crystal structure has confirmed that Cr^{3+} binds to the same protein-provided ligands of transferrin as Fe^{3+} (Peterson et al. 2020), although under crystrallization conditions with synergistic bicarbonte is replaced by malonate.

The binding of Cr^{3+} to transeferrin has been followed spectroscopically using two techniques: ultraviolet-visible spectroscopy and EPR spectroscopy (Figs. 3.3, 3.4 and 3.5), allowing rate constants and formation constants for binding of Cr^{3+} to be determined (Deng et al. 2015; Edwards et al. 2020b). In this case, electronic spectroscopy at the ultraviolet wavelength of 245 nm can be used to follow the increase in the extinct coefficient of the π–π^* transition of the π system of the tyrosine ligands provided by the metal-binding sites of transferrin upon binding of Cr^{3+}. This increase in extinct coefficient arises from the increased rigidity and delocalization of the π systems upon binding Cr^{3+}.

Currently, such assays can be performed with cultured cells, e.g., 3T3-L1 cells or C2C12 myoblasts, to eliminate the requirement for living vertebrate animals. For example, the peptide EEEEGDD, corresponding to the first seven amino acids of LMWCr, when added concurrently with Cr^{3+} to mouse C2C12 myoblasts results in an increase in the ability of insulin to stimulate glucose uptake by the cells (Arakawa et al. 2016); the addition of Cr^{3+} or the peptide separately had no effect. When the cells are rendered insulin resistant by treating them for 24 h with 25 mM glucose, the additon of the combination of Cr^{3+} and the peptide, but neither by itself, was albe to restore most of the ability of insulin to activate glucose uptake. Intravenous

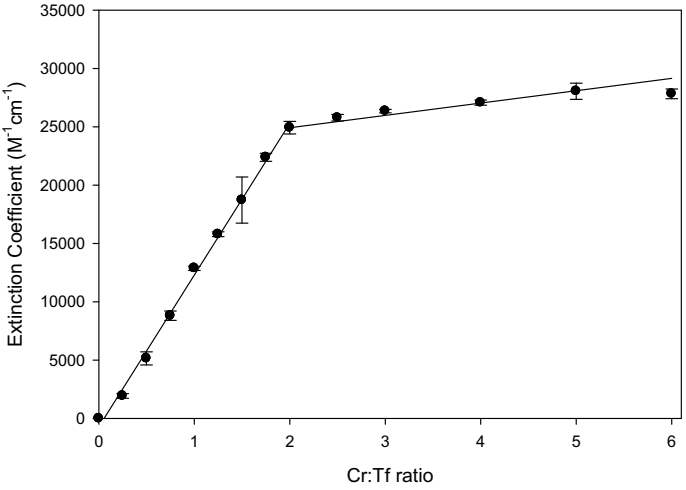

Fig. 3.3 Titration of human serum apo-transferrin with Cr^{3+} in 25 mM HCO_3^-, 0.1 M HEPES, pH 7.4. The time between additions of Cr^{3+} was 3 h. Adopted from Deng et al. (2015)

adminsitraton of Cr^{3+} and the peptide to mice resulted in lowering of the area under the curve (AUC) for glucose in glucose tolerance tests, consistent with the results of the cell studies.

In buffered water with its ambient bicarbonate concentration, Cr^{3+} binds to transferrin slowly, requiring approaching two weeks to be fully loaded with Cr. In contrast ultraviolet spectroscopy studies reveal as a result of the binding being first order in synergistic bicarbonate that Cr^{3+} is fully loaded into transferrin in less than 10 min at 25 mM bicarbonate, the concentration in blood (Fig. 3.4) (Deng et al. 2015). Thus, the binding of Cr^{3+} to transferrin in the bloodstream can take place in a physiologically relevant timeframe.

