The Bioinorganic Chemistry of Chromium

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Preface

Two oxidation states of chromium, Cr^{3+} and Cr^{6+}, are generally considered biologically and environmentally relevant and stable, that is, they are stable in the presence of air and water. Chromium(III) complexes are both kinetically and thermodynamically stable. However, chromium(VI) complexes are kinetically stable but unstable thermodynamically. In the presence of appropriate reducing agents, Cr^{6+} can readily be reduced via Cr^{4+} and/or Cr^{5+} intermediates ultimately to Cr^{3+}.

The biochemistries of both Cr^{3+} and Cr^{6+} have controversial histories. The public is generally more familiar with the chemistry of Cr^{6+} (or chromate) because of its toxicity. Chromium(VI), d^0, is most commonly encountered as the intensely coloured chromate, $[CrO_4]^{2-}$, or dichromate, $[Cr_2O_7]^{2-}$, anions. These two species are interconvertable in water. Chromate occurs at basic pH values and has a distinctive yellow colour; $PbCrO_4$ has been used as the pigment in paint used for yellow highway lines. Below pH 6, chromate is in equilibrium with yellow–orange dichromate. Acidic dichromate solutions are potent oxidants. The coordination environment of chromium in both the chromate and dichromate anions is tetrahedral. The intense colour of both anions results from ligand to metal charge transfer bands. Mixed ligand complexes of Cr^{6+} with oxides and halides or oxides and amines are well known, as are Cr(VI) peroxo complexes. The diamagnetic Cr^{6+} centre does not give rise to ESR (electron spin resonance) spectra, while NMR (nuclear magnetic resonance) studies of Cr(VI) complexes with oxo, peroxo and halo ligands are of limited utility.

While Cr(VI) complexes are known to be potent carcinogens and mutagens when inhaled, a serious debate has arisen with regards to the effects of the oral intake of these complexes, as illustrated in recent years by the popular movie *Erin Brokovich*. Chromium(VI) complexes could

give rise to these effects through a number of mechanisms, including oxidation by the complexes or the subsequently generated Cr^{4+} and Cr^{5+} intermediates, reactions of reactive oxygen species (ROS) generated as by-products of these oxidations, reactions of organic radicals generated in these processes and the binding of the ultimately generated Cr^{3+} to biomolecules. The relative importance of these mechanisms is far from being explained.

However, while the chemistry of Cr^{6+} and Cr^{3+} may be intertwined to some degree and this intertwining cannot simply be dismissed, this book focuses on the biochemistry of Cr^{3+}, particularly in terms of its potential use as a nutritional supplement, nutraceutical agent or pharmaceutical agent. (The coordination of Cr^{3+} ions to DNA as a result of Cr^{6+} reduction is beyond the scope of this work, and the nature and significance of this binding is a current topic of much debate.)

Coordination complexes of Cr^{3+} are nearly always octahedral. Consequently, the chromic centre has a d^3 electron configuration with three unpaired electrons ($S = 3/2$) in each of the t_{2g} orbitals. This configuration is responsible for the kinetic inertness of Cr(III) complexes, where ligand exchange half-times are generally in the range of hours. The hexaaquo ion of chromium, $[Cr(H_2O)_6]^{3+}$, is purple in aqueous solution. Solutions of the ion are acidic; at neutral and basic pH the ion readily oligomerizes to give hydroxo-bridged species starting with the $[(H_2O)_5Cr(\mu\text{-}OH)_2Cr(H_2O)_5]^{4+}$ ion. The commonly used commercial form of $CrCl_3{\cdot}6H_2O$ is actually *trans*-$[Cr(H_2O)_4Cl_2]Cl{\cdot}2H_2O$. Dissolution of this green solid initially yields green solutions of the $[Cr(H_2O)_4Cl_2]^+$ cation. The Cr^{3+} ion has a large charge to size ratio and is considered as a hard Lewis acid, preferring oxygen and nitrogen coordination. With common biomolecules, coordination to anionic oxygen-based ligands such phosphates and carboxylates would be expected.

The magnetic and spectroscopic properties of chromium(III) complexes do not readily lend themselves to providing much information on the coordination environment of chromic centres in biomolecules. For mononuclear complexes, a magnetic moment close to the spin-only value for an $S = 3/2$ centre (3.88 BM) is generally observed. While ^1H and ^{13}C nuclear magnetic resonance spectra can be obtained on Cr(III) complexes, the spin 3/2 centre results in greatly broadened and shifted resonances in NMR spectra. The structure of the complex must generally be known in order to interpret the NMR spectra, rather than the reverse. In contrast, Cr(III) complexes can give rise to sharp features in ESR spectra (ESR is also known as electron paramagnetic resonance

(EPR) spectroscopy); however, the ESR spectra of biomolecules have often proved to be quite broad, providing limited information. ESR spectroscopy is probably a significantly underutilized technique in characterising chromium in biological systems. Cr^{3+} as an impurity in the Al_2O_3 matrix of emeralds and rubies gives rise to the green and red colour of these gems; yet, the electronic spectra of chromium-containing biomolecules are usually very simple. Three spin-allowed d→d transitions are expected; two usually occur in the visible region, while the third is expected in the ultraviolet region (where it can be hidden by ligand based features). No charge transfer transitions generally occur while the visible absorption bands have extinction coefficients of typically less than 100 M^{-1} cm^{-1}. Thus, only relatively concentrated solutions of Cr^{3+} have appreciably observable colour. Cr(III) complexes are generally stable against oxidation or reduction.

Although chromium as the Cr^{3+} ion was proposed to be an essential element about 50 years ago, its status is currently in question, as recent experiments appear to demonstrate that the element can no longer be considered essential. Supplemental nutritional doses of Cr^{3+} have been proposed to result in body mass loss and lean muscle mass development, leading to an appreciable nutraceutical industry being built around chromium. However, these claims have been thoroughly refuted. Chromium has also been suggested to be a conditionally essential element whose supplementation could lead to improvements in carbohydrate and lipid metabolism under certain stress situations, including type 2 diabetes and the effects of shipment of farm animals; this is currently an area of intense and hotly debated research with recent findings suggesting that beneficial effects from Cr^{3+} supplementation are pharmacologically, not nutritionally, relevant. At the same time, supplementation of the diet with at least certain Cr(III) complexes has been proposed to have potentially deleterious effects.

Chapter 1 examines the current status of chromium as defined by various government agencies or public foundations. Chapter 2 reviews the evidence that chromium is an essential trace element. Chapter 3 explores the history of nutritional studies on chromium(III) complexes. The ability of chromium(III) complex supplementation to generate body composition changes is covered in Chapter 4, while potential pharmacological effects of chromium supplementation, particularly for type 2 diabetic subjects, is reviewed in Chapter 5. Chapter 6 explores the mechanisms by which chromium might have pharmacological effects. Chapters 7 and 8 review chromium supplements that are commercially available or under development and the use of chromium supplements in farm animal

nutrition, respectively. The potential toxicity of chromium supplementation is examined in Chapter 9.

This work is by far the most exhaustive treatment of the biochemistry and related nutritional and pharmacological effects of Cr^{3+}. It presents the views of the author at the time of writing. Surprisingly after more than two decades of research personally in the field, these views are continually being revised as more experimental results are reported. Much that was learned 20 years ago has had to be 'unlearned' and reassessed. The basics of the field as understood 20 years ago has been entirely inverted by recent experimental results. Clearly while more than five decades old, the field of chromium biochemistry is not a mature field. Major gaps in our knowledge remain to be filled. For example, no biomolecule has been shown unambiguously to bind chromium and be responsible for its effects *in vivo*. Recent research has led to a reassessment of much of what was believed two decades ago and suggests that major advances may be on the horizon. Hopefully this work will inspire additional research that can fill these holes.

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1

Introduction – The Current Status of Chromium(III)

When a member of the general public thinks about chromium and health, unfortunately the first thing to come to mind is probably one or more of the following claims:

- reduces body fat;
- causes weight loss;
- causes weight loss without exercise;
- causes long-term or permanent weight loss;
- increases lean body mass or builds muscle;
- increases human metabolism;
- controls appetite or craving for sugar; or
- 90% of US adults do not consume diets with sufficient chromium to support normal insulin function, resulting in increased risk of obesity, heart disease, elevated blood fat, high blood pressure, diabetes, or some other adverse effect on health.

In other words, most people think of chromium in terms of weight loss and lean muscle mass development as a result of nutraceutical product marketing. Yet the Federal Trade Commission (FTC) of the United States ordered entities associated with the nutritional supplement chromium picolinate to stop making each of the above representations in 1997 because of the lack of 'competent and reliable scientific evidence' [1]. This ruling is now well over a decade old; however, the situation has

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changed little. In fact in 2000, products containing chromium picolinate had sales of nearly a half a billion dollars [2]. The FTC currently has pending law suits against entities associated with chromium picolinate-containing products, while the scientific support for most of these claims has completely eroded [3]. For example, recently the National Institutes of Health sponsored a study where male and female rats and mice were given diets containing up to 5% chromium picolinate by mass for up to two years; no effects were observed on body mass or food intake [4]. Studies of the effects of chromium picolinate will be presented in Chapter 4.

The basis for the use of chromium as a nutritional supplement stems from chromium being on the list of essential vitamins and minerals under examination by the National Research Council of the National Academies of Science, USA since 1980 [5], after initially being proposed as an essential element in 1959; (the history of the status of chromium as a trace element is reviewed in Chapter 3) [6]. In 2001, the National Academies of Science established an Adequate Intake (AI) of chromium of 35 μg/day for men and 25 μg/day for women [7]. AI is defined as 'the recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate.' The AI 'is expected to cover the needs of more than 97–98% of individuals' [7]. Thus, almost all Americans are believed to be chromium sufficient, and little if any need exists for chromium supplementation. The bases for this determination are rather limited. Anderson *et al.* have established that self-selected American diets contain on average 33 μg Cr/day for men and 25 μg Cr/day for women [8], while nutritionist-designed diets [9] contain on average 34.5 μg Cr for men and 23.5 μg Cr/day for women. Offenbacher *et al.* have found that men (two subjects) could maintain their chromium balance when receiving 37 μg Cr/day [10]. Bunker *et al.* have shown for 22 elderly subjects consuming, on average, 24.5 μg Cr/day that 16 were in chromium balance, 3 were in positive balance and 3 were in negative balance [11]. The situation is likely to be similar in other developed nations; for example, pre-menopausal Canadian women eating self-selected diets have been found to have an average daily intake of 47 μg of chromium [12]. Currently, as discussed in Chapter 2,whether chromium is an essential element is at best an open question, and it probably should not currently be considered to be an essential element. If chromium is an essential element, it must interact specifically with some biomolecules in the body

and serve a specific function; attempts to identify such a molecule and a role in the body will be discussed in Chapter 6.

In addition to the purported use to reduce body mass and build muscle, chromium supplements have also been touted to alleviate the symptoms of type 2 diabetes and related cardiovascular disorders, in addition to other conditions. While administration of chromium(III) complexes has positive effects in rodent models of type 2 diabetes and other conditions, the situation in humans is currently ambiguous (see Chapter 5 for a thorough discussion). According to the American Diabetes Association in its 2010 Clinical Practices Recommendations, 'Benefit from chromium supplementation in people with diabetes or obesity has not been conclusively demonstrated and therefore cannot be recommended' [13]. The American Diabetes Association dropped any mention of chromium in its 2011 and 2012 recommendations.

In December 2003, Nutrition 21, the major supplier of chromium picolinate, petitioned the US Food and Drug Administration (FDA) for eight qualified health claims:

1. Chromium picolinate may reduce the risk of insulin resistance.
2. Chromium picolinate may reduce the risk of cardiovascular disease when caused by insulin resistance.
3. Chromium picolinate may reduce abnormally elevated blood sugar levels.
4. Chromium picolinate may reduce the risk of cardiovascular disease when caused by abnormally elevated blood sugar levels.
5. Chromium picolinate may reduce the risk of type 2 diabetes.
6. Chromium picolinate may reduce the risk of cardiovascular disease when caused by type 2 diabetes.
7. Chromium picolinate may reduce the risk of retinopathy when caused by abnormally high blood sugar levels.
8. Chromium picolinate may reduce the risk of kidney disease when caused by abnormally high blood sugar levels [14].

After extensive review, the FDA issued a letter of enforcement discretion allowing only one (No. 5) qualified health claim for the labelling of dietary supplements [14, 15]: '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 small study was performed by Cefalu *et al.* [16]. This study was a

placebo-controlled, double-blind trial examining 1000 μg/day of Cr as chromium picolinate on 29 obese subjects with a family history of type 2 diabetes; while no effects of the supplement were found on body mass or body fat composition or distribution, a significant increase in insulin sensitivity was observed after four and eight months of supplementation [16]. Mechanisms by which chromium has been proposed to potentially have an effect on type 2 diabetes and associated conditions will be discussed in Chapter 6.

A safety assessment was also part of the FDA evaluation of chromium picolinate [14]. As reviewed in Chapter 9, the safety of chromium picolinate has been questioned after cell culture and developmental toxicity studies in fruit flies have shown that the compound could be mutagenic and carcinogenic. However, the FDA determined that the 'use of chromium picolinate in dietary supplements . . . is safe' [14]. The European Food Safety Authority (EFSA) recently also determined that chromium supplements in doses not exceeding 250 μg Cr per day are safe [17,18]. The safety of chromium picolinate as a nutritional supplement has been confirmed by a study commissioned by the National Toxicology Program of the National Institutes of Health. The study examined the effects of chromium picolinate comprising up to 5% of the diet (by mass) of rats and mice for up to two years and found no harmful effects on female rats or mice and, at most, ambiguous data for one type of carcinogenicity in male rats (along with no changes in body mass in either sex of rats or mice) [4]. The reasons behind the discrepancies between the toxicology studies will be examined in Chapter 9.

Chromium(III) complexes are often used as animal feed supplements, in addition to being a popular human supplement. The use of chromium as an animal feed supplement was evaluated in the mid-1990s by the Committee on Animal Research, Board of Agriculture of the National Research Council [19]. In general the available data were insufficient for conclusions to be drawn; for example, no conclusions could be reached about the need for supplemental chromium in the diets of fish, rats, rabbits, sheep and horses. Specific recommendations could not be made about the diets of poultry, swine and cattle, although chromium was determined possibly to have a beneficial effect for cattle under stress and improve swine carcass leanness and reproductive efficiency [19]. Chromium was, however, found to be safe as a food additive. As is reviewed in Chapter 8, the situation with regard to chromium dietary supplementation in animals has changed little in the last decade.

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2

Is Chromium Essential? The Evidence

Support for chromium being essential comes primarily from: (i) studies attempting to provide rats with chromium-deficient diets, (ii) studies examining the absorption of chromium as a function of intake, (iii) studies of patients on total parenteral nutrition and (iv) studies looking for an association between insulin action and chromium movement in the body (Table 2.1).

Chromium levels in tissues, food components and other biological samples reported prior to circa 1978 are problematic and should be ignored [12, 13]. Improvements in analytical techniques revealed several problems, including appreciable contamination of biological samples (as these samples were often homogenized in a stainless-steel blender); in fact, measured Cr levels reflected the levels of contamination not the actual tissue or fluid Cr concentrations, which were extremely small. Another major problem in atomic absorption experiments prior to 1978 was that workers were attempting to measure a tiny signal against a large background; a linear correspondence was actually found to exist between background absorbance and the 'apparent Cr content' of samples [12]. Currently, analyses of human blood and urine samples with Cr concentrations above 1 ppb should be considered suspect, unless the subjects are taking chromium supplements. Consequently, studies prior to 1978 utilizing patients who were believed to be Cr deficient based on Cr tissue or fluid concentrations and that reported Cr levels in tissues, foods or fluids of one order to several orders of magnitude too high must be

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Table 2.1 Evidence used to support an essential role for chromium.

Evidence	Ref.	Complications
Rats fed a Cr-deficient, high-sucrose or high-fat diet develop resistance, reversed by Cr administration	1	Diets not shown to be deficient in Cr; insulin pharmacological doses of Cr utilized
Patients on TPN develop diabetes-like symptoms, which are responsive to Cr	4 (review), 5 (review)	TPN solutions often rich in Cr; pharmacological doses of Cr utilized
Absorption of dietary Cr is inversely proportional to intake	6	Highly suggestive, requires reproduction
Factors that affect glucose metabolism alter urinary Cr loss	7–10, 11 (review)	May reflect insulin-sensitive movement of Fe(III), also may simply reflect increases in absorption associated with diabetes and insulin-resistance

discarded. Thus, with the exception of some ^{51}Cr-labelled tracer studies, the field of chromium nutritional biochemistry really began in the late 1970s. At present, chromium levels in tissues and biological fluids are usually determined by graphite furnace atomic absorption spectrometry, although neutron activation analysis and inductively coupled plasma-mass spectrometry(ICP-MS) can also be used [12]. Neutron activation and ICP-MS have been utilized with stable isotopes of Cr for determining Cr levels in tracer studies, in addition to the continuing use of radioactive ^{51}Cr.

Chromium is ubiquitous in foods but at very low concentrations, however, while processing, particularly in stainless-steel equipment, the concentration appears to increase; in fact, most of the Cr in some foods may come from processing [14]. Foods particularly rich in Cr (i.e. >100 ppb) include broccoli and black pepper [14] and certain beers [15]; however, values for vegetables must be considered carefully because of the variable amount of chromium that comes from soil contamination [16]. The low concentrations of chromium in food, the ease of contamination and the low adequate intake(AI) established for chromium make preparation of a low-chromium (or chromium-deficient if chromium is essential) diet difficult.

2.1 'CHROMIUM-DEFICIENT' DIET STUDIES WITH RATS

Prior to 2011, the most notable efforts of work with rats to generate a chromium-deficient diet had been reported by Anderson and co-workers. Rats in plastic cages (with no access to metal components) were given a diet consisting of 55% sucrose, 15% lard, 25% casein and vitamins and minerals, and providing 33 ± 14 μg Cr/kg diet [1]. The sucrose levels were provided in a theoretical attempt to induce Cr deficiency; dietary carbohydrate stress leads to increased urinary chromium loss (see below). To compromise pancreas function, low copper concentrations (1 mg/kg) were employed for the first 6 weeks; high dietary iron concentrations were used throughout to potentially aid in obtaining Cr deficiency. A supplemented pool of rats were given water containing 5 ppm $CrCl_3$; unfortunately, the volume of water consumed was not reported so that the Cr intake of the rats cannot be determined. Over 24 weeks, body masses were similar for both groups. At 12 weeks, Cr-deficient rats had lower fasting plasma insulin concentrations and similar fasting plasma glucose levels compared with supplemented rats; yet, both concentrations were similar after 24 weeks. In intravenous glucose tolerance tests after 24 weeks on the diet, plasma insulin levels tended to be higher in Cr-deficient rats; rates of excess glucose clearance were statistically equivalent. Glucose area above basal was reported to be higher in Cr-deficient rats; however, at every time point in the glucose tolerance test, the plasma glucose concentrations of each pool of rats were statistically equivalent, suggesting that the difference in area arises from a mathematical error. (These workers reported another study utilizing a high-sucrose diet in 1999, in which the plasma insulin levels were again observed to be elevated; however, the plasma glucose area was not [2].) Thus, a high-sucrose diet can lead to hyperinsulinemia, possibly reflecting defects in peripheral tissue sensitivity to glucose [1]. This research group also obtained similar results using a high-fat diet that contained 33 mg Cr/kg diet [3]. This diet also contained an altered copper content in the first six weeks. After 16 weeks on the diet alone, rats had higher fasting plasma insulin levels, but not fasting glucose levels, compared with rats also receiving drinking water containing 5 ppm Cr [3]. Similar results were obtained when the fasting insulin and glucose levels of the rats on the diet alone were compared with rats on a normal chow diet. Insulin and glucose areas after a glucose challenge were equivalent [3]. Thus, the high-fat diet appears to induce increased fasting insulin levels, which can be corrected with chromium administration.

Some calculations are in order to put this work into perspective. Humans lack signs of Cr deficiency with a daily intake of 30 μg Cr; assuming an average body mass of 60 kg, 30 μg per day correspond to 0.5 μg Cr/kg body mass per day. A 100 g rat eats about 15 g of food a day [17]. Now, 15 g (0.015 kg) of food containing 33 μg Cr/kg food provides approximately 0.5 μg Cr. Thus, 0.5 μg Cr/day for a 0.100 kg rat is 5 μg Cr/kg body mass per day, *ten times what a human intakes*. Thus, the 'low-Cr' diets provided by Striffler and co-workers [1–3] cannot be said to be deficient at all unless rats require more than ten times the chromium that humans do on a per kilogram body mass basis. Consequently, the effects of the diet cannot be attributed to Cr deficiency; the high doses of Cr in the Cr-supplemented rats can only be considered as having a pharmacological role on those rats whose physical condition were impaired by the high-sucrose or high-fat diets and/or the other mineral stresses.

The author's laboratory has recently tested the effects of a purified diet (AIN-93G without Cr in the mineral supplement, <30 μg Cr/kg diet) on male Zucker rats in metal-free cages for 16 weeks [18]. The rats also received the normal AIN-93G diet (i.e. with 1000 μg Cr added/kg diet) and the normal diet supplemented with an additional 200 or 1000 μg Cr/kg diet. The diets had no effect on body mass gain or food intake. The diets also had no effect on fasting plasma glucose levels or in 2-h glucose tolerance tests. A trend existed in fasting plasma insulin levels, although the only statistical difference was that insulin levels for rats on the AIN93-G diet without any added chromium was greater than that of the group receiving the greatest amount of Cr. This difference was also present in plasma insulin levels 30 and 60 min after a glucose challenge. Thus, with no added stresses, a purified diet with as little chromium content as possible does not result in symptoms that can be attributed to chromium deficiency, although effects on insulin sensitivity can be observed at supra-nutritional/pharmacological doses of chromium [18].

Establishing whether chromium is an essential nutrient is apparently not feasible from traditional nutrition studies, because developing a chromium-deficient but otherwise sufficient diet appears not to be possible. This could be because chromium is not an essential element or uniquely because such low amounts of chromium are required that generating a deficiency is not possible. Similarly, no genetic disorder that alters chromium transport or distribution or other function has been identified; the study of such disorders for other metals has pointed to the essential nature of those elements and the biomolecules which utilize the metal. How then can the potential essential nature of chromium (or other elements such as silicon, vanadium, arsenic or boron that have

been proposed to be essential but in such miniscule amounts that generating a dietary deficiency is impossible) be established? The most obvious method is establishing that a biomolecule containing the element *in vivo* performs an essential biological function at physiological levels of the element. For chromium, this has proved less than easy, as several mechanisms for chromium action at a molecular level have been proposed while none have been adequately established *in vivo* (see below). In fact, major questions shroud each proposal, and open scientific discussions of these issues and the other issues described above have proved to be quite difficult given the financial implications of the outcomes (see below).

2.2 TOTAL PARENTERAL NUTRITION

Evidence for an essential role for chromium in humans has also been taken from studies of patients on total parenteral nutrition(TPN) (reviewed in references [19] and [20]). Patients on TPN have developed impaired glucose utilization [19] or glucose intolerance and neuropathy or encephalopathy [20–22]. The symptoms were reversed by chromium infusion and not by other treatments. While limited to five individual cases, these studies have been interpreted as providing evidence of clinical symptoms associated with chromium deficiency that can be reversed by supplementation. Another patient on TPN who developed symptoms of adult-onset diabetes and hyperlipidaemia but died had low tissue chromium levels [23]. These incidences have been reviewed [5, 24]. Recently the effects of chromium supplementation on five patients on TPN requiring a substantial amount of exogenous insulin have been examined. Three subjects displayed no beneficial response while two showed a possible beneficial response to chromium supplementation [25]. Subjects received TPN containing 10 μg Cr/day followed by supplementation with an additional 40 μg Cr/day for 3 days and then restoration of the normal TPN.

Curiously the development of symptoms that were reversible by chromium supplementation does not correlate with serum chromium levels [24], indicating that either serum chromium levels are not an indicator of chromium deficiency or that another factor is in operation. Additionally, these incidences of diagnosed potential chromium deficiency have been questioned recently as they lack consistent relationships between the chromium in the TPN, time on TPN before symptoms appear, serum chromium levels and symptoms [26].

For this discussion, the most notable features of these studies are the level of Cr administered. In the cases where deficiencies were reported, the TPN solutions provided 2–10 μg Cr/day. For comparison, all the Cr in the TPN is introduced into the bloodstream, while only 0.5% of Cr in the regular diet is absorbed into the bloodstream. Thus, 30 μg of Cr in a typical daily diet present only ~0.15 μg Cr to the bloodstream. The TPN solutions are hence presenting 13–67 times the required amount of chromium; thus, based on these data, the TPN solutions cannot be considered Cr deficient. Subjects were, in turn, treated with 40–250 μg Cr/day added to the TPN solution to alleviate their conditions, clearly pharmacological doses as the largest dose provided 1.7×10^3 times more chromium than a standard diet. Consequently, the results with the insulin-resistant TPN patients can only be considered as providing evidence for a pharmacological role of chromium. The data are not relevant for examining whether chromium is an essential element.

Recently, three additional reports of beneficial effects from intravenous chromium administration have appeared [27–29]. The doses required were again large (200–240 μg/day [27], 60 μg/day [28] and 12 μg/day [29]) when one considers that this administration corresponds to the equivalent of 100% absorption so that the values should be multiplied by 100 for comparison against oral studies. Treatment resulted in improved glucose control and reduction in insulin needs. This is again consistent with a pharmacological role for chromium.

Not surprisingly, as TPN provides ten or more micrograms of chromium per day, TPN patients are accumulating chromium in their tissues [30,31]. Calls are appearing for the re-examination of the chromium levels in TPN solutions in terms of a need to reduce recommended levels [32].

2.3 CHROMIUM ABSORPTION VERSUS INTAKE AND THE TRANSPORT OF CHROMIUM BY TRANSFERRIN

The mechanisms of absorption and transport of chromic ions are poorly elucidated. Notably, little is known of the fate of Cr^{3+} taken orally. For example, essentially no data exist on the forms of chromium(III) in food as a result of its very low concentration. Similarly, the fate of dietary chromium at a molecular level in the digestive tract is also poorly known, although >98% passes through without being absorbed. This lack of knowledge is in stark contrast to that for the ferric iron (Fe^{3+}), with a similar charge to size ratio as Cr^{3+}. Absorption of iron takes place

in the proximal portion of the duodenum (reviewed in reference [33]). Dietary iron is probably primarily in the ferric form. Unlike Cr^{3+}, whose reduction potential is such that it should not readily be reduced under the conditions in the gastrointestinal tract, ferric ions can be reduced chemically or enzymatically by a brush border ferrireductase of the enterocytes. Subsequently, iron enters the enterocytes as ferrous iron via the transmembrane protein DMT1(divalent metal transporter 1); DMT1 is probably responsible for the entry of a variety of divalent metal ions. The transported iron is then stored or is released from the enterocyte by the basolateral transmembrane protein ferroportin [33]. Iron probably passes through the enterocyte in the ferrous state (Fe^{2+}) and is returned to the ferric state (Fe^{3+}) by ferroxidases for transport in the bloodstream by the protein transferrin (see below). Release of iron by ferroportin is carefully controlled; it is enhanced by interaction with ceruloplasmin and its membrane-bound analogue hephaestin [1]. Another protein, hepcidin can bind to ferroportin, targeting it for degradation; high body iron levels lead to increases in the production of hepcidin [33]. The failure of Cr^{3+} ions to be reduced requires a unique absorption system to be present for chromium, compared with other proposed essential metals ions, *if* chromium is actively absorbed and essential. However, the preponderance of evidence suggests that chromic ions are passively absorbed, that is, absorbed via simple diffusion.

Only a small percentage (<2%) of dietary Cr is absorbed, while the remainder is excreted in the faeces. Inorganic chromium salts are generally used to mimic dietary chromium and are absorbed to a similar extent, that is <2%. The most commonly used salt is chromium(III) chloride hexahydrate, which is actually the chloride salt of the trans isomer of the $[CrCl_2(H_2O)_4]^+$ cation (Figure 2.1). Chromium supplementation of the diet results in an increase in urinary chromium loss, and most of the absorbed chromium is rapidly excreted (see, for example, reference [34]).

Dowling *et al*. have examined the absorption of Cr^{3+} from an intestinal perfusate with added Cr as $CrCl_3$ [35]. A double perfusion technique was utilized in which the intestinal vasculature, from the superior mesenteric artery to the portal vein, and the intestinal lumen,

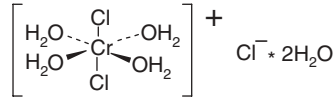

Figure 2.1 Structure of $CrCl_3 \cdot 6H_2O$.

from the duodenum to the ileum, were perfused simultaneously. Over a 10^2-fold range of Cr^{3+} concentrations (10–1000 ppb), Cr absorption was found to be a nonsaturable process. An average of 5.90% of the chromium of the intestinal perfusate was taken up, while 5.52% was transported into the vascular perfusate and 0.38% was retained in the small intestine. These results led to the conclusion that the Cr^{3+} was 'absorbed by the nonmediated process of passive diffusion in the small intestine of rats fed a Cr-adequate diet [35].' Previous, but methodologically much less rigorous, studies have generally come to similar conclusions. Studies by Donaldson and Barreras found for human subjects that 0.1–1.2% of an oral dose of Cr as $CrCl_3$ was absorbed while intestinal absorption of chromium in intestinal perfusion studies was so small (0.2–1.0%) that $CrCl_3$ could be used as a 'nonabsorbable' marker [36]. Mertz and co-workers found that gavage doses of $CrCl_3$ (0.15–100 μg Cr/kg body mass) were absorbed to the same extent by rats [37]. Mertz and Roginski had also reported the results of perfusion studies [38]. Absorption of chromium as $CrCl_3$ through the time course of the studies never reached equilibrium, suggesting a diffusion process. However, a dose dependence was observed with the greater doses leading to reductions of the rate of transport [38]. Yet, this study utilized an *in vitro* technique using inverted gut sacs in which substances of interest must follow an alternate, non-physiological route of absorption. Recently, a study in which rats were gavage dosed with $CrCl_3$ found ~0.2% absorption of Cr over a 2000-fold range of doses (0.01–20 μg Cr) [39]. Thus, the absorption of simple inorganic chromium(III) salts appears to occur via diffusion and not active transport.

However, the fate of dietary chromium(III) organic complexes could be different to that of the inorganic chromium salts if the complex (or some product thereof) absorbed is different from the form absorbed using inorganic chromium(III) salts. For example, the presence of added amino acids, phyate (high levels) and oxalate in the diet reportedly alter Cr uptake [40,41], as does ascorbic acid [42]. Low levels of phyate appear to have no effect on absorption [43]. Yoshida *et al*. have recently reported a comparative study on accumulation and excretion of chromium from $CrCl_3$ compared with chromium picolinate [44]. Rats were fed diets containing 1, 10 or 100 μg Cr as the chromium-containing compounds for 28 days. No effects were seen on body mass although 100 μg Cr as chromium picolinate lowered liver mass. Chromium accumulation was similar in liver and kidney but greater in the femur for $CrCl_3$. Chromium excretion increased with dietary chromium content. The rate of urinary excretion of chromium was constant with

diet content with chromium picolinate but fell with increases of dietary $CrCl_3$. Chromium picolinate, but not $CrCl_3$, raised serum aminotransferase levels. Yet none of these studies disproves that absorption is passive, only that the extent of chromium available for diffusion may be altered by the addition of these potential chelators to the diet and that the potential chelators may alter steps subsequent to absorption (see below). The possibility that complexes of chromium with organic ligands may have altered the absorption properties is part of the impetus behind the various Cr(III) complexes used or proposed as nutritional supplements; these supplements and their properties will be discussed in more detail in Chapter 7.

The absorption of Cr as a function of intake by humans is still widely cited as evidence for the essentiality of chromium, although this is based on a single study. Anderson and Koslovsky have reported an inverse relationship between dietary chromium intake and degree of absorption observed in human studies [6]. The data suggest that absorption of Cr varies approximately from 0.5 to 2.0% for Cr intakes of ~15–50 μg per day. This difficult to perform study is far from definitive and desperately requires repeating. For example, a distinct difference is found if the data are separated into male and female subjects (Figures 2.2 and 2.3).

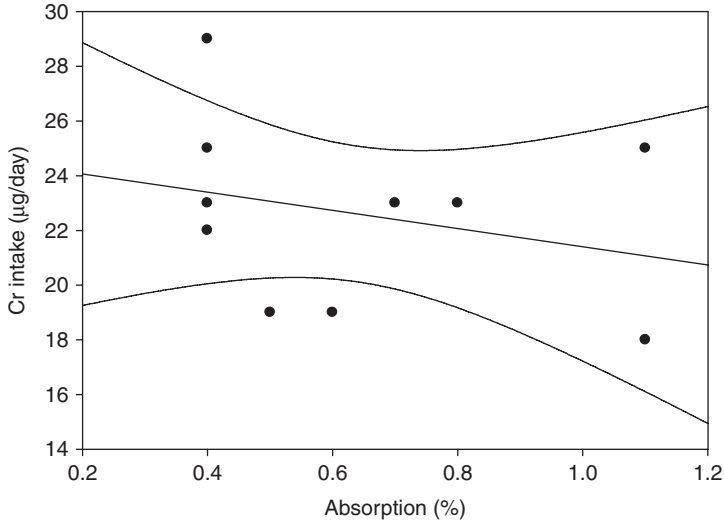

Figure 2.2 Chromium absorption by adult male subjects at varying Cr intakes. Data represent 7-day averages and are adapted from reference [6]. The curves represent the 95% confidence limit for best-fit line. Reproduced from [6] © 1985 ANS Journals.

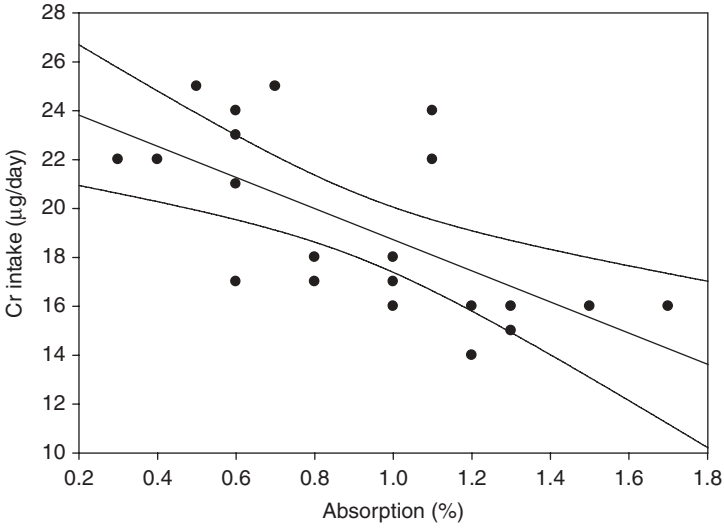

Figure 2.3 Chromium absorption by adult female subjects at varying Cr intakes. Data represent 7-day averages and are adapted from reference [6]. The curves represent the 95% confidence limit for best-fit line.

For males, no statistical variation occurs for chromium absorption as a function of intake, while an apparent inverse trend is observed for the female subjects. However, these data are in striking contrast to this same lab's studies reported two years earlier [34]. Chromium absorption was determined to be ~0.4% for free-living individuals; when Cr intake was increased by over fourfold, urinary chromium excretion increased over fourfold while maintaining ~0.4% absorption of chromium for both males and females. The difference between the two studies lies in the range of Cr intakes of ~15–50 µg per day for the former and ~60–260 µg per day for the latter, suggesting that an inverse relationship between Cr intake and absorption, if it exists, exists only at the lowest portion of the range of intakes.

The authors of the human study have also examined absorption of chromium by rats [45]. The observed results, consistent with other researchers utilizing rats, displayed no effect of intake on absorption. The authors proposed that Cr homoeostasis was maintained at the level of excretion, not absorption, and suggested Cr uptake by rats may be different from that in humans. However, the assumption that chromium is in a state of homoeostasis is unproven. In fact, the extent of absorption appears to determine the amount of urinary excretion. As absorption

appears to be directly determined by intake (as the percentage absorption is constant), the amount of chromium intake determines the amount of chromium excretion. No evidence for chromium homoeostasis can be derived from these studies (with the exception of the data for female humans). Studies on the absorption and excretion of chromium by insulin-resistant and diabetic rats support these conclusions (see Chapter 5).

Thus, in summary, Cr appears to be absorbed by diffusion, not actively transported. The process of chromium absorption could possibly be different in humans (i.e. at least females) from that in rats, but this would seem unlikely and would require more research for this to be firmly established. If chromium intake by humans is active, this transport system would only play a significant role at low chromium intake and would be overwhelmed by diffusion at higher intakes. Clearly, chromium absorption in rodents is not inversely proportional to intake, distinct from that of other reported essential elements. Based on absorption studies, at least in rodents, chromium does not appear to be an essential element; and this probably extends to other classes of mammals.