When Cr^{3+} is allowed to bind for prolonged periods of time, Cr^{3+} in each metal-binding site of the generated $Cr^{3+}{}_2$-transferrin gives rises rise to distinct EPR signals for the human serum protein (Aisen et al. 1969; Edwards et al. 2020b). Cr^{3+} in the N-terminal lobe metal-binding site of human serum transferrin gives rise to EPR signals at $g \sim 5.1$ and 5.6, while Cr^{3+} in the C-terminal lobe metal-binding site gives rise to signal at $g \sim 5.4$ and 2; thus, the amount of Cr^{3+} bound in each lobe can be followed using a window of g between 5 and 6. Combining ultraviolet and EPR studies has revealed that the binding of Cr^{3+} to human serum transferrin is surprisingly coimplicated (Fig. 3.5). Initially, Cr^{3+} binds rapidly to transferrin resulted in a large increases in the molar absorbtivity of the absorbance at 280 nm; this is accompanied by the appearance of an EPR signal centered at $g \sim 2$ from the newly bound trivelent chromic ions, but not the signals between $g = 5$ and 6. With time, the signal at $g \sim 2$ is replaced by a signal at $g \sim 5.2$ resulting from chromic ions in *both* metal binding sites. This signal in turn looses intensity with time as new signals at $g \sim 5.1$ and 5.6 appear. Thus, as a function of time for human serum transferrin, $Cr^{3+}{}_2$-transferrin

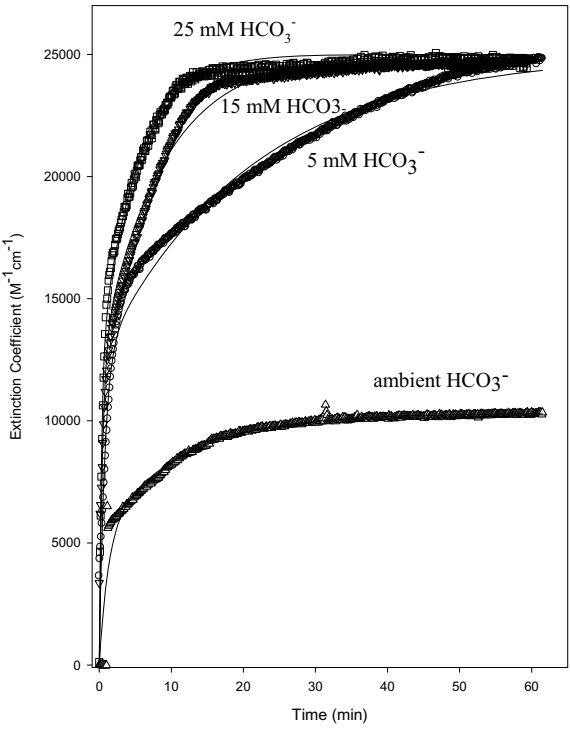

Fig. 3.4 Binding of Cr^{3+} to human serum apo-transferrin as a function of time in 0.1 M HEPES, pH 7.4 with ambient HCO_3^- (squares), 5 mM HCO_3^- (large circles), 15 mM HCO_3^- (small circles), and 25 mM HCO_3^- (triangles). Lines represent fits of the time dependence of the Cr binding to apo-transferrin to the equation $\Delta\varepsilon = a(1 - e^{-bx}) + c(1 - e^{-dx})$ where a and c are the extinction coefficients for each metal binding and b and d are the rate constants. Adopted from Deng et al. (2015)

exists in three different confirmations, giving rise to different EPR signals (and also different extinction coefficients at 280 nm). The middle confirmation is the most biologically relevent one as it is the major conformation from 15 min to few hours after Cr^{3+} addition (Edwards et al. 2020b). In contrast, the addition of chromic ions to bovine transferrin results with time in the formation of the conformation with chromic ions in both metal-binding sites giving rise to the EPR feature at $g \sim 5.4$. The third conformation formed as a function of time for the human protein does not form for the bovine protein.

The exact changes in three-dimensional structure corresponding to the conformation changes are not known. One conformational change can be eliminated. Apotransferrin exists in an open confirmation, where protein-provided ligands in the metal-binding sites are farther apart than in the closed confirmation when metals with large charge to size ratios are bound in the metal-binding sites. Metal ions (or metal-bicarbonate complexes) are believed to first bind to the tyrosine residues, which results in a conformation change, as the protein holds along a hinge in each lobe containing the metal-binding site bringing the aspartate and histidine ligands into close proximity to the metal ion, completing coordination about the metal. This also leavces the metal less exposed than in the open conformation. Three-pulse ESEEM (electron spin echo envelope modulation) spectra of human serum $Cr^{3+}{}_2$-transferrin in each of the three conformations reveals are extremely similar (Edwards et al.