Another interesting conclusion that can be drawn from the intestinal perfusate studies of Dowling and co-workers is that chromium appears to be actively transported out of the intestinal cells, as ~94% of the chromium entering the cells was cleared from the cells (leaving only ~6% behind to be stored). As mentioned above, no transporter is known for chromium. However, a potential candidate has recently been examined [46]. This possibility is that Cr^{3+} bound to some chelating ligand is transported by monocarboxylate transporters (MCT) as occurs for chelated Al^{3+} (a non-essential metal ion) [47,48]. Chromium complexes with free carboxylates are known; for example, the complex formed between Cr^{3+} and EDTA(ethylenediaminetetraacetate) is the violet [Cr(Hedta)(H_2O)], where one of the carboxylate groups is protonated [49]. More importantly, the common complex of Cr^{3+} and citrate possesses a free carboxylate in the solid state [50] and in solution [51]. This idea is also supported by later perfusate studies by Dowling *et al.* [41]. When amino acids were left out of the solutions perfused through the intestinal lumen, less chromium was transported into the vascular perfusate while additional chromium was retained by the intestinal mucosa. Thus, potential ligands for the chromium may need to be transported into the intestinal cells for chromium to be efficiently transported out of the cells [41]. However, the recent studies showed that monocarboxylate transporters are not involved in Cr^{3+} transport from endosomes in hepatocytes [46].

The fate of chromium in the bloodstream is somewhat better elucidated. *In vivo* administration of chromic ions to mammals by injection results in the appearance of chromic ions in the iron-transport protein transferrin. In 1964, Hopkins and Schwarz established that $^{51}CrCl_3$ given by stomach tube to rats resulted in $\geq$99% of the chromium in blood being associated with non-cellular components [52]; 90% of the Cr in blood serum was associated with the β-globulin fractions; 80% immunoprecipitated with transferrin [52]. *In vitro* studies of the addition of chromium sources to blood or blood plasma also result in the loading of transferrin with Cr(III), although under these conditions albumin and some degradation products also bind chromium [53, 54]. *In vitro* studies suggest that transferrin may be important for transport from the intestines [55]; Dowling *et al*. found that including transferrin in the vascular perfusate of their double perfusion experiments increased transport of chromium from the intestines. Similar results were also obtained with albumin [55]. One must be careful to distinguish experimental design when examining chromium binding to serum proteins. When chromium is added to blood *in vitro*, chromium binds to both transferrin and albumin; *in vivo* only transferrin binds appreciable quantities of chromium (see for example reference [56]).

Transferrin is an 80 000 Da blood serum protein that tightly binds two equivalents of ferric iron at neutral and slightly basic pH values. The protein exhibits amazing selectively for iron(III) in a biological environment because the metal sites are adapted to bind ions with large charge to size ratios, and it is primarily responsible for the transport of iron through the bloodstream. In humans, transferrin is maintained only approximately 30% loaded with iron on average and consequently has been proposed to potentially carry other metal ions [57]. The similar charge and ionic radii of chromic ions to ferric ions suggests that chromic ions should bind relatively tightly to the protein. *In vitro* studies of the addition of chromic ions to isolated transferrin reveal that chromium(III) readily binds to the two-metal-binding sites, resulting in intense changes in the protein's ultraviolet spectrum [58–64]. Two equivalents of (bi)carbonate are concomitantly bound. The amount of bicarbonate has been determined by measuring the release of CO_2 after the addition of acid, resulting in 1.09 equivalents being released per bound Cr^{3+} for the human protein [58]. The changes in the ultraviolet spectrum suggest that each chromic ion binds to two tyrosine residues from the protein, strongly indicating that the chromic ions bind in the two ferric ion binding sites. For human serum transferrin, the dichromium protein has an ultraviolet absorbance maximum at 293 nm [58]. The binding of tyrosine residues has been

confirmed by Raman spectroscopy [61]. Human serum transferrin with two bound chromic ions is pale blue in colour with visible maxima at 440 and 635 nm [58]; dichromium lactoferrin (milk transferrin) has been described as grey–green in colour and has maxima at 442 and 612 nm ($\varepsilon = 520$ and 280 M^{-1} cm^{-1}, respectively) [62]. The visible spectra indicate that the d^3 chromic centres are in pseudo-octahedral environments. Variable temperature magnetic susceptibility studies confirm that the chromium centres are trivalent (i.e. $S = 3/2$) [58].

These ultraviolet spectral changes have been used to determine the effective binding constants of chromium to conalbumin (chicken egg white transferrin), $K_1 = 1.42 \times 10^{10}$ M^{-1} and $K_2 = 2.06 \times 10^5$ M^{-1} [65]. Notably the binding of chromium to one of the two sites on transferrin is five orders of magnitude higher than for the other. The two chromium-binding sites in human transferrin can be distinguished by electron spin resonance(ESR) spectroscopy (frozen solution at 77 K), and only chromic ions at one site can be displaced by iron at near neutral pH [60, 61]. Below pH 6, only one site binds chromium [62]. Unlike the binding of other metal ions to transferrin, Cr^{3+} apparently binds first to the C-terminal metal binding site, rather than the N-terminal site (for a review see reference [66]). Mixed metal complexes (and their EPR spectra) with Cr^{3+} in its tight binding site and Fe^{3+} and VO^{2+} in the other metal-binding site have been described [60]. Given the binding constants for Cr^{3+} and that transferrin is maintained on average only 30% loaded with ferric ions, the protein appears to be primed to be able to transport chromium through the bloodstream.

Recent reports on the effects of insulin on iron transport suggest that transferrin is actually the major physiological chromium transport agent. Plasma membrane recycling of transferrin receptors is sensitive to insulin; increases in insulin result in a stimulation of the movement of transferrin receptor from vesicles to the plasma membrane [67]. The receptors at the cell surface can bind metal-saturated transferrin, which subsequently undergoes endocytosis. The metal ions are released when the pH of newly formed vesicles drops as protons are pumped into the endosomes. Based on these results, a mechanism for chromium transport has been proposed (see Chapter 6) [68]. Chromium-loaded transferrin has been demonstrated to transport chromium *in vivo* [69, 70]. Injection of ^{51}Cr–transferrin into rats results in incorporation of ^{51}Cr into tissues. Injection of labelled transferrin and insulin results in a several fold increase in urinary chromium [70]. Thus, transferrin, in an insulin-dependent fashion, can transfer Cr to tissues from which it is excreted in the urine.

Cr_2–transferrin serves as an inhibitor for the binding of Fe_2–transferrin to the surface of reticulocytes [71], presumably at the site of binding transferrin to transferrin receptor. The Cr-loaded human transferrin is a better inhibitor than apotransferrin or Cu^{2+}-loaded transferrin but not as good as mono- or diferric transferrin [71].

The movement of chromium from the gastrointestinal tract through the bloodstream can be summarized in Figure 2.4. Cr^{3+} bound to water or other small ligands from the diet is absorbed into cells lining the intestines by passive diffusion. Around 5% of the absorbed chromium may be stored in the cells while most chromium is actively transported from the cells by an unidentified transporter, presumably as a Cr(III)–chelate complex. In the bloodstream, Cr^{3+} is bound to transferrin. The fate of Cr^{3+} from the bloodstream to the tissues and ultimately to the urine will be examined in Chapter 6.

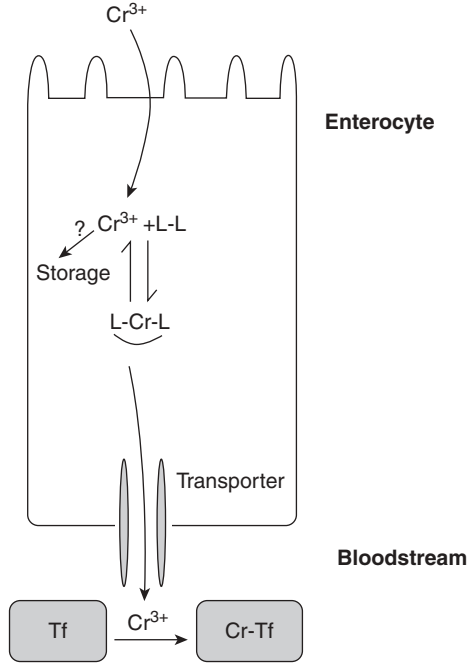

Figure 2.4 The movement of chromium from the gastrointestinal tract to the bloodstream. Cr^{3+} from the diet is absorbed into enterocytes lining the intestines by passive diffusion. Chromium presumably binds to small chelating ligands (L-L) and possibly other cell components. Around 5% of the chromium may be stored in the cells, while most chromium is actively transported from the cells by an unidentified transporter, presumably as a Cr(III)–chelate complex, $Cr(L\text{-}L)_x$. In the bloodstream, Cr^{3+} is bound to transferrin (Tf) to form a chromium–transferrin complex (Cr–Tf).

2.4 CHROMIUM MOVEMENT RELATED TO STRESSES

A role for Cr in the body is also suggested by an association between insulin action and increased urinary Cr loss, although other explanations may be possible. In human euglycemic hyperinsulinemic clamp studies, Morris *et al.* have shown that increases in blood insulin concentrations following an oral glucose load result in significant decreases in plasma chromium levels; a subsequent infusion of insulin led to further chromium losses [7]. Within one and one-half hours after the increases of blood insulin concentrations, blood chromium levels started to recover. Patients also showed increased urinary chromium losses during the course of the experiments, with the amount of chromium lost roughly corresponding to the amount of chromium estimated to be lost from the intravascular space [7]. Thus, increases in glucose, which result in increases in plasma insulin concentration, lead to a movement of chromium from the bloodstream ultimately to the urine; chromium in response is more slowly moved from body stores to the bloodstream. Numerous studies have demonstrated that chromium is released in urine within 90 min of a dietary stress, such as high sugar intake [8, 72–76]. As glucose tolerance resulting from repeated application of carbohydrate stress decreases, the mobilization of chromium and resulting chromium loss have been shown to decrease [74].

As previously described, chromium from the diet apparently is transported from the bloodstream to the tissues by the iron transport protein transferrin. A pool of the iron transported by transferrin is insulin-sensitive. Is chromium(III) mobilization in response to insulin simply the result of its similarity to iron(III), and thus its binding to transferrin, or is this physiologically relevant? This clearly is an area in need of further investigation.

Recent studies have made a link between adult-onset diabetes and chromium. Studies examining serum and urine chromium concentrations of healthy and diabetic subjects (using analytical techniques developed after circa 1980 and reporting serum or urine chromium levels of healthy subjects less than approximately 0.5 $\mu g\ l^{-1}$) [77] demonstrate that the chromium levels of diabetics are distinctly different from those of healthy individuals. Morris and co-workers have reported that serum chromium levels of adult-onset diabetics are approximately one-third lower than those of healthy subjects, while urine chromium concentrations are about twice as high [78–80]. In the first two years of the onset of diabetes, serum chromium levels inversely correlated with plasma glucose concentrations, but this trend disappeared for patients

with the disease for longer duration. (Ding *et al.* have examined the serum and urine chromium concentrations of elderly patients with diabetes for 20–30 years (type of diabetes was not indicated) and observed lower serum chromium levels and lower urine chromium levels when patients were 60–70 or more than 70 years of age [81]. However, elsewhere in the article the authors reported lower urine chromium levels for diabetics than controls and have more patients reported in some tables than are indicated in the experimental section, making the article noninterpretable.) Increased urinary output in adult-onset diabetics would be consistent with studies of healthy individuals that indicate increased urinary chromium output and decreased serum chromium levels in response to increases in serum glucose or insulin [8, 72–76]. Notably, Anderson *et al.* have shown in a double-blind crossover, placebo-controlled study that serum chromium levels reflect chromium intake but reported no effect by a glucose challenge [82]. However, they only used serum glucose values 90 min after glucose treatment; in studies with multiple time points from 0 to 180 min after glucose treatment, serum chromium decreases but is restored to near original concentrations by 90 min [7, 75, 82]. Rat models of diabetes excrete greater amounts of chromium than their healthy counterparts [83].

Thus, type 2 diabetes is associated with abnormally low serum chromium and high urine chromium levels, possibly related to elevated serum glucose and insulin levels. Recent studies have shown that type 2 diabetic rats and type 1 diabetic rats have increased urinary chromium loss as a result of their diabetes but this increased urinary chromium loss is offset by increased absorption of chromium [9,10]. In fact, given, as described previously, that chromium absorption in rats is diffusion controlled, increases in absorption resulting from the diabetes probably directly account for the greater urinary chromium loss. (How diabetics have more movement of chromium across the intestinal lining is an area worthy of study.) Thus, any increases in urinary chromium loss associated with insulin resistance or diabetes are offset by increased absorption. In other words, increased chromium absorption in diabetics results in greater urinary chromium loss. Consequently, supplementing the diet with nutritionally relevant quantities of chromium is not anticipated to have any beneficial effects. Similarly, beneficial effects on plasma variables such as cholesterol, triglycerides and insulin concentration, from supra-nutritional doses of Cr(III) complexes should not arise from alleviation of chromium deficiency. These beneficial effects must arise from pharmacological effects of high doses of Cr(III) (see Chapter 5).

Three other conditions reported to result in increased urinary chromium loss are trauma, exercise and pregnancy. In the case of trauma [84, 85], urinary Cr excretion is very high but appears to decrease rapidly. Effects of chromium intake are difficult to analyse as intake varies dramatically depending on patient treatment. The appropriate level of chromium in TPN solutions has been an issue of debate and may become one again (see above). Acute exercise-induced changes associated with increased glucose utilization have been found to result in increased urinary chromium excretion in several human studies [86–90], but not all [91,92]. In a recent review, Clarkson determined that insufficient evidence of any beneficial effects existed to recommend chromium supplementation for athletes [93].

Unfortunately, data on any potential relationship between chromium and pregnancy and especially gestational diabetes are sparse, especially after 1980 when reliable analytical techniques to determine tissue and fluid chromium concentrations began to be utilized. Patients in the first half of pregnancy have been reported to have higher chromium excretion [94]. Patients in the second half of pregnancy had urinary chromium levels 30% higher than controls, but the difference was not significant. The concentration of chromium in hair from women with gestational diabetes appears to be lower than that of controls [95]. Another recent report indicates that blood and scalp hair chromium levels are lower in gestational diabetes mothers and their infants and that urinary excretion of chromium is higher. Other metals, such as manganese and zinc, were examined, and similar effects were observed [96]. At the International Symposium on the Health Effects of Dietary Chromium, Jovanovic presented results of chromium supplementation of women with gestational diabetes [97]. Chromium was reported to lower glucose and insulin levels compared with controls. If the results of this study are reproduced by additional studies, this could have significant implications for treatment of this condition. Padmavathi *et al*. have reported that providing a chromium 'restricted diet' to pregnant rats led to increased body mass and fat percentage in offspring; addition of 1 mg Cr/kg diet alleviated the effects [98]. However, the diet contained 0.51 mg Cr/kg diet, similar to the content of standard rat chow; thus, the rat diet was not restricted, and the observed effects were from chromium supplementation at a pharmacological level [99]. Padmavathi *et al*. have also claimed that chromium 'restriction' to pregnant rats may irreversibly impair muscle development and glucose uptake by muscles of offspring [100], but the study suffers from the same flaw – the investigators were examining the effects of pharmacological doses of chromium to rats given a sufficient diet.

But, is chromium essential? What is actually meant by essential must first be examined. The definition of an essential element, as reviewed by Nielsen [101], has changed over time. Before the 1980s, the consensus definition for an element to be essential was 'a dietary deficiency must consistently and adversely change a biological function from optimal, and this change is preventable or reversible by physiological amounts of the element'. By this definition, the evidence for essentiality of chromium is ambiguous at best. Not only are Americans not chromium deficient, but generating a chromium-deficient diet (for humans or animal models) has proven extraordinarily difficult, as chromium is ubiquitous in foods at very low concentrations. Additionally, because physiological/dietary levels of Cr are so low, studies in which Cr has been added to the diet add supra-nutritional levels, suggesting pharmacological effects of Cr rather than nutritionally relevant effects. Thus, without additional and conclusive evidence, chromium cannot be considered an essential element by this definition.

Another definition for essentiality has appeared recently, which requires demonstration of a biological role for an element at a molecular level. That is, the element presumably must bind specifically to a biomolecule(s) to have an effect. If the biomolecule(s) can be identified and the mode(s) of action of the biomolecule containing the element established at physiologically relevant levels of the element, then the element is essential. Also by this definition, chromium cannot be considered an essential element (see Chapter 6) as evidence at a molecular level currently is insufficient at best. However, significant research effort has been focused in this area in the last 20 years and may lead ultimately to a resolution of this issue. Thus, by a nutritional definition or a biochemical definition, chromium cannot be considered an essential element at this time.

Nielsen [102] has also promoted some additional terms to address 'possibly essential elements': conditional essentiality, pharmacologically beneficial and nutritionally beneficial. These terms can be of use in describing the status of chromium. Conditionally essential refers to an element that is only indispensable under certain pathological conditions. Pharmacologically beneficial elements alleviate a condition other than nutritional deficiency of that element or alter biomolecules in a therapeutic manner. Nutritionally beneficial elements are similar in action to pharmacologically beneficial ones; however, amounts closer to the dietary intake are required for the beneficial effect. While chromium at doses can have beneficial effects in diabetic and insulin-resistant rodent models, data do not support it being essential in these models. The

dose required for these beneficial effects is clearly physiological, rather than nutritional. Thus, following these definitions, chromium is probably pharmacological beneficial, although further research could potentially demonstrate that Cr is essential. (Nielsen [102] has recently drawn his own conclusions on the status of chromium and stated that 'Chromium should not be classified as an essential element' and 'the best classification for chromium is that it is a nutritionally or pharmacologically beneficial element.')

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3

The Story of Glucose Tolerance Factor (GTF)

3.1 THE 'IDENTIFICATION' OF GTF

The fields of chromium nutrition and biochemistry recently celebrated their 50th anniversary [1]. Yet, this anniversary was bittersweet, as the status of chromium as an essential nutrient is probably about to be retracted. Unfortunately, from the vantage point of hindsight, much of the original data on which chromium was designated an essential element were ambiguous or seriously flawed methodologically. The problems in this area and how things have reached the current point of understanding requires a thorough review of the history of these particular fields.

The field of chromium biochemistry started in 1955 when Mertz and Schwarz reported feeding rats a *Torula* yeast-based diet that resulted in the rats apparently developing impaired glucose tolerance in response to an intravenous glucose load (in addition to previously identified necrotic liver degeneration) [2]. Rats on the diet with *Torula* yeast as the sole protein source had a clearance rate of excess glucose of 2.8% per minute in contrast to rats on a basal diet that have a rate of 4.1%. (Excess glucose is defined as glucose above background concentrations before giving the glucose challenge.) The challenge consisted of administering an intravenous injection of 1250 mg glucose per kg body mass as a 50% aqueous solution after an 18 h fast. The glucose intolerance was at first assumed to be a symptom of the liver disease. Shortly thereafter a dietary factor (selenium) was discovered that could reverse the liver

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disorder but not the glucose intolerance; thus, the authors believed they had identified a new dietary requirement absent from the *Torula* yeast-based diet and responsible for the glucose intolerance, which they coined glucose tolerance factor or GTF [3].