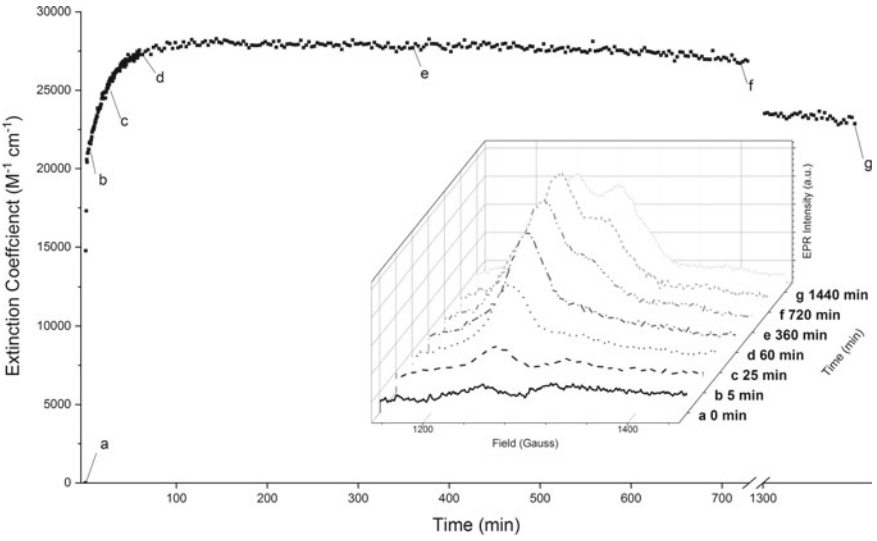

Fig. 3.5 Change in extinction coefficient at 245 nm as a function of time corresponding to the formation of conformations of $Cr^{3+}{}_2$-transferrin following the addition of Cr^{3+} to apo-transferrin in 100 mM HEPES with 25 mM HCO_3^-, pH 7.4, at 37 °C. Insets: EPR spectra of aliquots were taken at prescribed intervals concurrent with the UV measurements. Adopted from Edwards et al. (2020b)

2020b) (Fig. 3.6). All three spectra display coupling of the chromic centers to a nitrogen atom(s), consistent with the chromic ions being bound to histidine; thus, all three conformation ciorrespond to different closed conformations.

When $^{51}CrCl_3$ is administered by gavage to rats, 80% immunoprecipitates with transferrin. This result led to suggestions that the transferrin-bound Cr^{3+} represents a stage in chromium transport or represents a deadend, where transferrin ties up Cr^{3+} to detoxify the ion. For the latter proposal, which would be consistent with the low ligand replacement at trivalent chromium centers, how the chromium-containing transferrin was subsequently to be degraded leading to chromium release and ultimately excretion from the body was not proposed. Intravenous administration of radiolabeled ^{51}Cr–transferrin to rats results in ready incorporation of ^{51}Cr into tissues (Clodfelder and Vincent 2005). Most of the injected ^{51}Cr is transported to the tissues within 30 min so that tissue levels of ^{51}Cr are maximal 30 min after injection. The loss of ^{51}Cr in the tissues with time is mirrored by the appearance of ^{51}Cr in the urine. Approximately 50% of the ^{51}Cr appears in the urine within 6 h of injection.

When ^{51}Cr-containing species found in the blood plasma and urine as a function of time after injection of ^{51}Cr–transferrin have been examined by size-exclusion chromatography, three primary phases are observed (Clodfelder and Vincent 2005). First, the injected ^{51}Cr–transferrin rapidly disappears from the bloodstream. Second, with time, a low-molecular-weight species chromium-contaiing species appears in the bloodstream. Finally, this appearance is rapidly followed by the appearance of a

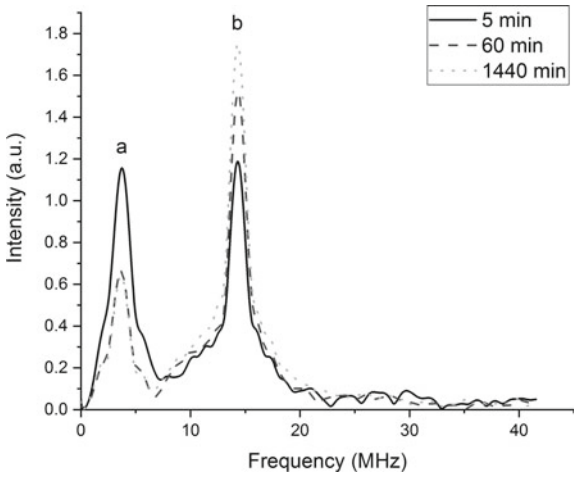

Fig. 3.6 Three-pulse ESEEM spectra of apo-transferrin incubated with 2.0 equivalents of Cr^{3+} at 5, 60, and 1440 min following the addition of Cr^{3+}. **a** Feature at 4 MHz corresponds to coupling between ^{14}N in the vicinity of the Cr^{3+}. **b** Feature at ~15 MHz corresponds to coupling between Cr^{3+} and nearby ^{1}H's. The intensities of these spectral features depend on the tau used during measurement. Adopted from Edwards et al. (2020b)

low-molecular-weight species in the urine. This establishes a clear pathway for the transport of chromium starting from movement of transferrin-bound chromium from the bloodstream into the tissues, followed by subsequent release and processing in the tissues to form a low-molecular-weight chromium-binding species, which is moved into the bloodstream. In the bloodstream, chromium as the low-molecular-weight species is rapidly cleared and ultimately excreted in the urine.

However, at physiological conditions and Cr^{3+} concentrations in the blood stream (~1 ppb), Cr^{3+}, despite its large binding constants for the formation of Cr^{3+}-loaded transferrin, does not load apparently appreciably to apotransferrin (metal-free transferrin) (Levina et al. 2022). However, most transferrin in the bloodstream is not in the form of apo-transferrin, but rather is half loaded with Fe^{3+} in either the C-terminal or N-terminal lobe. These half-loaded forms of Fe^{3+}-containg transferrin bind Cr^{3+} readily under physiologically relevant conditions (Levina et al. 2022). Cr^{3+} loss from mixed Cr^{3+}, Fe^{3+}-transferrins occurs at similar rates to that from Cr^{3+}-loaded transferrin (Vincent et al. 2022). Thus, conditions in the blood stream (e.g., the presence of monferric-transferrin and high bicarbonate concentrations) prime transferrin to be able to bind Cr^{3+}, even at its low concentrations, ultimately for elimination from the body.

Cr^{3+}-containing transferrin is taken up by tissue via endocytosis, similar to the uptake of Fe^{3+}-loaded transferrin, as binding of $Cr^{3+}{}_2$-transferrin to transferrin receptor is inhibited by Fe^{3+}-loaded transferrin (Kornfield 1969); transferrin receptor binds two equivalents of certain metal-loaded transferrin, including transferrin

containing Fe^{3+} and/or Cr^{3+} (Bonvin et al. 2017), although Cr^{3+}-containing transeferrins bind weaker to transferrin receptor than fully Fe^{3+}-loaded transferrin. Acidification of the endosome results in loss of metal ions from the transferrin/transferrin receptor complex, although loss of Cr^{3+} is sightly slower than Fe^{3+} (Edwards et al. 2021). This loss can readily be followed by ultraviolet spectroscopy of EPR spectroscopy (Fig. 3.7). The binding of transferrin to its receptor is crucial for the rapid loss of Cr^{3+} upon acidification; Cr^{3+} is lost significantly slower from transferrin, so slow that it could not be released appreciably during the endocytic cycle (Edwards et al. 2020a,b). Thus, the conformation of Cr^{3+}-containing transferrin is crucial for its release in the endosome. Also crucial for the removal of Cr^{3+} from the endosome before it fues with the cell membrane releasing transferrin back to the bloodstream is the oligopeptide low-molecular-weight chromium-binding substance (LMWCr), which binds the Cr^{3+} released from transferrin (Fig. 3.8).