These researchers [4] followed their report in 1959 by identifying the active ingredient of 'GTF' as Cr^{3+}. Inorganic compounds containing Li, Be, B, F, Ti, V, Mn, Co, Ni, Cu, Zn, Ge, As, Se, Br, Rb, Sr, Y, Zr, Mo, Ru, Rh, Pd, Ag, Cd, Sn, Sb, I, Cs, Ba, La, Ce, Ta, W, Os, Ir, Au, Hg, Tl, Bi, Th and U (200–500 μg/kg body mass) could not restore glucose tolerance, while several inorganic Cr(III) complexes (200 μg Cr/kg body mass) restored glucose tolerance from a $\leq$2.8% per minute rate of removal of intravenously injected glucose to the approximately 4% rate of the control rats. Brewer's yeast and acid-hydrolysed porcine kidney powder were identified as natural sources of 'GTF', and the active (i.e. effective in reversing the inability to handle the glucose load) ingredient could be concentrated from these materials by physical and chemical means [4]. When given by stomach tube (500–1000 μg/kg body mass), the intact materials and the concentrates could restore proper glucose metabolism in rats on the *Torula* yeast-based diet. Although the separation means used to isolate 'GTF' were not described in detail, 'GTF' was found to be water-soluble, extractable with phenol and isobutanol and absorbable on charcoal and ion-exchange resins.

From the benefit of 50 years of hindsight, these studies are deeply flawed, despite the success of similar studies in identifying other dietary requirements. Unfortunately, for example, the Cr content of the diet was not reported (although the experimental procedures at the time would not have likely produced the correct value). Additionally, the rats were maintained in wire-mesh cages, possibly with stainless-steel components, allowing the rats to obtain chromium by chewing on these components. Consequently, the actual Cr intake of the rats in these studies is impossible to gauge, putting into great question the suggestion that the rats were Cr deficient. The use of the large amounts of the metal ions is also of concern. As will be discussed in Chapter 5, large doses of chromium may have pharmacological effects, which may or may not be related to any nutritional requirements. These doses are probably about 10^3 times the typical daily content of a rat's diet. Questions about data handling and the significance of the effect observed from the chromium treatment have also been raised (see below). As a result the *Torula* yeast diet is no longer assumed to be a Cr-deficient diet or used in Cr research, caging should be carefully described and Cr analysis of diets should be

required (yet publications still appear without the Cr content of basal diets being reported).

Considerable confusion in terminology in terms of the use of 'glucose tolerance factor' has arisen and continues to this day. Studies over the 50 years have more often than not failed to distinguish the difference amongst the inorganic ion Cr^{3+}, Cr(III) complexes and the biologically active form of chromium (i.e. the naturally-occurring biomolecule(s), which has an inherent function when containing bound chromium(III), *if such exists*) and called each a glucose tolerance factor. As originally proposed in 1957, GTF is a substance that is involved in maintaining normal glucose, prevents and cures impairment of glucose removal when given in the diet or by stomach tube and which results in impairment of intravenous glucose tolerance when it is deficient in the diet [3]. If one *assumes* that chromium is related to GTF and follows this definition, then *GTF can only be the chromic ion*, Cr^{3+}. The chromic ion was the only species in common between the extracts and inorganic compounds added to the *Torula* yeast diets. In publications soon after 1959, Mertz and Schwarz equated GTF and trivalent chromium [5, 6]. However, thereafter 'GTF' takes on a different usage. As chromium must presumably interact with some organic biomolecule(s) to manifest an effect(s) in mammals, attempts were subsequently made to identify this (these) species. Unfortunately, the products of such attempts have also been termed 'GTF.' The situation was not helped in 1980s and early 1990s when nutritional supplements containing 'glucose tolerance factor' were widely marketed; these products generally contained yeast extracts and synthetic complexes of Cr^{3+} and nicotinate, adding to the confusion. To distinguish in the use in the literature between organic chromium(III) complexes termed 'GTF' and Cr^{3+} being referred to as GTF, 'GTF' when used to indicate a chromium(III)–organic ligand complex will be enclosed in quotation marks.

Research just prior to and in the 1960s was consistent with these early results of Schwarz and Mertz. Continuing studies using rats fed a variety of diets found that rats on some diets in addition to the *Torula* yeast-based diet apparently possessed low glucose removal rates; the glucose removal rates could be improved by addition of 'GTF' concentrates. The concentrates were prepared from dried Brewer's yeast, an enzymatic digest of Brewer's yeast, or dried defatted porcine kidney powder [7]. However, the methods of preparation of these materials were not described; while the amounts of the concentrates added to the diet where given, the amounts of Cr that these concentrates

contained were not included. Again the Cr contents of the diet were not reported. This leaves open the possibility that another agent other than chromium found in the concentrates and lacking in the diets could be responsible for the effect. A study with glucose intolerant rats on the *Torula* yeast diet found that chromic chloride ($Cr(III)Cl_3 \cdot 6H_2O$ or actually *trans*-dichlorotetraaquachromium(III) chloride dihydrate) dissolved in the drinking water (5 ppm Cr) or as an aqueous solution given intravenously (1 μg Cr/kg body mass) could reverse the condition, emphasizing the apparent association between Cr^{3+} and GTF [8]. An eye lesion was identified as a common symptom of rats fed a diet considered to be Cr deficient (<100 μg Cr/kg) [9].

Cr^{3+} (as a solution of hexaureachromium(III) chloride or a neutralized solution of chrome alum) in the presence of insulin was reported *in vitro* to enhance glucose uptake by epididymal fat tissue of 'GTF'-deficient rats in a concentration dependent fashion [5]. A commercial rat chow was used in this study as it was found to lead to reduced excess glucose removal rates in glucose tolerance tests similar to the *Torula* yeast diet [7]. (A conclusion which requires the assumption that only 'GTF' deficiency can lead to the effect in glucose tolerance tests that is reversible by addition of Brewer's yeast to the diet.) Similarly, the uptake of galactose by the fat tissue was enhanced by Cr^{3+} (chrome alum) [10], and the production of CO_2 by the fat tissue was found to be potentiated by insulin and Cr^{3+} (hexaureachromium(III) chloride) [11]. This led in part to the postulation of an association between chromium and insulin and the conclusion that 'GTF' bound to insulin and insulin receptor will form a ternary complex, stimulating insulin's ability to signal cells [12, 13].

Other evidence came from studies of the effects of chromium and insulin on mitochondria [14, 15]. Polarographic studies with *millimolar* concentrations of chromic chloride indicated that chromium forms complexes with insulin and mitochondria [14]; these complexes, however, formed at non-physiological concentrations of Cr^{3+} far above physiological concentrations (as was indicated by the authors), making any relevance to any natural system unlikely at best. Furthermore, the observation that the combination of insulin and Cr^{3+} (as a chromic chloride solution) causes swelling of rat liver cell mitochondria [15] raises some caution. Hydrated chromic ions or polynuclear complexes of the type $[Cr_x(OH)_y(H_2O)_z]^{n+}$ (which form when chromic ions are dissolved in water at near neutral pH) must form a complex with insulin and/or a component of mitochondria to be the biologically active form of chromium for this study to be physiologically relevant. However, neither appears to be the case.

3.2 BREWER'S YEAST 'GTF'

Nearly all of the attempts to isolate and characterize 'GTF' have involved Brewer's yeast, identified as a source of 'GTF' in the 1959 study. 'GTF' from Brewer's yeast, isolated by 'extraction with ethanol and subsequent chromatography on activated charcoal', was shown by Evans, Roginski and Mertz to bind to insulin [16]. Acetylation of insulin, but not 'GTF', impaired binding.

The next attempts at further characterizing isolated Cr complexes from Brewer's yeast were reported by Evans and co-workers in 1973 [17]. Brewer's yeast grown in a ^{51}Cr-containing media was extracted by stirring for 4 h with an equal volume butanol–H_2O mixture. The aqueous component containing 90% of ^{51}Cr from the isolated yeast was freeze-dried. The powder was extracted with water, and the soluble components were separated by G-25 size-exclusion chromatography or ion-exchange chromatography. The ^{51}Cr was associated with a 400–600 molecular weight, anionic species, whose organic component appeared to be synthesized by the yeast. Infrared studies suggested that the material contained a carboxylate functionality, and the material gave a positive ninhydrin test, suggesting the presence of free primary amine groups, such as amino acids or the amino terminus of a peptide. The material was also found to be comprised of at least six amino acids tentatively identified as leucine or isoleucine, proline, valine, alanine, serine and either glutamic or aspartic acid. The material when administered orally to rats (2 ng Cr) resulted in better chromium absorption than when chromic chloride was given [17].

Mertz and co-workers reported the details of the isolation of Brewer's yeast 'GTF' in 1977 nearly 20 years after the initial report [18]. Brewer's yeast was extracted with *boiling* 50% ethanol. The ethanol was removed under vacuum, and the aqueous residual was applied to activated charcoal. Material active in bioassays (see below) was eluted from the charcoal with a 1 : 1 mixture of *concentrated ammonia* and diethyl ether. After removal of the ammonia and ether under vacuum, the resulting solution was hydrolysed by *refluxing for 18 h in 5 M HCl*. Finally, the HCl was removed under vacuum, the solution was extracted with ether and the pH of the solution was adjusted to 3. The cationic orange–red material was further purified by ion-exchange chromatography [18]. Unfortunately, these incredibly harsh conditions would have destroyed any proteins, peptides, complex sugars or nucleic acids that initially could have been associated with the chromium. Thus, the possibility that the form of chromium recovered after the treatment resembles the

form in the yeast is remote at best. Yet, these studies are even further complicated.

The isolated 'GTF' possessed a distinct feature at 262 nm in its ultraviolet spectrum, while mass spectral studies (no data presented) indicated the presence of a pyridine moiety [18]. This led to the identification of nicotinic acid as a component of 'GTF'; nicotinic acid was sublimed from the material (no experimental information given) and identified by extraction with organic solvents (no data presented). Consequently, no data were actually presented that nicotinic acid is associated with 'GTF'; this should be remembered when studies of Cr nicotinate and Cr picolinate are described elsewhere in this volume. Amino acid analyses indicated the presence of glycine, glutamic acid and cysteine as well as other amino acids, although the relative amounts were not reported. The results were remarkably interpreted to indicate that 'GTF' was a complex of Cr, nicotinate, glycine, cysteine and glutamate.

The properties of 'GTF' were compared with those of a synthetic complex(es) prepared from Cr^{3+} and nicotinic acids and the above amino acids [18]. The material was found to possess an approximate composition of 1 Cr : 2 nicotinate : 2 glycine : 1 glutamate : 1 cysteine, but these components only accounted for 88% of the material. The synthetic material and the yeast material possessed similar ultraviolet and infrared spectra. The synthetic material and the material from Brewer's yeast behaved similarly upon ion-exchange and size-exclusion chromatography (unfortunately no estimate of molecular weight was given), as the major component of each co-eluted. In paper chromatography experiments, the materials gave several bands, only one of which from each material was active in the bioassays and migrated with the same R_f value. The chromium in these active bands represented 11 and 6% of the total chromium for the synthetic and Brewer's yeast materials, respectively. Note that only 6% of the bulk material upon which the characterization studies were performed is the apparently active component of the bulk material; no chemical characterization studies were performed on the active species from the paper chromatography.

Interpretation of this work is simply impossible. Characterization of bulk materials of which only a tiny minority is active does not allow for deciphering the composition of the active component(s). Thus, one cannot assume that the amino acid analysis of the bulk product reflects the 'active' component. If nicotinate was detected in the bulk, it is not necessarily in the 'active' component. Because of the destructive isolation procedure used to obtain the chromium material from Brewer's yeast (that destroyed proteins from which the observed amino acids were

probably generated and also contained conditions under which nicotinate could have been chemically generated from other compounds), the nature of the form of chromium in Brewer's yeast was not successfully determined. The orange–red colour associated with both materials is also notable and could suggest that ammonia is present as a chromium ligand.

Based on this work, 'GTF' has been proposed to be a Cr(III)–glutathione–nicotinate complex as glutathione is a tripeptide of glutamate, glycine and cysteine; synthetic complexes made from the combination of Cr^{3+}, nicotinate and glutathione have been reported to have similar biological activity to Brewer's yeast 'GTF' and to bind tightly to insulin [19]. A three-dimensional structure has also been proposed for Brewer's yeast 'GTF' that has two *trans N*-bound nicotinic acid ligands and amino acids occupying the remaining four sites of an octahedral around the chromic centre [13]. This proposal, which has been reiterated numerous times in reviews and textbooks as the structure of the biologically active form of chromium, 'GTF,' has absolutely no basis. Yet, this study dominated the understanding of the apparent nature of the biologically active form of chromium for the next two decades.

The results of the 1977 study of Mertz and co-workers have not been reproduced in several laboratories [20–24], although conditions were varied in many of the later studies. Kumpulainen *et al*. [20] extracted Brewer's yeast with 50% ethanol, removed lipids by extraction with petroleum ether and then isolated Cr-containing species by size-exclusion, ion-exchange and thin-layer chromatography. The material isolated by Mertz and co-workers could not be found. None of the isolated components, whether they were anionic or cationic, bound insulin.

Mirsky *et al*. isolated a yellow, cationic chromium-containing species from commercial yeast extract powder [24]. The powder was initially extracted with a 1 : 1 butanol–H_2O mixture before being subjected to dialysis and DEAE-cellulose and Dowex 50W $\times$ 8 chromatography. The material had a molecular weight below 3500, an ultraviolet absorption maximum at 262 nm and stimulated fermentation by three strains of yeast. It was also assumed to be the yeast 'GTF' reported in 1977 by Mertz and co-workers even though no other efforts to determine the composition of the material were reported. Subsequently this material was shown to affect fermentation by facilitating glucose uptake [25]. The material also reportedly increased decarboxylation of pyruvate by yeast [26] and decreased blood glucose and fatty acids in rats with streptozotocin-induced diabetes without addition of exogenous insulin [27]. The 'GTF' also apparently increased 2-deoxyglucose incorporation

and increased growth in the yeast *Saccharomyces cerevisiae* in a fashion similar to mammalian insulin, leading to a proposal of a common mechanism regulating metabolic processes in yeast and mammals [28, 29]. Recent studies support the possibility that this yeast shares some parts of the signalling cascades regulating oxidative and non-oxidative glucose metabolism in response to glucose and insulin with insulin-sensitive mammalian cells [30–32].

Gonzalez-Vergara and associates also attempted to isolate 'GTF' from Brewer's yeast [21]. A single Cr-containing fraction was found; it was yellow but possessed an ultraviolet spectrum distinct from nicotinic acid. Spectral characteristics suggested the presence of tryptophan or a derivative; the authors concluded that 'GTF' is not a Cr–nicotinate complex. A follow-up study [22] found that a species with activity in the rat adipocyte assay for biological activity could be separated from the chromium-containing species. The chromium species possessed Cr(III) in a pseudo-octahedral environment of oxygen (possibly from phosphate ligands) and a molecular weight of 900 and was anionic. The active material possessed an ultraviolet feature at 254 nm and little more than the background level of chromium. The authors concluded that the effect of Brewer's yeast on insulin action in adipocytes might not necessarily be due to Cr-containing species.

Another attempt to identify 'GTF' from Brewer's yeast separated 11 apparently homogeneous Cr-containing species from the yeast [23]. The species were amphoteric, anionic and cationic. The four cationic species and one anionic species displayed some ability to stimulate yeast fermentation. Further investigation [33], however, revealed that all 11 species were artefacts formed between components of the growth media and chromic ions added to the media. The authors proposed that 'GTF' could no longer be regarded as a chromium complex.

Holdworth, Neville and co-workers have examined Brewer's yeast extracts extensively [34–37]. They too have been able to distinguish between Cr-containing fractions (either cationic or anionic) and a fraction active in *in vitro* biological activity assays using rat adipocytes or rat hepatocytes from control rats, Cr-deficient rats and rats on Cr-deficient diets supplemented with chromium and yeast. They concluded that the enhanced response in the presence of insulin comes from gamma-aminobutyric acid in the yeast extracts. No evidence was found that 'GTF' aided the binding of insulin to insulin receptor, such as by the formation of a ternary complex. The 'GTF'-like activity from non-Cr-containing fractions using the adipocyte assay required the cells to be from rats on a *Torula* yeast diet.

Most recently Hwang *et al.* [38] and also Simonoff *et al.* [39] showed that fractions of Brewer's yeast that stimulate glucose oxidation by rat adipocytes in the presence of insulin were distinct from fractions containing chromium. The rats were raised on a standard (i.e. Cr adequate) diet.

Efforts to look at this issue have continued. Beran *et al.* [40] attempted to use less harsh techniques than used originally to isolate the yeast 'GTF'; yeast were ruptured by sonication and separations were performed by reverse phase high performance liquid chromatography following absorbance at 260 nm and Cr by atomic absorption spectroscopy. The chromium compounds in the yeast could not be separated. Zetic *et al.* in 2001 reported an attempt at isolation of 'GTF' [41]. They extracted Baker's yeast with 0.1 M ammonium hydroxide. Unfortunately, they only used G-75 gel-filtration chromatography to attempt to purify the 'GTF,' resulting in only a crude separation of components such that their results are not interpretable in terms of the effects of any particular component of the yeast, regardless of the presence or absence of chromium.

3.3 BIOLOGICAL ACTIVITY ASSAYS

Up to this point in time, biological activity of a chromium-containing species referred to the ability of the species to potentiate the action of insulin to stimulate *in vitro* the metabolism of epididymal fat tissue from 'chromium-deficient' rats. 'Chromium-deficient' rats in this definition means rats on the *Torula* yeast diet or another diet giving rise to similar effects when rats are given glucose tolerance tests (which, as discussed earlier, have essentially no data supporting the rats actually being chromium deficient). The reproducibility and sensitivity of the assay for biological activity was improved in 1978 by Anderson *et al.* who showed that adipocytes isolated from epididymal fat tissue of 'Cr-deficient' rats could be utilized [42]. Replicate assays at a series of insulin concentrations could now be readily performed, allowing for detailed kinetics experiments. These assays showed that Brewer's yeast extracts and the synthetic Cr–nicotinate complexes described above potentiated the ability of insulin to stimulate glucose oxidation at a variety of insulin concentrations. The degree of stimulation also depended on the chromium concentrations of either the extract or synthetic complexes, leading to the conclusion that these compounds contain biologically active forms of chromium. However, further analysis of these results has

shown that the original interpretation is incorrect [43]. Addition of the chromium sources without adding insulin stimulated the metabolism of glucose; the stimulation due to insulin above this increased background was actually decreased. The chromium sources in fact made insulin less effective, consistent with components of the extracts binding to insulin (as described above) and not allowing the complexed insulin to bind to its receptor. An alternative explanation has been pointed out, in that the isolated adipocytes may not have been washed sufficiently to remove traces of insulin [44]; however, extrapolation of the insulin dose response curves indicates that insulin levels would have to be far too high in these preparations for this to be possible.