LMWCr was first reported in 1981 by Yamamoto and coworkers (1981). The oligopeptide is about 10 or 11 amino acids in length and comprosed of glutamate, aspartate, glycine, and cysteine. From mammalian, avian, and reptilian liver and human urine, teh oligopeptide N-terminus starts with the sequence EEEEGDD (Chen et al. 2011). The oligopeptide tights binds four equivalents of Cr^{3+} ($K_f \sim 10^{21}$) and cooperatively (Sun et al. 2000) in an anion bridged assembly (Jacquamet et al. 2003).

The anionic LMWCr oligopeptide has recently been shown to be able to dock to the the acidified transferrin/transferrin receptor complex where it fits into a cationic channel beside each metal binding site in transferrin (Edwards et al. 2021). The oligopeptide with its affinity for chromic ions has little propensity to bind Fe^{3+} (Kircheva et al. 2022), making it able to tie up Cr^{3+} released from transferrin in the transferrin/transferrin receptor complex.

After LMWCr is loaded with Cr^{3+} about half exits the endosome and enters the cell, while the remainder fails to exit the cell before the endosome fuses with the cell membrance, releasing its contents to the bloodtsream. Currently, LMWCr is believed to serve as ionophore, exiting the cell while carrying its Cr^{3+} (Edwards et al. 2021).

These EPR and electronic spectroscopy and related studies allow for a detailed model of chromium transport from the bloodtsream to the urine (for ultimate elimination from the body) to be established (Fig. 3.9). After Cr^{3+} enters the bloodstream via passive diffusion from the gastrointestinal tract, the Cr^{3+} binds relatively quickly to monoferric transferrin; the resulting metal-saturated transferrin is incorporated into cells via endosytosis. Acidification of the endosome releases Cr^{3+} from the transferrin/transferrin receptor complex where it is bound by apoLMWCr. About half the Cr^{3+}-loaded LMWCr does migrate out of the endosome rapidly enough to prevent it being carried out of the cell into the bloodstream upon fusion of the endocsoe with the cell membrane. The other half of the Cr^{3+}-loaded LMWCr, serving as an ionophore for Cr^{3+}, crosses the endosomal membrane to enter the cell, where it migrates from the cell into the bloodstream. From the bloodstream, LMWCr is readily eliminated from the body via the urine. LMWCr was low tubular reabsorption rates in the kidneys (Wada et al. 1983). The rate constants determined for the binding and release of Cr^{3+} to transferrin and from the transferrin/transferrin receptor complex can be used in