The use of the microbiological assay (yeast fermentation assay) to determine biological activity is interesting but also problematic. The yeast fermentation assay reportedly yields results that parallel rat fat pad assays when using Brewer's yeast 'GTF' as a Cr source [45]. However, as the active component of yeast extracts can be separated from chromium, what exactly this assay measures in regard to chromium is questionable.

3.4 PORCINE KIDNEY POWDER 'GTF'

The other source of GTF in addition to Brewer's yeast originally proposed by Schwarz and Mertz [4] was porcine kidney powder, which has received much less attention. In one of only two reports on isolating 'GTF' from porcine kidney or kidney powder after 1960, Haylock *et al.* identified one cationic, chromium-containing species from kidney powder [23]. The species was isolated and concentrated by ion-exchange chromatography (without a hydrolysis step). The material was extremely active in 'biological activity' assays that used the rate of fermentation of chromium-deficient yeast. In the second study, an oligopeptide termed low-molecular-weight chromium-binding substance (LMWCr) (see below) was isolated from porcine kidney and porcine kidney powder [46]; hydrolysis of this material produces species similar in properties to 'GTFs' from Brewer's yeast and kidney powder, suggesting the original reports of these 'GTFs' are on artefacts.

3.5 OTHER QUESTIONS REGARDING 'GTF'

The reproducibility and interpretation of other results associated with research on 'GTF' have been questioned. Woolliscroft and Barbosa have

examined the effects of a normal and a *Torula* yeast diet in intravenous glucose tolerance tests in rats [47]. They reproduced the results of Mertz and Schwarz; yet, observation of a significant difference in glucose metabolism between the two groups of rats depended on the method used to present the data, that is using total plasma glucose concentrations versus using excess plasma glucose concentrations. (Excess plasma glucose concentration refers to the total plasma glucose concentration minus the fasting plasma glucose concentration.) The effect was only statistically significant when excess plasma glucose was used. As calculating the excess plasma introduces error (this error was not considered in the statistical analyses), use of actual measured plasma glucose is the accepted practice. This places the observations by Schwarz and Mertz of an effect from the *Torula* yeast diet on glucose clearance rates into serious doubt.

Shepherd *et al*. examined the *in vitro* production of carbon dioxide and fatty acids from glucose by adipocytes from rats on a control diet or diets using either *Torula* yeast, Brewer's yeast or casein as the sole protein source [48]. Glucose utilization increased only for fat cells from rats fed the *Torula* yeast diet when the material isolated from Brewer's yeast, as described by Mertz and co-workers in 1977 [18], was added to the assay. The levels of amino acids and 22 out of 23 trace elements were comparable in all diets. Manganese content was low in all diets but especially low in the *Torula* yeast diet. These workers proposed that Mn deficiency might be responsible for the effects of the *Torula* yeast diet. This suggestion would appear to be contradicted by the results of the study by Schwarz and Mertz in 1959, where no effect on 'Cr-deficient' rats was observed when the diet was supplemented by Mn [4]. The *Torula* yeast diet of Shepherd *et al*. possessed 0.6 μg Cr/g dry mass of diet [48]. A *Torula* yeast diet used by Mertz, Anderson and co-workers has been reported to contain no greater than 0.040 μg Cr/g dry mass [43], a difference in Cr content of greater than 15-fold. Irregular intakes of minerals other than chromium can lead to changes in glucose tolerance or insulin resistance and can be a concern in experimental diets. Thus, this emphasizes the problem of the lack of knowledge of the Cr content of the original *Torula* yeast diet of Schwarz and Mertz.

3.6 CONCLUSIONS ABOUT GTF

Over five decades ago, Schwarz and Mertz reported that Cr^{3+} (or GTF) is a nutrient for mammals; inorganic chromium(III) complexes apparently

could restore the glucose tolerance of rats fed a *Torula* yeast-based (supposedly Cr-deficient) diet. Major portions of these studies, which were considered the pioneering work in the field of chromium biochemistry and nutrition, have been effectively refuted. The diet used by these workers has not been demonstrated to be chromium deficient; thus, any effects from supplementing the diet with quantities of Cr several fold larger than the normal dietary intake does not establish an essential requirement. Similarly, proposals that Cr acts by binding to insulin are without foundation.

The biologically active form of chromium is not 'GTF', reportedly a Cr(III)–nicotinate–amino acid (or glutathione) complex. In fact, the composition of the artefact from yeast isolated by Mertz and co-workers was actually not established, and proposals for the three-dimensional structure of 'GTF' are simply science fiction. The consensus is that the effects of materials isolated from Brewer's yeast observed by Mertz and co-workers [18] were probably serendipitous, as Cr^{3+} has been demonstrated repeatedly to be separable from agents in yeast responsible for *in vitro* stimulation of glucose metabolism in adipocytes. Yet, even the detailed results of the most recent studies of yeast 'GTF' by different laboratories still cannot be reconciled completely. *Given the considerable confusion over the names GTF and 'GTF' and the history of the quest for 'GTF,' the author recommends that the use of the term glucose tolerance factor or GTF (and 'GTF') be terminated.*

One unexplained item related to these studies is the accumulation of chromium by Brewer's yeast. The yeast may accumulate chromium for a reason. The possible reasons for the accumulation, for example, possibly include that fermentation by the yeast may be accelerated by chromium, and the form of the accumulated chromium are interesting bioinorganic questions worthy of a systematic study, and potentially could reconcile or explain some of the results described above.

3.7 THE RACE TO SYNTHESIZE A MODEL OF 'GTF'

The proposed identification of nicotinate (3-carboxypyridine) in 'GTF' stimulated an interest in the synthesis of chromic–nicotinate or –nicotinic acid ester complexes starting in 1981 [49–59]. (For a review see reference [60]). However, the inability of the biologically active form of chromium to be elucidated (i.e. the problems with the isolation of Brewer's yeast 'GTF') and the lack of successful attempts to synthesize and characterize the synthetic 'GTF' of Mertz and co-workers [18] led to a rapid decline

in studies after 1985. In well-characterized complexes, nicotinate coordinates in a variety of manners to Cr(III): bridging through the carboxylate (μ-*O*,*O*′) [50], monodentate through a carboxylate oxygen [53], or monodentate through the pyridine nitrogen [58]. Legg and co-workers developed a clever method for determining whether nicotinate (either as the anion or zwitterion) is bound through the oxygen(s) or nitrogen using ^{2}H NMR [55, 58, 61]. The ^{2}H NMR signal of 2-d-nicotinate compounds is shifted to approximately –70 to –75 ppm if the ligand is bound through the nitrogen and to $+6$ to $+10$ ppm if bound through the oxygen. Recently all the nicotinate proton or deuteron resonances of a number of chromium(III)–nicotinate complexes have been assigned [62]. (The assignment for Cr(III)–nicotinate complexes described by Broadhurst *et al.* [63] are incorrect as no paramagnetically-broadened resonances were observed [62].)

A number of studies have examined a material characterized as '$Cr(nicotinate)_2(OH)(H_2O)_3$' (subsequently to be referred to as chromium nicotinate or chromium polynicotinate for consistency with the nutrition literature), which is the product of the reaction of two or three equivalents of nicotinic acid with chromic ions in aqueous solution at elevated temperatures [54, 62–65]. The complex has not yet been crystallized, and NMR studies suggest it is more complex than the proposed formula would indicate [62]. The original interest in the complex arose from its ability to stimulate CO_2 production in Cr-deficient yeast [54, 56]. However, the problems with this assay were described above. Because this chromium–nicotinate material is supposedly absorbed better than dietary chromium by mammals [64, 66], it has gained substantial use as a nutritional supplement, especially before the rise in the popularity of chromium picolinate. The use of chromium nicotinate as a nutritional supplement will be discussed in Chapter 7.

3.8 RELATED ANIMAL STUDIES

While studies were being performed to attempt to identify GTF, related studies on the potential effects of chromium and supposed chromium deficiency were being performed on animals, primarily on rats. These studies need to be analysed carefully in terms of diets provided to the rats. For example, as noted above, the chromium content of the *Torula* yeast diet used by Mertz and Schwarz was not analysed for chromium content. Given the low (if one even exists) dietary requirements for chromium, the low concentration of chromium in food sources and the ready potential

for contamination from contact with metal, the content and preparation of the diets need to be carefully established. This has been well stated by Anderson and co-workers: 'It should be noted that the mineral content of the low-Cr *Torula* yeast–sucrose diets used in the early studies [2, 3, 7] and in more recent studies [8, 34, 36, 47, 67] on Cr deficiency were not completely defined and differed considerably from diets prepared using purified materials or commercial rat chow. Diets used in the earlier studies resulted in undefined dietary stresses' [68]. These studies are also complicated by the lack of an indicator of Cr status in rats, other than the supposed or apparent effects of chromium deficiency being uniquely reversed by chromium supplementation. Also for the study to be nutritionally relevant, a nutritional, not pharmacological dose of chromium should be provided. Unfortunately, researchers during this period of time had limited data at best on what a nutritionally relevant dose of chromium might be. Also, chromium sources added to the diet should be well characterized so that any chemistry involved in the experiments might be more readily elucidated and understood. This review of animal studies will not describe studies with chromium-enhanced yeast, an ill-defined form of chromium supplement. For example, does growing yeast in the presence of chromium alter the yeast in manners other than increased chromium content?

The first studies by Mertz and Schwarz [4], that used well-characterized chromium(III) complexes, utilized rats fed a commercial laboratory chow that was deemed to be 'GTF' deficient, as it gave rise to reduced glucose removal rates in intravenous tolerance tests as had the *Torula* yeast diet (30% yeast, 59% sucrose, 5% vitamin E-free lard and vitamins and minerals) [2, 3, 69]. As noted previously, the Cr content of the diet was not reported. Rats fed the chow had an excess glucose removal rate of 2.8% (or lower) administered glucose per minute after an intravenous injection of 1250 mg glucose /kg body mass [4]. Significant improvement (even restoration of normal rates, that is ~4%) resulted when rats were given several chromium(III) complexes (200 μg Cr/kg body mass) by stomach tube 18 h before the glucose tolerance tests. Chrome alum was subsequently shown to be effective in increasing excess glucose removal rates in rats on the *Torula* yeast diet when given at levels of 2.5 mg Cr/kg body mass (or greater) by intravenous injection 2 h before the glucose tolerance tests [11]. The rats were kept in wire mesh cages; the times for the durations of feeding the diets were not given (other than that the diet was used long enough to give the depressed glucose removal rates). Woolliscroft and Barbosa (see above) gave 6-week old rats a diet comprised of 30% *Torula* yeast, 50% sucrose, 15% lard

without antioxidants and vitamins and minerals for 6 weeks. For the glucose tolerance tests, rats were given 1250 mg gluose/kg body mass. For comparison with control rats, Cr-supplemented rats were given 5 ppm Cr as chromium chloride in their drinking water over the six weeks on the diet. Glucose removal rates varied significantly between the control and supplemented rats only if excess glucose rates were used rather than the more commonly used total glucose removal rates [47].

Schroeder and co-workers performed similar studies during the 1960s and 1970s. The diet was comprised of 60% seed rye, 30% dried skim milk, 9% corn oil and 1% NaCl with added vitamins and minerals and contained 100–200 μg Cr/kg. In the first of these studies, rats received 5 ppm chromium in their drinking water. After 360 days of treatment, mortality was low, while growth of male rats, but not that of females, was stimulated [70]. For supplementation in another study, rats were given water containing 2 ppm Cr as chromic acetate [71]. Male and female rats were fed the diet or diet supplemented with chromium for 300–650 days after weaning. 'Chromium-deficient' (i.e. rats on the diet without added chromium) rats had higher fasting (18 h) serum glucose levels. Other rats were fed the *Torula* yeast diet; chromium-supplemented rats were found to grow more rapidly than rats not supplemented with Cr [71]. Elevated serum glucose was not observed as in the earlier work by Schwarz and Mertz. Mertz and Schroeder collaborated on examining glucose removal rates for rats on the seed rye diet (Cr content ~100 μg/kg wet weight) [8]. Cages were acrylic with stainless-steel covers. Rats were fed the diet from weaning until age 456–752 days and then maintained on the *Torula* yeast diet for only 4 days. Pre-supplementation glucose tolerance tests were performed on six rats, and four of these rats were given chromium as chromium chloride (5 ppm) in the drinking water and received an intravenous dose of chromium (1 μg Cr/kg body mass). Glucose tolerance tests were given 2 h after the injection and again after 4–7 days. Supplemented rats showed significant increases in glucose removal rates [8]. While not stated, excess glucose rates appeared to have been used. Schroeder returned to comparing the results of the *Torula* yeast diet and rye seed diet in 1966 [72]. Rats on both diets possessed higher serum glucose concentrations than rats on the diet supplemented with chromium.

In 1968 and 1969 Schroeder also examined the effects of chromium on cholesterol levels [73, 74]. In the first study, rats from weaning to 11–30 months of age received 1–5 ppm Cr as chromium acetate, while control rats received ~80 μg Cr/kg body mass daily via food and water. Although results are somewhat difficult to compare because of the range

of number of animals, length of treatment and amount of Cr provided, male rats on 1 ppm Cr appeared to possess lower serum cholesterol levels, while females required 5 ppm Cr to suppress cholesterol levels [73]. In the later study, weanling rats were provided a *Torula* yeast diet containing 50% sucrose [74]. A portion of the rats received 5 ppm chromium in their drinking water as chromium acetate. After 318 or 322 days of treatment, male and female rats, respectively, on chromium had lower serum cholesterol levels, while no effect was observed after 151 days for the male rats. Female rats were not tested at the earlier time. Fortunately, these studies by Schroeder and co-workers finally analysed the chromium content of the diet.

Some other studies before 1970 are notable. Staub *et al.* found that rats on a high cholesterol diet (added cholesterol and cholic acid) with either sucrose or potato starch as the carbohydrate source had higher serum cholesterol concentrations than rats on the diets supplemented with 5 ppm Cr (as chromic acetate) in the drinking water [75]. The diets were reported to contain 310 μg Cr/kg (sucrose) or 380 μg Cr/kg (starch). Similar results had been obtained previously by Schroeder and Balassa, but other metals (Cd, Pb) also elicited a similar effect [76]. Roginski and Mertz examined the effects of a high sucrose diet (77% sucrose, 10% soy protein, 8% distilled lard and vitamins and minerals) [77, 78]. Rats in plastic cages on the low Cr diet (<100 μg/kg) had moderately depressed growth, which could be exacerbated by exercise and had their fasting blood glucose levels decreased more by insulin when compared with supplemented rats (2 ppm Cr in drinking water). In 1978, O'Flaherty and McCarty reported the use of a purified 'low Cr' diet (800 μg Cr/kg diet) comprised of 62.4% corn starch, 3.0% cellulose, 20% egg white and vitamins and minerals [79]. A portion of the rats were supplemented with 5 ppm Cr (as chromic acetate) in their drinking water. Supplementation did not affect growth, but rats on the purified diet had higher mean fasting glucose levels. The higher plasma glucose levels were maintained during glucose tolerance tests after 8 weeks on the diet. Unfortunately as discussed in Chapter 2, diets containing 300–800 μg Cr/kg cannot be considered to be Cr deficient; in fact, commercial rat chows now generally have Cr concentrations in this range.

Between 1980 and 1995, few studies on rats were executed. Anderson and Polansky reported that male rats on the *Torula* yeast diet had decreased sperm counts and decreased fertility at 8 months of age [80]. Anderson and co-workers found no change in growth rate for rats given a purified low Cr, high sucrose diet versus that of Cr-supplemented rats (2 ppm Cr in drinking water) [81]. Jain *et al.* [82] fed rats in plastic

cages a diet containing 80 μg Cr/ kg diet for 11 weeks. Compared with rats supplemented with Cr by adding chromic acetate to the drinking water (1.140 mg Cr/ml), rats fed the Cr-deficient diet had similar mean body mass and similar responses during intravenous glucose tolerance tests. However, the unsupplemented rats had higher fasting blood glucose. Donaldson *et al.* [83] found that rats kept in plastic cages and fed a synthetic high sucrose (68.4%), high cholesterol (2%) diet 'low in Cr' (60–100 μg/kg) from weaning until 18 months of age had normal growth. Plasma cholesterol and triglyceride levels in the rats did not differ from those of rats given supplemental chromium (5000 μg Cr as potassium chromium sulfate per kg diet). At age 4 and 8 months, one-hour postgavage (2500 mg glucose/kg body mass) plasma glucose concentrations were statistically higher in the low-Cr rats, but this difference disappeared at 12 months. Flatt *et al.* [84] fed rats a 'low-Cr diet' (30 μg Cr/kg) or a Cr-containing diet (1000 μg/kg) from weaning to 53 days of age. The diets were neither high in sucrose (<9% sugars) nor fat (~5%). At 53 days, the rats had similar body masses, food intake, glycosylated haemoglobin levels, and plasma glucose and insulin concentrations. Plasma glucose levels of the rats responded in an identical fashion to intraperitoneal glucose and insulin.

Results of these last two studies have questioned whether the effects of the previous studies were due to the element chromium or to other dietary factors. For example, was the Torula yeast diet actually low in chromium? Interpretation of the results of these approximately 35 years of studies is definitely complicated by the variety of experimental conditions. When studies using the problematic yeast diet and studies using analytical techniques that cannot guarantee the accuracy of the Cr contents of the 'low-Cr' diets (before circa 1980) are eliminated from consideration, of the studies described above, only the studies by Jain *et al.* [82], Donaldson *et al.* [83] and Flatt *et al.* [84] remain (plus those of Striffler and co-workers reviewed in Chapter 2). The effects of these 'low Cr' diets (30–100 μg/kg) are equivocal at best. No beneficial effect from Cr supplementation is observed in common between any two of the studies.

The other factor that must be discussed is that none of these studies actually provide a low-chromium diet to the rats. Repeating from Chapter 2, humans lack signs of Cr deficiency with a daily intake of 30 μg Cr; assuming an average body mass of 60 kg, 30 μg per day correspond to 0.5 μg Cr/kg body mass per day. As a 100-g rat eats about 15 g of food a day, 15 g (0.015 kg) of food containing 30 μg Cr/kg food provides approximately 0.5 μg Cr. Thus, 0.5 μg Cr per day for a 0.100 kg rat

is 5 μg Cr/kg body mass per day, *ten times what a human intakes*. The lowest concentration of chromium in the diets was that of Flatt *et al.* [84], in which no effects were observed. This diet did not contain any other stresses as did the diets of Striffler and co-workers (see below), and no effects from chromium supplementation were observed. Donaldson *et al.* [83] provided a 'low-chromium' diet with only 2–3 times more chromium than that of Flatt but with the stresses of high sugar and cholesterol content and observed no consistent effects. These results are in stark contrast to the often cited results of Striffler and co-workers discussed in detail in Chapter 2. The results of Flatt *et al.* are consistent with recent research in the laboratory of the Vincent group [85] that also failed to observe an effect of a purified diet with <30 μg Cr/kg on body mass, food intake, fasting plasma insulin and glucose concentrations and plasma glucose and insulin levels after a glucose or insulin challenge.