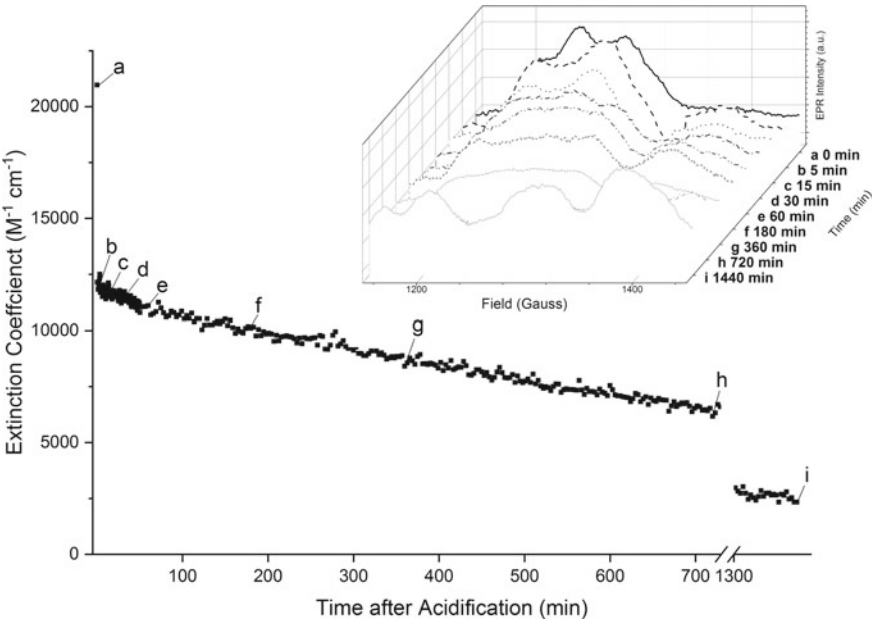

Fig. 3.7 Decrease of the extinction coefficient at 245 nm of Cr_2-transferrin blanked against apo-transferrin following lowering of pH to pH 5.5. Acidification initiated after 24 h of Cr^{3+} incubation with transferrin (2 Cr^{3+}:1 transferrin) at pH 7.4, 37 °C (Fig. 3.5). Insets: EPR spectra of aliquots were taken at prescribed intervals concurrent with the UV measurements. The features in the EPR spectrum at 1440 min after acidification correspond to Cr^{3+} weakly adhering to transferrin. Adopted from Edwards et al. (2020b)

association with this model to reproduce the behavior of Cr^{3+} from $Cr^{3+}{}_2$-transferrin injected via the tail vein into rats (Edwards et al. 2021).

Chromate because of its similarity to phosphate at neutral pH (i.e., HPO_4^{2-}) is transported into cells and subcellular compartments by phosphate and sulfate transporters and, thus, can readily enter cells.

3.3.3 Toxicity

Complexes of Cr^{6+} are well known to be potent carcinogens and mutagens when inhaled, and a current debate exists as to whether these complexes in drinking water pose similar risks. The mechanism(s) by which Cr^{6+} complexes give rise to these problems have not been clearly established but include oxidation by the complexes or more likely the subsequently generated Cr^{4+} and Cr^{5+} intermediates, reactions of reactive oxygen species (ROS) generated as by-products of these reactions, reactions of organic radicals generated in these processes, and the binding of the ultimately generated Cr^{3+} to biomolecules (Levina et al. 2003).

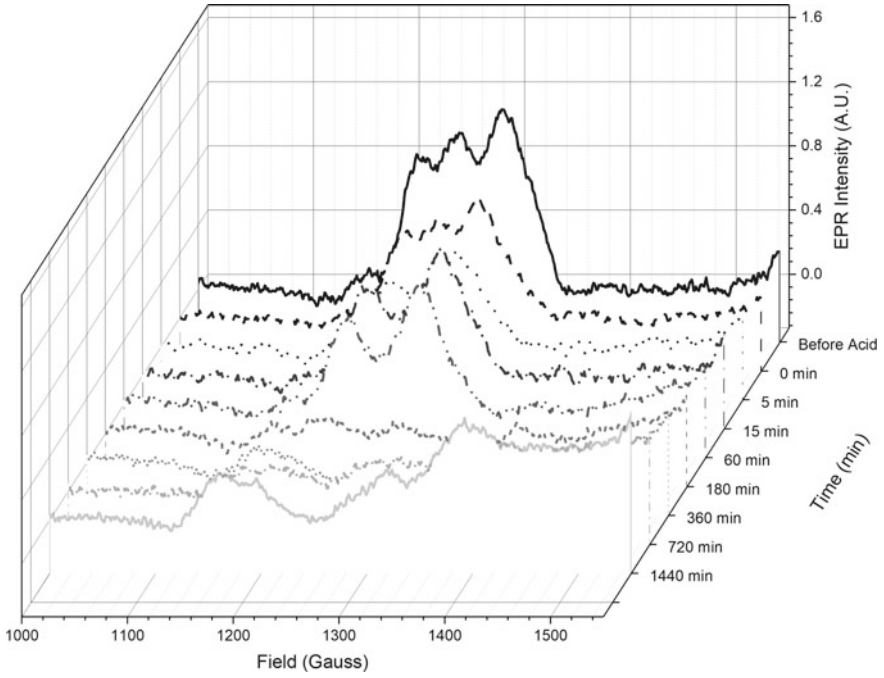

Fig. 3.8 EPR spectra of $Cr^{3+}{}_2$-transferrin in 100 mM HEPES with 25 mM HCO_3^- at 37 °C at various times after the addition of acid to change the pH to 5.5 in the presence of 0.48 mM apoLMWCr. Features at 1210 and 1285 G (g = 5.1 and g = 5.6) correspond to Cr^{3+} in the N-lobe metal-binding site. The feature at 1245 G (g = 5.4) corresponds to Cr^{3+} in the C-lobe metal-binding site. The broad rising feature starting at ~1400 G corresponds to LMWCr. Adopted from Edwards et al. (2021)