Thus, a review of nutritional studies utilizing rats, so that the diet and environment can be rigorous controlled compared with human studies, fails to identify any reproducible symptoms associated with chromium deficiency: additional evidence that chromium cannot currently be considered an essential element. The studies also provide stark evidence, in addition to the human studies (see Chapter 4), that chromium supplementation has no significant effects on body mass and body composition, even when given in pharmacological doses, several times greater doses than used in the human studies.

Since the rat studies described in this chapter were carried out, studies have moved from focusing on the potential effects in healthy rats to attempting to treat symptoms of rat models of diabetes and related conditions with chromium; these studies also generally use commercially available chromium–organic complexes or such chromium complexes proposed for use as nutraceuticals that have or have not yet made it to the marketplace. The results of these studies, their potential beneficial effects and toxicity, will be the subject of subsequent chapters.

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4

Is Chromium Effective as a Nutraceutical?

4.1 CHROMIUM PICOLINATE ABSORPTION

In addition to attempts to synthesize and characterize chromium(III)–nicotinate complexes, the products of reaction of chromium(III) sources and the related pyridinecarboxylic acids picolinic acid (2-carboxypyridine) and isonicotinic acid (4-carboxypyridine) [1] have also been studied in some detail. Chromium(III) picolinate, $[Cr(pic)_3]$ (Figure 4.1), has been the most thoroughly studied of these synthetic products and has become a very popular nutritional supplement; products containing $[Cr(pic)_3]$ generate over $100 million in sales annually, as the supplement is available over-the-counter in numerous forms including pills, chewing gums, sports drinks and nutrition bars. One of the commonly repeated myths about $[Cr(pic)_3]$ (one into which the author has fallen) is that it is a well absorbed form of chromium. Chromium picolinate appears to be absorbed in rats with the same ~0.5–2% efficiency compared to dietary chromium and inorganic chromium salts such as $CrCl_3$ [2, 3].

Fortunately, a somewhat detailed description of the fate of $[Cr(pic)_3]$ after entering the stomach is possible. $[Cr(pic)_3]$ is fairly stable in aqueous solution [4], such that it should not break down when slurried or dissolved in buffered water. Gammelgaard *et al.* have reported that the compound is stable in artificial gastric juice [5]; (however, the method used would not have actually revealed whether the compound was stable as opposed to being converted into a similar small molecular

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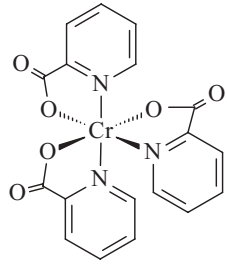

Figure 4.1 Structure of chromium picolinate.

weight species). Similarly, the Research Triangle Institute found that $[Cr(pic)_3]$ was stable when added to excised stomach contents of rats for 2 h and when added to excised small intestine contents for 4 h [6]. Thus, dissolved and solid $[Cr(pic)_3]$ should be stable. As the pH of a solution of $[Cr(pic)_3]$ decreases upon entering the stomach, $[Cr(pic)_3]$ should precipitate from solution as the pH of its environment is reduced. The solubility of $[Cr(pic)_3]$ decreases below neutral pH [6] until a pH is reached where the complex starts to hydrolyse releasing the picolinate ligands) [7, 8]. Only when the compound is in close contact with the stomach mucosa with the mucosa's very low pH should it hydrolyse, releasing picolinic acid. This would allow the free acid and the chromium to be absorbed separately. The absorption of the picolinate and chromium would then potentially be quite different; this has been observed by Research Triangle Park [6] and by Vincent *et al.* (unpublished results). The picolinic acid is absorbed much more efficiently than the chromium. A small percentage of chromium picolinate is absorbed intact. The former researchers found that ~1% of absorbed chromium was in the form of $[Cr(pic)_3]$ [6]. Once chromium is released from chromium picolinate, the form of chromium present in the stomach (and subsequently throughout the gastrointestinal tract) should be similar to that when other forms of chromium break down in the stomach (e.g. chromium chloride and chromium nicotinate). In fact, this explains very nicely the almost identical measured absorption of gavaged aqueous suspensions of $[Cr(pic)_3]$, chromium nicotinate and chromium chloride in rats [9,10] when the extent of absorption is followed in the urine and body tissues, not just the urine. In fact, the bioavailability of chromium from $[Cr(pic)_3]$ is simply not superior to that of $CrCl_3$ [11].

The intact chromium picolinate in the body could also come from simple absorption (diffusion) by the small intestines, although absorption must be very low in this form. Consequently, studies that have

examined the fate (and toxicology (Chapter 9) of *intravenously* administered [$Cr(pic)_3$] are relevant only to the fate of the tiny proportion of chromium picolinate that is absorbed from the stomach or the intestines intact. Kareus *et al*. showed that microsomal extracts could break down the compound efficiently [12], although this would require the compound to be absorbed intact and subsequently enter tissues intact. The organic product of this break down was *N*-1-methylpicotinamide [12]; however, the primary product of the breakdown of gavaged [$Cr(pic)_3$] is *N*-picolinoylglycine [6]. This difference in product as a function of administration is more evidence that [$Cr(pic)_3$] breaks down along the stomach mucosa before absorption. This mechanism of Cr absorption from [$Cr(pic)_3$] is also consistent with the similar tissue distribution and tissue concentration of Cr from gavaged [$Cr(pic)_3$], chromium nicotinate and chromium chloride [9, 10]. In fact, the bioavailability of [$Cr(pic)_3$] is not greater than that of $CrCl_3$ when tissues and body fluids are both considered, as tissues can retain a considerable portion of the absorbed chromium at given periods of time [11]. Thus, comparative absorption studies of different forms of chromium supplements examining only urinary chromium loss should be treated with some scepticism [13–15]. Chromium picolinate is made from $CrCl_3$; therefore, the use of $CrCl_3$ (if the use of a chromium supplement was warranted) would be considerably more cost effective.

[$Cr(pic)_3$] has been proposed to be the biologically active form of chromium [16]; yet, [$Cr(pic)_3$] has neither been shown to possess intrinsic biological activity nor to occur naturally in mammals or other organisms. No reason exists to expect it to exist naturally *in vivo*, especially since chromium and picolinic acid levels in tissues are low, making its generation unlikely at best.

4.2 HISTORY OF CHROMIUM PICOLINATE AS A NUTRITIONAL SUPPLEMENT

The study of the effects of chromium picolinate on mammals has been extremely contentious. This has been stated well in a review by Hellerstein [17]:

> 'To an outsider reviewing literature on chromium and diabetes/obesity, the field is most striking for two features: its nearly complete lack of biomedical or clinical understanding and its high degree of polarization . . . As in all fields with more heat than light, the reason has been

> the incomplete ability to measure and test key factors . . . The high degree of politicization and polarization in this field is characterized by unproven claims and counterclaims and suspicion among investigators. Concerns about possible commercial bias and potential conflict of interest have naturally emerged. Reports of benefits of supplementation (e.g. that lean tissue is increased and fat decreased by chromium in athletes in training) [18] that were not confirmed by several subsequent studies [19–21] have furthered these concerns.'

The history leading to the first publication of beneficial effects from chromium picolinate in 1989 has been excellently summarized by Forrest Nielsen of the USDA (United States Department of Agriculture) (Grand Forks Human Nutrition Research Center) [22]. While examining the use of zinc picolinate to treat children with acrodermatitis enteropathica, a genetic disorder that results in the inability to absorb zinc from cow's milk, Gary Evans, employed by the Grand Forks Human Nutrition Research Center, found that zinc and other metal picolinates were absorbed better than the corresponding mineral salts. Evans patented the process of synthesizing coordination complexes of picolinic acid [23]. Nutrition 21 licensed the patent from the USDA in 1986 and supported Evans research leading to the 1989 publications. 'In other words, contrary to what many advertisements touting chromium picolinate led many to believe, the USDA patent is not specific to chromium, nor does it mention that chromium picolinate has any beneficial effects claimed for this form of chromium supplement' [22].

In 1989, Evans [18] found that in double blind crossover studies that volunteers taking 200 μg per day chromium as $[Cr(pic)_3]$ (compared with those taking placebos) had decreased total cholesterol, LDL cholesterol and apolipoprotein B and increased apolipoprotein A1 [18, 24]. In a similarly designed study, 8 out of 11 adult-onset diabetic patients displayed positive effects with chromium supplementation (see below). Two studies by Evans with young men participating in weight training resulted in significant losses in body fat and increases in lean muscle mass with chromium supplementation [18]. These human studies were followed by studies with rats and humans [16, 25, 26]. Studies of cultured rat skeletal muscle myoblasts found increased insulin internalization and accompanying glucose and leucine uptake by cells in media containing 1 μM $[Cr(pic)_3]$ but not containing the same concentration of chromic chloride, chromium nicotinate or zinc picolinate [16]. The results were postulated to result from increased membrane fluidity. In another study, rats were fed a diet containing $[Cr(pic)_3]$ or chromium nicotinate

(1 μg/g diet for 200 days); only rats fed the [$Cr(pic)_3$]-containing diet had decreased plasma glucose and glycated haemoglobin [25]. In another study, similar results were observed after 1000 days on the diet; also 80% of rats on [$Cr(pic)_3$] were alive after 41 months, while all rats fed equivalent amounts of chromium nicotinate or chromium chloride had died [26]. The increase in average life span suggested by these results is simply ludicrous.

Two of the studies of Evans mentioned above reported examined body mass and composition of healthy human subject and are of particular interest here (Table 4.1). In the first study [18], 10 males between 18 and 21 years of age were involved. Half of these student subjects received a supplement containing 200 μg Cr as [$Cr(pic)_3$] for 40 days; the other half received a placebo. The subjects engaged in 40-min exercise periods twice a week. Body composition was estimated by measuring thickness of skin folds and bicep and calf circumferences. Subjects on the supplement on average gained 2.2 kg body mass, had no significant change in percentage body fat and gained 1.6 kg of lean body mass. In contrast, subjects on the placebo on average gained 1.25 kg body mass, had an increase in body fat of 1.1% and increased their lean body mass 0.04 kg. The increase in lean body mass for the subjects receiving chromium was said to be statistically greater than that for the control (or placebo) group ($P = 0.019$) [18].

In the second study [18], 31 (of an initial 40) college football players completed a 42-day program. Half the players were given 200 μg Cr as [$Cr(pic)_3$] while the other half received a placebo. The subjects exercised 1 h per day for 4 days per week. Body composition was estimated by measuring thigh, abdomen and chest skin folds and thigh, bicep and calf circumference. After 14 days, subjects receiving Cr on average lost 2.7% of their body fat and had an increase of lean body mass of 1.8 kg, while no changes were observed in the control group. After 6 weeks, the chromium group on average lost 1.2 kg, lost 3.6% (or 3.4 kg) of their body fat and had an increase in lean body mass of 2.6 kg, while the control group had a loss of 1 kg of body fat and a 1.8 kg increase in lean body mass. Both the loss of body fat and the increase in lean body mass were said to be significantly greater for the chromium group ($P = 0.001$ and $P = 0.031$, respectively). No method to determine compliance of subjects was indicated. The standard deviation of the data was not presented.

This area has received much attention since these initial reports, and the effects of chromium nutritional supplements on body composition and body mass in these reports have not been reproduced. The next study

Table 4.1 Effects of $[Cr(pic)_3]$[a] on body mass or composition.

No. of subjects	μg Cr/day	Time (weeks)	Exercise programme[b]	Compliance monitored[b]	Measurement technique[b]	Results	Ref.
10 (male)	200	5.7	Yes	No	Skin-fold thickness, circumference	Increase in lean body mass	Evans [18]
31 (male)	200	6	Yes	No	Skin-fold thickness, circumference	Increase in lean body mass loss in body fat mass	Evans [18]
32	200	6	No	Yes	—	No effect on body mass	Press *et al.* [27]
59 (37 male) (22 female)	200	12	Yes	Yes	Skin-fold thickness, circumference	Males no effects and females increase in body mass	Hasten *et al.* [28]
24 (12 male) (12 female)	400 200	12	Yes	No	Resistivity	No effects[c]	Evans *et al.* [25]
21 (male)	200	6	Yes	Yes	Underwater weighing	No effects	Clancy *et al.* [19]
95 (79 male) 16 female)	400	16	Yes	Yes	Circumference	No effects	Trent *et al.* [30]
16 (male)	200	12	Yes	Yes	Underwater weighing, skin-fold thickness, circumference	No effects	Hallmark *et al.* [20]
36 (male)	172	8	Yes	Yes	Dual X-ray absorptiometry, skin-fold thickness	No effects	Lukaski *et al.* [21]

154	200 or 400	10.3	No	Yes	Underwater weighing	?[d]	Kaats *et al.* [32]
40 (20 male) (20 female)	400	24	Yes	No	Underwater weighing	Increased lean body mass decreased fat body mass decreased % body fat	Bulbulian *et al.* [33][e]
43 (female)	200	9	Yes (but not all subjects)	Yes	Underwater weighing	Non-exercising subjects increased body mass	Grant *et al.* [34]
18 (male)	1000	12	Yes	Yes	—	No effects	Campbell *et al.* [35]
21	200	26	No	?	Skin-fold thickness	Increase in lean body mass	Bahadori *et al.* [36]
20 (male)	200	14	Yes	Yes	Underwater weighing, skin-fold thickness, circumference	No effects	Walker *et al.* [38]
122 (17 male) (105 female)	400	12.9	No	Yes	Dual X-ray absorptiometry	No effects except decrease in fat mass[d]	Kaats *et al.* [39]
35	1000	13	Yes	Yes	Skin-fold thickness, circumference	No effects	Boyd *et al.* [40]
18 (male)	924	13	Yes	Yes	Underwater weighing, skin-fold thickness, circumference	No effects	Campbell *et al.* [41]

(*continued*)

Table 4.1 (*Continued*)

No. of subjects	μg Cr/day	Time (weeks)	Exercise programme[b]	Compliance monitored[b]	Measurement technique[b]	Results	Ref.
35 (17 male) (18 female)	924	12	Yes	Yes	Underwater weighing, circumference	No effects	Joseph *et al.* [42]
29 (14 male) (15 female)	1000	38	No	Yes	MRI, skin-fold thickness, circumference	No effects	Cefalu *et al.* [43]
19 (10 male) (9 female)	1000	8	No	Yes	Dual X-ray absorptiometry	No effects	Amato *et al.* [44]
15 (female)	500	6	Yes	Yes	Underwater weighing	No effects	Livolsi *et al.* [45]
44 (female)	400	12	Yes	Yes	Underwater weighing, circumference	No effects	Volpe *et al.* [46]
17 (female)	924	12	Yes	?	Underwater weighing	No effects	Campbell *et al.* [47]
83 (female)	200	12	No	Yes	Dual X-ray absorptiometry	No effects	Lukaski *et al.* [48]
42 (female)	1000?	8	No	Yes	Dual X-ray absorptiometry	No effects	Anton *et al.* [50]
63 (33 male) (30 female)	1000?	16	No	Yes	Bioimpedence	No effects	Iqbal *et al.* [51]
80 (40 male)	1000?	24	No	?	Bioimpedence	No effects	Yazaki *et al.* [52]

[a]Studies are included only if $[Cr(pic)_3]$ is the sole supplement utilized. For example, Table 4.1 does not include studies by Kaats *et al.* [29]; Krieder *et al.* [31]; Pasman *et al.* [37] and Diaz *et al.* [49].
[b]Exercise programme refers to any type of regulated exercise programme. Compliance may be either through determining supplement left after study, notebook maintenance, or interview and is specifically indicated. Measurement technique used for body composition determination.
[c]After statistical concerns are addressed (see text).
[d]Cannot be determined from data presented with statistical concerns (see text).
[e]Published abstract.

to appear on the effects of chromium picolinate on body composition by Evans and co-workers was reported in 1990 [27]. The study was double-blind, crossover in design. Groups received supplements or placebos for 42 days, then received neither for 14 days and finally received for 42 days the opposite (placebo or supplement) from that in the original 42 days. Subjects varying from 25 to 80 years in age were utilized. Compliance was monitored by capsule count. While the study was designed to look at serum cholesterol and apolipoprotein levels, body mass data were also collected. [$Cr(pic)_3$] supplementation, 200 μg/day, had no effect on body mass.

In 1992, Hasten *et al*. reported a larger study on the effects of [$Cr(pic)_3$] supplementation [28]. College students ranging from 18 to 36 years of age were involved in the 12-week study; 37 males and 22 females completed the study. Students were not involved in physical training 2 years prior to the study or during the study outside of the conditioning class. Students were in 40-min exercise classes 3 days a week. The study was double-blind with the supplement group receiving 200 μg Cr a day as [$Cr(pic)_3$]. Compliance was determined by capsule count. Body composition was determined by skin fold and circumference measurements. For male subjects, [$Cr(pic)_3$] had no effect on strength, body measurements, or body mass. For females, no effect was observed on strength or body measurements; however, females increased body mass on average by 2.5 kg compared with the control, a significant change ($P = 0.0048$). Approximations of lean body mass and percentage body fat were also not significantly different. In this work the same concerns about estimating body compositions from skin-fold measurements mentioned above still hold, although the authors were careful to refer to the approximate nature of the calculations. The authors also suggested that the increase in body mass in females was probably primarily from lean body mass increases; the increase in the latter was almost statistically significant.

Also in 1992, Kaats *et al*. reported the effects of a diet supplemented with a high-fibre cookie and a capsule containing [$Cr(pic)_3$] and L-carnitine [29]. Some positive effects on body composition were noted, but as the subjects received numerous vitamins and minerals, fibre and carnitine in addition to [$Cr(pic)_3$], it is impossible to determine the effect of [$Cr(pic)_3$].

In 1993, Evans reported a study of the effects of [$Cr(pic)_3$] on body composition [25]. The 12 males and 12 females were involved in a weekly aerobics class. Subjects were 25– 36 years of age. Males received 400 μg Cr/day as [$Cr(pic)_3$] or 400 μg of Cr as chromium nicotinate.

Females received half as much of either Cr source. Lean body mass was measured by resistivity. Data were presented with standard errors. For males receiving [$Cr(pic)_3$], the lean body mass increased 2.1 kg and was statistically equivalent to the initial value and to the values of the group receiving chromium nicotinate. For females, the lean body mass increased 1.8 kg and was statistically equivalent to the initial value and to those of the group receiving chromium nicotinate. Yet, Evans claimed that despite this, the change in lean body mass for both males and females on [$Cr(pic)_3$] was significant ($P < 0.01$). The statistical analysis, which indicated that while final and initial values were equivalent but that the difference between them was significant, failed to incorporate the error in both the initial and final values in the calculation of the error of the difference.

The results of the first human studies by Evans and coworkers [18, 25] were very soon questioned by Lefavi [53] and Lefavi, Anderson *et al.* [54]. Lefavi [53] pointed out that 'It is likely that reviewers well-read in exercise physiology would find the notion of a 4.6-lb *lean* body mass (LBM) increase in males and a 4.0-lb LBM increase in females resulting from 12 weeks of a weekly aerobics class preposterous. A LBM increase that dramatic is not typically seen in subjects who are weight-training three times per week for 12 weeks, no matter what they are taking. ... Investigators familiar with this type of research would suggest either (a) that was one great aerobics class, or (b) people in Bemidji, MN, respond in a highly unusual manner to aerobic exercise and/or are extremely chromium deficient, or (c) Dr. Evan's group is consistently having difficulty accurately measuring LBM.' (See reference [53] page 121.) Body composition by skin-fold measurements and circumference measurements is only an indirect estimation, especially in young males [55]; more accurate techniques such as underwater weighing were available in 1989. These measurements can give rise to values that are 'statistically different yet functionally meaningless' [54]. Subsequent studies of body composition have generally used underwater weighing, dual X-ray absorptiometry, or magnetic resonance imaging to measure the fat and lean body content and consequently are more accurate than the initial studies using chromium picolinate. Curiously none of the studies described above reported a source of funding; funding sources are presented so that readers can note any potential conflicts of interest.

Clarkson and co-workers [19] in 1994 followed 36 college football players for nine weeks. Twenty-one completed all or most of the study. Half the players received 200 μg Cr as [$Cr(pic)_3$]. The players trained the first and last four-week periods of the study with spring break in the

middle; each week's training consisted of weightlifting on four days and running on two days. Compliance was monitored. Body composition was determined using underwater weighing; skin fold and circumference measurements were also performed. No changes in body composition or strength were found to result from Cr supplementation. The source of funding was not given. In 1995 Trent and Thieding-Cancel [30] studied US Navy personnel (79 men, 16 women) with a mean age of 30.3 years. Subjects received 400 μg [$Cr(pic)_3$] or placebo daily; (the authors probably meant 400 μg of Cr as [$Cr(pic)_3$]). A conditioning programme consisting of exercise (at least 30 min) three times a week (average 4.5 h per week) was employed; compliance was monitored. Body composition was estimated by circumference measurements. No statistically significant changes from Cr supplementation were found for body mass, percentage body fat, or lean body mass. Funding was provided by the US Navy and Nutrition 21.

Four studies of relevance appeared in 1996. Hallmark *et al.* [20] followed 16 untrained males (23 ± 4 years of age) through a 12-week resistive training programme consisting of training 3 days a week. Half received 200 μg Cr/day as [$Cr(pic)_3$]. Underwater weighing was used to determine body composition. While the strength of individuals increased on average over the course of the study, [$Cr(pic)_3$] had no effect on body mass, percentage body fat, lean body mass, or skinfold thicknesses. Compliance was monitored; [$Cr(pic)_3$] was donated by Nutrition 21. Krieder *et al.* [31] examined the effects of 800 μg of Cr as [$Cr(pic)_3$] as the commercial product GainersFuel. While the study was well designed and body composition was determined by X-ray absorptiometry, no changes arose from the product versus the control in subjects on a resistance-training programme. Drawing conclusions specifically on the effect of the [$Cr(pic)_3$] cannot be done as it is conceivable that some ingredient in the product could have cancelled any beneficial effects from [$Cr(pic)_3$]. Thirty-six men aged 19–26 years participated in a study by Lukaski *et al.* [21]. Participants received a placebo or 172 μg Cr as [$Cr(pic)_3$] for 8 weeks. Subjects exercised 5 days a week, and compliance was monitored. Body composition was measured by dual X-ray absorptiometry, and skin-fold thicknesses and body mass and height were measured. No effects on body composition, body mass, skin fold, or strength were found from supplementation. Nutrition 21 provided the supplements. Interestingly, Lukaski and co-workers found that urinary Cr loss increased for control subjects during weeks 2, 3, 5, 7 and 8 of the exercise program. However, whether Cr absorption changed as a result of increased losses is not known. Thus, homoeostatic adaptation

could take place, preventing the increased urinary chromium loss from changing the chromium status of the body.

Kaats *et al.* [32] examined 154 free-living subjects who received a placebo or 200 or 400 μg Cr daily as [$Cr(pic)_3$] in a protein–carbohydrate nutritional drink; 218 subjects started the study, which was 72 days in length. Body composition was determined using underwater weighing. The significance of this study is difficult to determine as the authors describe their results in terms of a calculated 'body composition improvement' (BCI) index rather than on measured parameters. The BCI index was calculated by adding 'losses in body fat and increases in [free fat mass] as positive changes' and 'increases in body fat and decreases in [free fat mass] as negative changes' [32]. The BCI of subjects on either amount of [$Cr(pic)_3$] improved significantly while the extent of improvement was equivalent for both quantities. In terms of changes in measured parameters, non-significant changes were observed in non-fat mass. The group receiving 400 μg Cr, but not 200, had lower body mass. Fat mass and percentage body fat also appeared to drop significantly with either amount of supplementation. The statistical treatment is based on comparing changes rather than comparing initial values against final values; the changes are fairly small. The post-study values were not presented with their standard deviations, preventing further statistical analysis. Lukaski [56] has criticized the design of this study because of a lack of control of Cr intake and failure to maintain constant energy intake and expenditure. Subjects were not asked about their diet or exercise during the study, only about how much of the drink they consumed each day. The study was funded in part by Nutrition 21.

Also in 1996 Bulbulian *et al.* reported the effects of 400 μg Cr as [$Cr(pic)_3$] daily for 24 weeks on 20 male and 20 female swimmers [33]. Using hydrodensitometry, supplementation after 24 weeks led to decreased fat mass and percentage fat mass and increased lean body mass, with the magnitude of the changes being greater in women than those in men. No significant effects were observed after 12 weeks. Unfortunately, the work was only presented in an abstract, such that the same scrutiny given other studies reviewed here is not possible. For example, the actual mass changes, standard deviation, compliance measures, and so on, are not reported; therefore, this preliminary report will not be considered further.

In 1997, four studies appeared. Grant *et al.* [34] examined 43 obese female subjects ranging from 18 to 35 years of age. Over 9 weeks, the subjects each received either two placebo capsules or two capsules containing 200 μg of Cr as [$Cr(pic)_3$]. Compliance was monitored by counting

returned capsules. Subjects consuming [$Cr(pic)_3$] and involved in cross training had no changes in body mass, percentage body fat, fat mass, or fat free mass compared with those consuming placebos and exercising. However, subjects consuming the supplement but not exercising gained body mass compared against a non-exercising control group, while no effects were observed on percentage body fat, fat mass, or fat free mass. Body composition was determined by underwater weighing. Funding was provided by Shaklee, USA, Inc., who also provided the supplements. Campbell *et al.* have examined the effects of [$Cr(pic)_3$] on moderately overweight men [35]. Eighteen men aged 56–69 years participated in the 12-week study. All were involved in a twice-weekly resistance-training programme, while half received 1000 μg Cr as [$Cr(pic)_3$] daily and the other half received a placebo. Compliance was monitored by counting returned capsules. The supplement had no effect on body mass or strength. The study was supported in part by Nutrition 21. Bahadori *et al.* [36] examined 21 obese subjects. The average age of the subjects was 44 years. Subjects consumed a very low calorie diet for 8 weeks followed by an 18-week maintenance period and received either a placebo or 200 μg Cr as [$Cr(pic)_3$] or Cr-enhanced yeast. Body composition was unfortunately measured by skin-fold thickness. [$Cr(pic)_3$] significantly increased lean body mass compared with the lean body mass of the other two groups ($P < 0.029$). Body mass and percentage fat mass were not significantly affected by [$Cr(pic)_3$]. Use of skin-fold measurements places these results in question. Pasman *et al.* [37] examined the effects of [$Cr(pic)_3$] on 33 female obese subjects, although, unfortunately in terms of analysis of the effects of [$Cr(pic)_3$], fibre and caffeine were used in conjunction with 200 μg of [$Cr(pic)_3$] daily. The supplement had no effect on body mass or body composition (estimated from circumference measurements). Novartis Nutrition Ltd. provided funding.

Studies of the effects of [$Cr(pic)_3$] continued to appear at a brisk pace in 1998 (three studies) and 1999 (three studies). Walker *et al.* [38] followed 20 wrestlers whose average age was 20 years. In a double-blind, placebo-controlled study, the wrestlers consumed 200 μg Cr as [$Cr(pic)_3$] for 14 weeks. Compliance was monitored. Body composition was determined from underwater weighing, and skin folds and circumferences were also measured. No effects from [$Cr(pic)_3$] were found on body mass, lean body mass, percentage body fat, fat mass, or strength. Funding was provided by the Gatorade Sports Institute. Kaats *et al.* [39] examined 17 men and 105 women averaging 42.3 years of age. Participants received either a placebo or 400 μg [$Cr(pic)_3$] daily for 90 days. Body composition was determined by X-ray absorptiometry.

Compliance was monitored by having subjects keep a notebook and by interview. No significant changes from supplementation were found for body mass, percentage body fat, or fat-free mass; however, the loss of fat mass was significantly larger ($P = 0.023$) from [$Cr(pic)_3$] supplementation (although concerns about how the statistics for differences were calculated again hold for this work as with the earlier work by Kaats *et al*). The authors also indicate that when the data are corrected for physical activity and caloric intake that changes in body mass, percentage body fat and fat mass (but not free fat mass) all become significantly greater for the supplemented group ($P < 0.001$). The validity of this 'correction' is highly questionable. Notably the data from this study were not corrected this time for BCI as in Kaats and co-workers previous 1996 report. The lack of statistical changes in measurable variables (except fat mass) with supplementation directly contradicts the results of their work in 1996. The study was funded by Nutrition 21. Boyd *et al*. [40] studied 35 students for 13 weeks. The students received either a placebo or 1000 μg [$Cr(pic)_3$] (probably meant to indicate 1000 μg Cr as [$Cr(pic)_3$]) daily. Students also underwent a 50-min exercise class twice a week. Circumferences were measured, and skin folds were measured to estimate body composition unfortunately. No significant changes in strength or lean body mass were noted either from the exercise programme or from the programme in combination with supplementation. [$Cr(pic)_3$] was provided by Nutrition 21.

Campbell *et al*. [41] also found that [$Cr(pic)_3$] had no effect on fat free mass, whole body muscle mass and muscle strength in a 13-week study with 18 men aged between 50 and 75 years. However, one type of muscle power increased in the placebo group and not in the chromium picolinate group. This could equally well be restated as [$Cr(pic)_3$] appeared to prevent muscle power increase in one case. Each man received a placebo or 924 μg Cr/day. Compliance was monitored, and body composition was determined by underwater weighing. Participants also were involved in a twice-weekly resistance training programme. The research was funded by NIH (National Institutes of Health) and independently by Nutrition 21. Many of these same researchers were involved in another study which appeared at about the same time [42]. In this other study, 35 men and women aged 54–71 years were given a placebo or 924 μg Cr as [$Cr(pic)_3$]. Participants were involved in resistance training twice a week for 12 weeks. Body composition was determined by underwater weighing. No effects from [$Cr(pic)_3$] were observed on body mass, percentage body fat, fat free mass, or strength. The research was funded by the same sources. Thus in these two studies 53 subjects were given

large (924 μg) amounts of Cr daily for 12–13 weeks without effect. Also in 1999, Cefalu *et al.* [43] examined 14 men and 15 women who received 1000 μg of Cr daily as [$Cr(pic)_3$] for 8 months. Compliance was monitored, and body composition was monitored by MRI (magnetic resonance imaging). No effect was observed on body mass, abdominal fat distribution, or body mass index. No funding source was indicated.

In 2000 and 2001, four other studies using [$Cr(pic)_3$] were reported; none observed any effects from [$Cr(pic)_3$] supplementation on body mass or composition. Davis *et al.* [57] examined the time taken for 8 men to become fatigued during high-intensity shuttle running. The men consumed flavoured water, a drink containing carbohydrates and electrolytes or a drink containing these ingredients plus 400 μg Cr as [$Cr(pic)_3$]. The men then underwent an exercise programme before being tested for fatigue. While the drink had beneficial effects, the addition of Cr resulted in no additional effects beyond those of the original drink. No source of support was indicated. Amato *et al.* [44] examined 9 women and 10 men between the ages of 63 and 77 years. Part of the subjects received 1000 μg per [$Cr(pic)_3$] (probably meant to be 1000 μg Cr as [$Cr(pic)_3$]). Compliance was monitored, and body composition was determined by dual-energy X-ray absorptiometry. No change in body mass index or body composition was found from supplementation. [$Cr(pic)_3$] was provided by Nutrition 21. Fifteen female softball athletes were used in a study by Livolsi *et al.* [45]. The women between 17 and 21 years of age received 500 μg Cr as [$Cr(pic)_3$] or a placebo for 6 weeks. During this time the women trained for 2 h on 3 days a week. Body composition was determined by underwater weighing. No effect was observed from Cr on muscular strength, body mass, percentage body fat, or lean body mass. Nutrition 21 provided the [$Cr(pic)_3$]. Finally, Volpe *et al.* [46] examined the effects of 400 μg Cr as [$Cr(pic)_3$] on moderately obese women; 44 women aged 27–51 years received the supplement or a placebo for 12 weeks. During this time, the women participated in weight training 2 days a week. Body composition was determined by underwater weighing. No change was found from the [$Cr(pic)_3$] on body mass, body mass index, waist or hip circumferences, percentage body fat, lean body mass, or fat mass. The project was supported by Nutrition 21.

Since 2002, several additional papers have appeared on the effects of chromium picolinate on body mass loss of healthy or obese, non-diabetic subjects, all observing no statistically significant effects from supplementation. Campbell *et al.* [47] examined the effects of resistive training and chromium picolinate on 17 women. Eight were randomly and blindly provided a placebo, while nine received 924 μg Cr as chromium

picolinate daily for 12 weeks. No effects were observed on strength, body composition, or skeletal muscle size [47]. The study was supported from grants from the National Institutes of Health and monetary funds from Nutrition 21. Lukaski *et al*. [48] examined 83 women for 12 weeks in a double-blind study. Subjects received a placebo, 200 μg Cr as chromium picolinate, or 1720 μg picolinic acid. The careful design of this experiment in which the ligand alone is used as a control should be noted. Body composition was examined by dual X-ray absorptiometry. Chromium picolinate supplementation did not affect body mass, body fat, or iron status. Nutrition 21 provided the chromium picolinate. The effects of chromium picolinate in combination with conjugated linoleic acid compared with a placebo were examined in 35 women for 12 weeks by Diaz *et al*. [49]. Subjects received 400 μg Cr as [$Cr(pic)_3$] daily with 1.8 g of the conjugated linoleic acid. Body composition was examined by dual X-ray absorptiometry. No effects were observed compared with the placebo on body mass or composition or a variety of blood variables. No funding source was indicated.

Anton *et al*. [50] examined the effects of 1000 μg chromium picolinate (probably meant to be 1000 μg Cr as chromium picolinate although this is not what is indicated) or a placebo on food intake and satiety in healthy overweight women reporting a craving for carbohydrates. Forty-two women participated in the 8-week study. While the supplement appeared to lower food intake and cravings, it had no statistically significant effect on body mass. The study was funded by the Pennington Biomedical Research Center. This study, demonstrating an effect on cravings and food intake, is interesting and worthy of continued study but is hard to reconcile with the results of numerous studies failing to demonstrate effects on food intake in rats. As part of a study to look at the effects of chromium picolinate on several measures of symptoms of metabolic syndrome in obese, non-diabetic adults, Iqbal *et al*. [51] also examined the effects of the compound on body mass. In a double-blind, placebo-controlled trial, 63 subjects were given 1000 μg chromium picolinate (probably meant to be 1000 μg Cr as chromium picolinate although this is not what is indicated) or placebos daily for 16 weeks. Chromium picolinate had no effect on body mass and several blood variables although it did result in an increased acute insulin response to glucose. The work was supported by the National Institutes of Health while the supplement was provided by Nutrition 21.

Finally, in 2010 Yazaki *et al*. [52] gave 1000 μg chromium picolinate (probably meant to be 1000 μg Cr as chromium picolinate although this is not what is indicated) or a placebo daily for 24 weeks to 80 otherwise

healthy, overweight subjects. Subjects received passive nutritional education at the 12-week point of the study. After 12 or 24 weeks, no effects were observed on body mass, body fat, blood pressure and a long list of blood variables including fasting plasma insulin, glucose, cholesterols and triglycerides. Body fat was measured by bioelectrical impedance. The study was funded by Nutrition 21.

In summary, other than the initial reports by the patent holder and one study with small but statistically significant effects on body mass loss or changes in body composition from chromium picolinate, all the studies with chromium picolinate have failed to observe statistically significant effects. While some of the earlier studies were not performed with all the desired controls and techniques, the subsequent well-performed studies almost uniformly indicate that chromium picolinate has no effect on body mass and composition of humans in doses up 1000 μg Cr/day. When combined with the recent the National Institutes of Health sponsored study where male and female rats and mice were given diets containing up to 5% chromium picolinate by mass for up to 2 years and no effects were observed on body mass or food intake [58], one can safely state that chromium picolinate is not effective as a nutritional supplement for body mass loss or changing body composition.

The increase in the number of studies in the mid- to late-1990s was accompanied by a sudden explosion of review articles. The themes of the reviews have a similar tone. Nielsen states in reference [22], page 229:

> 'To summarize, the data related to chromium changing lean body mass, strength gain, and athletic performance are mostly negative . . . in other words, the findings to indicate that the use of large amounts of chromium supplements, including the picolinate form, will not bring forth the over zealously touted propitious effects on muscle accretion, strength gain, or athletic performance. . . . In summary, there are no data from well-controlled studies to support the astonishing weight-loss claims with the use of chromium picolinate supplements. Thus, a high intake of chromium picolinate is unlikely to lead to significant weight loss.'

Clarkson writes in reference [59] on page 347:

> 'The preponderance of evidence shows that chromium supplements will not increase lean body mass or decrease fat mass, despite the widespread hype to the contrary . . . . the prudent course for athletes should be to ingest foods rich in chromium.'

Similarly according to Anderson in reference [60], pages 268–296:

> 'In our studies during the past 20 years of daily supplementation of 200 μg as Cr chloride and up to 1000 μg in the form of Cr picolinate ranging from 5 weeks to 4 months of supplementation, we have been unable to detect an effect of supplemental Cr on body weight... If Cr is to have any effect on body composition and over all weight, it is likely to be a long-term small effect and not a quick fix or panacea.'