The binding of Cr^{3+} to DNA has been proposed to arise to the formation of binary Cr^{3+}-DNA complexes, ternary small molecule-Cr^{3+}-DNA complexes, and Cr^{3+}-based intrastrand crosslinks; the latter two are proposed to be the potentially carcinogenic and mutagenic forms (Zhitkovich 2005). Using a combination of paramagnetic NMR, EPR and other spectroscopic methods, the structure of binary adducts has been determined (Brown et al. 2020). The chromic center in the species $[Cr(H_2O)_5]^{3+}$ binds to N-7 of guanines, while the hydrogens of the coordinated water molecules are hydrogen-bonded to the surrounding DNA. The broadening of the ^1H NMR signals from the hydrogens of the DNA in close proximity to the paramagnetic chromic center in both one-dimensional and two-dimensional NMR experiments (Fig. 3.10) allows for the general position of the Cr^{3+} to be elucidated. The binding site of the Cr^{3+} can be further elucidated using by measuring the T_1 relaxation rates of the ^1H NMR signals. The T_1 relaxation rates change as a function of $1/r^6$ where r is the distance between the chromic center and the hydrogen atom (Rehmann and Barton 1990).

No upper limit (UL) has been established for Cr^{3+} (Institute of Medicine 2001) as excess intake of supplemental chromium could not be associated convincingly with

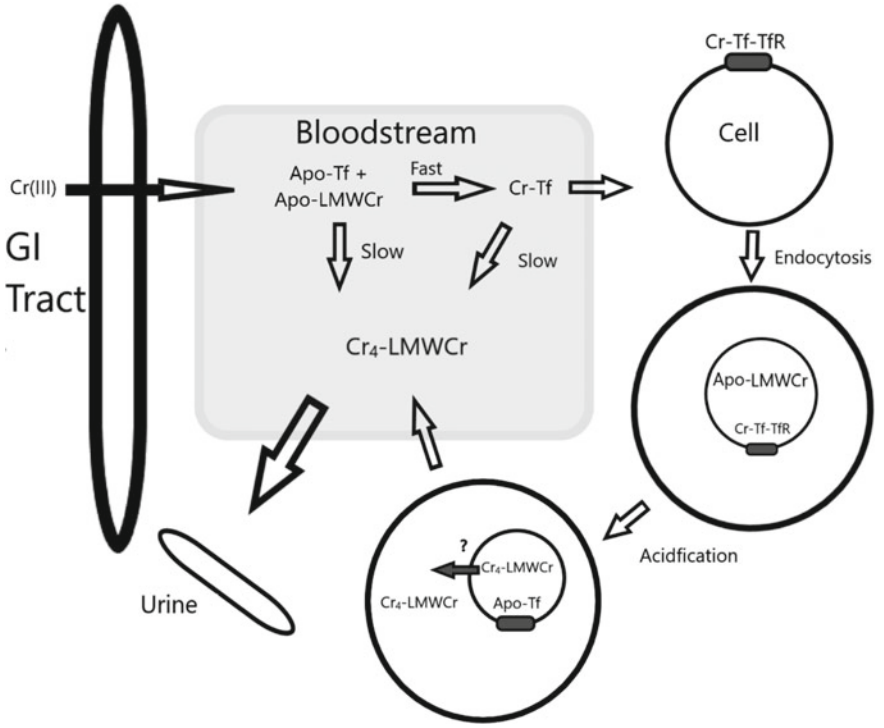

Fig. 3.9 Movement of Cr^{3+} from the gastrointestinal tract to the bloodstream, tissues, and urine. Cr^{3+} is absorbed by passive diffusion into the bloodstream where it binds rapidly at pH 7.4 ~ 25 mM HCO_3^- to transferrin (Tf). Cr^{3+} binding to apoLMWCr and transfer of Cr^{3+} from Cr^{3+}-transferrin to apoLMWCr is slow. Cr^{3+}-transferrin binds to the transferrin receptor (TfR) and undergoes endocytosis. Acidification of the endosome results in the rapid release of Cr^{3+} from the transferrin/transferrin receptor complex with its binding to apoLMWCr. The Cr^{3+}-loaded LMWCr then either passes through the endosome membrane via an unknown mechanism to deliver Cr^{3+}-loaded LMWCr to the cell, where the Cr^{3+}-loaded LMWCr is subsequently is expelled from the cell into the bloodstream or is delivered to the bloodstream by the fusing of the endosome with the cell membrane. Cr^{3+}-loaded LMWCr in the bloodstream is removed by the kidneys and the excreted from the body in urine. Adopted from Edwards et al. (2021)

any adverse health effects. As noted above, the FDA found chromium supplementation up to the highest amounts used in clinical trials (1 mg chromium daily) was safe (Food and Drug Administration 2005).

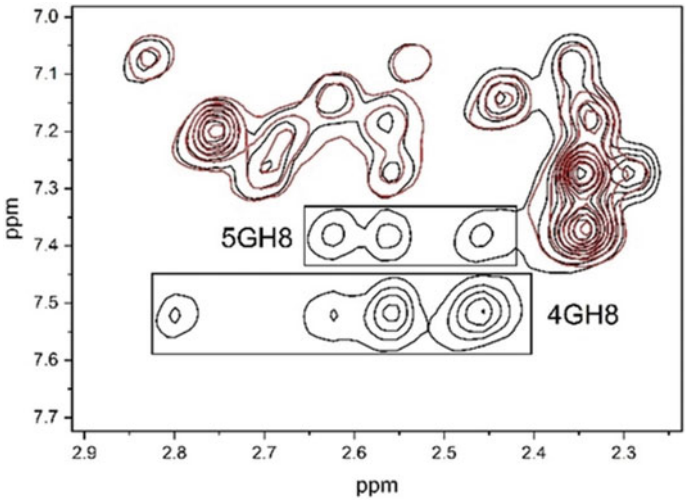

Fig. 3.10 NOESY (Nuclear Overhauser Enhancement Spectroscopy) NMR spectrum of oligonucleotide duplex with no Cr^{3+} (black) or 0.4 Cr^{3+} per oligonucleotide (red). Note the cross peaks remain largely unaffected except for those arising from each H-8 of the guanines whose relaxation times are altered due to binding to Cr^{3+} to the guanines. Adopted from Brown et al. (2020)

3.4 Biological Activity

Another point that arises out of the history of these studies with yeast and rodents is to ask what does it mean for a chromium-containing species to have biological activity or in other words how would one currently want to test a chromium-containing species or extract, such as a new plant extract, for biological activity. Clearly, assays involving Torula yeast should now be avoided. The one method that has seemed to be accepted for the decades is the insulin-dependent stimulation of glucose uptake and/or metabolism by isolated rat adipocytes. This assay was actually proposed initially to replace the use of fat tissue to test for GTF activity or GTF-like activity from Cr^{3+}-containing species other than GTF (Anderson et al. 1978). Curiously, the results of the assays using the rat adipocytes were originally misinterpreted; the assays actually showed that supposed GTF/yeast extracts increased glucose metabolism by the isolated fat cells in the absence of insulin but did not enhance the action of insulin as the GTF was supposed to do (Vincent 1994). This is consistent from hindsight with the subsequent demonstrations that the active species in the yeast extracts did not contain Cr^{3+}. In contrast, LMWCr activates glucose metabolism by rat adipocytes in an insulin-dependent fashion, consistent with activity after the binding of insulin to its receptor (Vincent 1994). A similar effect to adding LMWCr to adipocytes has been observed for healthy and diabetic rats administered pharmacologically relevant doses of Cr^{3+} daily by intraperitoneal injection (20 mg/kg body mass) for four weeks; addition of insulin to isolated adipocytes resulted in greater stimulation of insulin

action on glucose metabolism in Cr^{3+}-treated rats compared to controls (Yoshimoto et al. 1992).

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