According to Kreider in reference [61], page 105:

> 'It appears clear from these studies that short term chromium supplementation (i.e. chromium 200 to 800 mg/day, as chromium picolinate, for 4 to 12 weeks) does not promote muscle growth in healthy resistance-trained individuals. Consequently, in my view, chromium supplementation should not be recommended to athletes as a method of promoting muscle growth or fat loss.'

Lukaski on page 296 in reference [62] states 'CrPic supplementation with an exercise training program does not facilitate a preferential loss of FM [fat mass]. Thus, CrPic *per se* does not promote beneficial changes in body composition of humans.' Lukaski [63] also states in another review, on page 590S 'Thus, the limited studies to date indicate that chromium supplements do not promote general muscle gain and fat loss, as determined by various methods of body-composition assessment, nor do they facilitate regional or whole-body strength gain during resistance training.' Kobla and Volpe [64], page 304, concluded that 'Chromium supplements have gained popularity due to the belief that they may increase LBM and reduce percent body fat ... Chromium supplementation does not appear to increase LBM or decrease fat mass, even when combined with resistive training.' Perhaps most notable of all is that the US Federal Trade Commission in 1997 concluded that no basis existed for claims that chromium picolinate promotes body mass loss and fat loss in humans [65].

The most thorough previous review of this area was by Vincent [63] in 2004; this work serves as the foundation for this chapter. Vincent concluded that 'overwhelming evidence indicates that $Cr(pic)_3$ has no effects on body composition.' At about the same time, a review and meta-analysis from Pittler and Ernst appeared. In the review, these workers concluded that chromium picolinate 'could not be recommended for over-the-counter use' [66]. The meta-analysis found a relatively small

effect from chromium picolinate compared with placebo in body mass reduction; however, the clinical significance of the effect was indicated to be 'doubtful' as the study lacked robustness because of a single large study. The 1996 study by Kaats and co-workers (and the only one reporting a statistically significant effect) dominated the results of the meta-analysis (see reference Pittler *et al*. [67]). Thus, the American population and populations of other developed nations have wasted millions of dollars on chromium picolinate (and other chromium supplements) as body mass loss agents or muscle development agents while the supplements generate no discernible benefits.

4.3 CHROMIUM PICOLINATE TOXIC EFFECTS?

In 1995 questions arose about the safety of $[Cr(pic)_3]$ as a dietary supplement as Wetterhahn and co-workers showed that the compound caused clasotogenic damage in a Chinese hamster ovary (CHO) cell model [68]. 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. Wetterhahn and co-workers also suggested that taking $[Cr(pic)_3]$ supplements for 5 years could result in liver tissue concentrations of 13 μM [69]. The investigation of the potential toxic effects of the use of chromium picolinate or other chromium(III) compounds as nutritional supplements or pharmaceuticals has been an active and controversial area of investigation. The toxicology of chromium(III) is addressed in Chapter 9.

4.4 INORGANIC CHEMISTRY OF CHROMIUM PICOLINATE

The inorganic chemistry of chromium picolinate has a long history. The pinkish red crystalline compound is usually prepared by the reaction of simple chromium(III) salts (e.g. chromic acetate [70], chromic nitrate [71], chromic perchlorate [72], chromic chloride [73], or chromic sulfate) [74] and picolinic acid in water following the procedure of Ley and Ficken reported in 1917 [75]. It has also been prepared by the direct reaction of $Cr(OH)_3$ [76] and picolinic acid, the reaction of $[Cr(CO)_6]$ and the acid in methanol [77], or the oxidation of chromous ions by $[Co(NH_3)_5(pic)]^{2+}$ [78]. The preparation of the complex in water by the method of Ley and Ficken has long been recognized to result in the formation of a purple by-product of empirical formula '$Cr(III)(pic)_2$

(OH)' [70, 79], although it has been misidentified as an isomer of $[Cr(pic)_3]$ [80]. The by-product is actually the dinuclear complex $[Cr_2(\mu\text{-}OH)_2(pic)_4]$, which has been characterized by Stearns and Armstrong [71]. A mixture of monomer and dimer is also formed by the reaction of chromic salts with picolinic acid and aqueous hydroxide in ethanol [79]; the two products can be separated by their solubility in acetonitrile. Unfortunately, this procedure also gives rise to another intractable product, possibly polymeric chromic oxides, and the yields of monomer and dimer have not been reported. Stearns and Armstrong showed that increasing pH results in an increase in the yield of the dimer relative to monomer from aqueous solution, although only a 16% isolated yield of dimer was reported [71]. A high yield preparation of the dinuclear complex has also appeared [81]. The three dimensional structures of both the mononuclear and dinuclear complex have been determined by X-ray crystallography; this technique clearly reveals the red monomer to be the meridinal isomer [81], despite earlier claims based on electronic spectroscopy that it was the facial isomer (reviewed in reference [71]). The existence of the facial isomer in solution or solid phase has yet to be established.

Measuring the electronic and ^1H NMR spectra of $[Cr(pic)_3]$ has been problematic, but questions about the electronic spectrum have recently been resolved [81]. Because of the low solubility of the complex in water and other common solvents, electronic spectra were often measured in dilute mineral acid solution, in which the complex decomposes with time [71, 81]. Despite an original claim that no ^1H NMR signals could be observed for $[Cr(pic)_3]$ [82], the paramagnetic ^1H and ^2H NMR spectra have recently been obtained and assigned [83]. Unfortunately the low solubility of $[Cr(pic)_3]$ in H_2O and the broadness of the NMR signals prevent electronic and NMR spectroscopy from being useful in following the complex in biochemical or nutritional studies. The lipophilicity of $[Cr(pic)_3]$ has been measured and found to be surprisingly small [83], although $[Cr(pic)_3]$ has been proposed to act by affecting lipid bilayer fluidity [16].

The redox potential of $[Cr(pic)_3]$ has been reported [84]. The compound is considerably harder to reduce than previous estimates suggested. In DMF the Cr(II)–Cr(III) couple has an $E_{1/2}$ of -1.23 V vs. a normal hydrogen electrode, while the dinuclear compound $[Cr_2(\mu\text{-}OH)_4(pic)_4]$ is harder to reduce ($E_{1/2} = -1.45$ V) [84]. No features are observed at positive potentials until solvent decomposition occurs for either compound. The potential for the reduction of $Cr(pic)_3$ is slightly shifted in the positive direction in water (A. Pickering and

J. Vincent, unpublished results). The implications of this for the toxicology of [$Cr(pic)_3$] will be discussed in Chapter 9.

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5

Is Chromium(III) Effective as a Therapeutic Agent?

In July 2001, the Office of Dietary Supplements, National Center for Complementary and Alternative Medicine and the National Institute of diabetes and Digestive and Kidney Diseases of the National Institutes of Health (USA) issued the programme announcement 'Chromium as adjuvant therapy for type 2 diabetes and impaired glucose tolerance' [1]. The announcement was designed to encourage 'basic studies of chromium action on insulin secretory and signalling pathways' and 'clinical studies to assess the safety and efficacy of chromium as an adjuvant treatment of type 2 diabetes and/or impaired glucose tolerance.' The announcement was based on reports of beneficial effects from chromium supplementation of type 2 diabetic subjects, which were considered to be suggestive but not definitive. Methodological problems were noted as a concern for many of the studies, and the need for 'more rigorous, blinded and well-controlled studies' was stressed. The announcement coincided with increased use of claims of beneficial effects from chromium supplementation by the nutritional supplement industry. This raises the question of whether chromium(III) complexes can be used to treat the symptoms of type 2 diabetes, also called adult-onset or non-insulin-dependent diabetes.

Type 2 diabetes stems from insulin resistance in the tissues. As a result, blood glucose levels are above normal. The pancreas responds by producing and releasing more insulin that is not as effective as it should be, resulting in high blood insulin levels as well. As the production of

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insulin is stressful, the beta cells of the pancreas, which produce the vast majority of the insulin, wear out and die over time, resulting in patients being both insulin resistant and unable to produce adequate amounts of insulin. This contrasts with type 1 diabetes, which is usually first observed during childhood. In this case, an environmental response results in the body's immune system attacking the beta cells of the pancreas, destroying the body's ability to produce sufficient insulin.

The possibility that administration of pharmacological amounts of Cr could result in beneficial effects for subjects with altered metabolisms (that potentially could lead to altered Cr status) is in accord with current models of the biochemical role of chromium (although the correlation with Cr status is not) (see Chapter 6). Type 2 diabetes [2] and pregnancy [3] are examples of conditions leading to increased urinary Cr loss. Thus, initial researchers assumed that this increased urinary Cr loss could potentially lead, with time, to a decrease in Cr status, such that people with these conditions could potentially benefit from Cr supplementation, in contrast to healthy individuals. However, studies were not performed to establish whether increased urinary chromium loss was offset by increases in absorption (i.e. is there an increase in absorption that results in the increase in urinary chromium loss?).

Hence, whether the results of studies on the administration of chromium to type 2 diabetic subjects reflect restoration of Cr balance remains unknown for humans [4]. Loss of Cr balance would appear to be possible as, for example, type 2 diabetes patients have been found to possess ~33% lower plasma Cr and almost 100% higher urine Cr than healthy individuals [5]. Patients diagnosed with diabetes for several years had urinary Cr losses less than those of the control group. This study then suggests that insulin resistance (and associated increased non-fasting plasma insulin and glucose levels) could possibly result in increased mobilization of Cr from the blood with corresponding increased urinary Cr loss until Cr stores are depleted, resulting in decreased mobilization and urinary Cr loss [5]. Similarly, rat models of diabetes excrete greater amounts of chromium than their healthy counterparts [6]. This has been used to support postulates that Cr deficiency exacerbates the symptoms of diabetes and that Cr supplementation can thus reverse the symptoms to a degree.

Part of the difficulty in being more precise about a role for chromium under a stress, such as type 2 diabetes, is often that of determining cause and effect. For example, recent studies have found a relationship between chromium status and cardiovascular disease [7–10]. Does the disease or stress result in a mechanism by which the distribution of chromium is

altered, or is the loss of chromium from the body or are low Cr levels in part responsible for the symptoms of the disease?

However, absorption has been established in some rat models of diabetes. Vincent and co-workers [11] have examined chromium absorption in male Zucker lean, Zucker obese (an obese, insulin-resistant rat (see below)) and Zucker diabetic fatty (ZDF) (a type 2 diabetes model (see below)) rats. The rats were gavaged with a solution of $^{51}Cr^{3+}$ (3 μg/kg body mass) as ^{51}Cr chloride in water. Assuming a human has an average body mass of 65 kg, the dose corresponds roughly to a human receiving 200 μg Cr daily. Commercial Cr-containing nutritional supplements generally contain 200–600 μg Cr; thus, the dose is equivalent to that of a human taking a nutritional supplement. At least 90% of the administered dose cleared the stomach in 60–120 min for the lean and ZDF rats, while only ~80% of the dose past through the stomach after 2 h for the obese rats. The content of the label in the small intestine of all the groups reached a maximum in 1 h, although for the ZDF rats the amount in the intestine was statistically equivalent between 1 and 2 h. The maximum content of the label in the large intestine was reached at 6 h, although for the lean rats the amounts were equivalent at 6 and 12 h. This passage through the gastrointestinal tract was accompanied by increased loss of the label in the faeces with time. Nearly 100% of the administered label was lost in the faeces within 48 h of treatment for all three groups. Of the tissues examined, by far the most Cr was found in the skeletal muscle. The maximum retained dosage in the muscle was ~0.8% for the lean rats, ~0.7% for the obese rats and ~0.6% for the ZDF rats. For the lean and obese rats, the percentage of absorbed dose in any other tissue never exceeded ~0.06% of the applied dose, while over 0.15% of the applied ^{51}Cr appeared in the liver of the ZDF rats.

Besides the liver, skeletal muscle and gastrointestinal tract, only the kidneys of the harvested tissues possessed a notable amount of Cr, at maximum 0.01–0.02% of the applied dose for the lean and obese rats and ~0.04% for the ZDF rats. The disappearance of chromium from the blood and the tissues after approximately the first hour after administration of the radiolabel was accompanied by the rapid appearance of chromium in the urine. For the lean and obese rats, the amount of Cr in the urine rose to approximately 0.3–0.6% of the applied dose. In contrast, the amount of the radiolabel in the urine of the ZDF rats rose to about 1% of the applied dose. For the lean rats, ~1.1% of the applied dose is retained after 30 min and 1 h. For the Zucker obese rats, ~0.7% of the applied dose was retained after 30 min, and ~1.1% of the applied dose was retained after 1 h. Thus, other than the apparently slower

absorption process in the Zucker obese rats, the retention of chromium was similar between the Zucker lean and Zucker obese rats. In contrast, the ZDF rats retained appreciably greater amounts of Cr. Approximately 1.3% of the applied dose was retained after 30 min, and ~1.8% of the applied dose was retained after 1 h. Thus, the ZDF rats appear to absorb approximately twice as much Cr as their lean and obese counterparts. The ZDF rats have more ^{51}Cr appearing initially in the tissue and bloodstream, followed by a greater amount of Cr appearing in the urine.

Feng *et al.* [12] have examined the distribution of Cr in healthy and alloxan-induced diabetic rats, a type 1 diabetes model (see below). The form of chromium administered was not determined, but, given the preparation procedure, the form was probably $[Cr(H_2O)_6]^{3+}$. The Cr was administered intragastrically to fasted rats; thus, tissue and fluid Cr levels were influenced by absorption rates. Chromium content in the stomach, small and large intestines, faeces, urine, blood, liver, kidneys, muscle, femur, testes, heart, spleen, lung, pancreas and brain were measured 1, 2, 4, 8, 24, 48, 96 and 168 h after administration. The levels of Cr in the blood and liver were maximal 1 h after administration and similar in diabetic and control rats. In contrast, Cr levels in the muscle were maximal 4 h after administration and were almost four times higher than the level in controls (which were maximal at 1 h). For the diabetic rats, levels of Cr in the kidney, liver and femur rose from 8 to 24 h after administration, again potentially representing Cr movement into the medium-exchanging pool. After the Cr content of the blood and tissues had risen initially and as the Cr content began to drop, the Cr content of the urine increased, with the Cr content of the urine being twice as high for the alloxan-treated diabetic rats after 48 h and over four times higher after 168 h. The alloxan-treated diabetic rats had greater Cr absorption and greater urinary Cr excretion than the controls.

Thus, the results of Vincent and co-workers and of Feng and co-workers indicate that type 2 diabetic rats and type 1 diabetic rats have increased urinary Cr loss as a result of their diabetes; however, this increased urinary Cr loss is offset by increased absorption of Cr. The urinary loss of Cr reflects the increased absorption of Cr, consistent with Cr absorption being a diffusion controlled process. Insulin resistant, obese rats have alterations in the rates of Cr transport and distribution compared with lean rats but have similar urinary Cr loss and Cr absorption. Thus, any increases in urinary Cr loss associated with insulin resistance or diabetes are offset by increased absorption. Given that dietary chromium is normally absorbed with only ~1% efficiency, suitable

Cr exists in the diet so that a standard diet possess sufficient Cr to allow for the increases in absorption associated with diabetes. Consequently, supplementing the diet with nutritionally relevant amounts of Cr is not anticipated to have any beneficial effects.

The increased loss of Cr in the urine associated with diabetes cannot be taken as evidence for the essentiality of Cr, especially as supra-nutritional doses of Cr are required for any beneficial effects to potentially be observed (see below). These effects (to the extent they occur) cannot be derived from the restoration of Cr sufficiency from a Cr-deficient state but must come from a pharmacological mechanism. Similarly, altered Cr tissue and body fluid concentrations appear to be the result of diabetes and not a cause. In other words, increased Cr absorption in the diabetic rats appears to result in the increased urinary Cr loss, so that the urinary Cr loss is a result of the diabetes, not a cause.

Further research is required to determine whether nutritional doses of chromium might be beneficial to subjects with gestational diabetes. Pregnant women are reported to have greater urinary Cr loss than non-pregnant women, although more insulin-resistant pregnant women tended to excrete more Cr than pregnant women with less insulin resistance [13]. Women with gestational diabetes have increased levels of Cr in hair [14]. Women with gestational diabetes have equivalent levels of plasma Cr compared with non-diabetic pregnant women [15], yet the concentration of plasma Cr does not correlate with the glucose tolerance of these women [16]. Data are needed on Cr absorption in pregnant subjects to determine whether they can maintain Cr balance. Chromium absorption and excretion in lactating women has been reported to be similar to those of non-pregnant, non-lactating women [17].

5.1 HUMAN STUDIES

5.1.1 Type 2 Diabetes

An examination of the evidence for beneficial effects from administration of Cr to type 2 diabetics is now in order. Unfortunately, studies examining the effects of chromium supplementation of type 2 diabetic subjects vary considerably in quality and utility, as suggested by the National Institutes of Health. The first study of the effects of chromium in type 2 diabetics appeared in 1966; Glinsmann and Mertz administered six patients varying amounts of Cr for varying periods of time. The study was neither blind nor placebo-controlled, and the results are, thus,

ambiguous [18]. No funding source was provided. Sherman *et al.* followed in 1968 by administering 150 μg of Cr as $CrCl_3$ daily to seven subjects in a double-blind, placebo-controlled, crossover experiment lasting a total of 17 weeks. No effects were observed on fasting glucose or in glucose tolerance tests (GTT) (0–180 min after glucose administration) [19]. The study was supported by NIH.

No studies appeared for another decade when they started appearing at an appreciable rate. Nath *et al.* reported in 1979 on the effects of administering chromate reduced to Cr^{3+} with ascorbic acid [20]. The study involved 12 subjects but was not placebo-controlled. Subjects received 500 mg Cr daily for 60 days. After this period, phospholipid and triglycerides levels were unchanged while cholesterol was significantly lower. Improvement was also observed in insulin and glucose concentrations.

Six studies appeared in the 1980s. Not all provided much information on the effects of Cr on type 2 diabetics, unfortunately. Studies by Offenbacher and Pi-Sunyer [21] and by Elias *et al.* [22] used Cr-rich yeast as a Cr source. This source of Cr is not chemically well defined, leaving questions as to what, if any, observed effects might be attributable. For example, several groups have shown that an insulin-activating factor thought to contain Cr can actually be separated from the Cr in Brewer's yeast (reviewed in Chapter 3). Grant and McMullen provided 37 type 2 diabetic subjects 1600 mg of Brewer's yeast (containing 1.28 μg Cr) or a placebo for 7 weeks in a double-blind crossover study [23]. The yeast resulted in an increase in serum HDL (high-density lipoprotein) and a decrease in glycated haemoglobin; no effects were observed on total cholesterol, triglycerides, fasting blood glucose, or the area under the curve in a glucose tolerance test. In this study, how one could differentiate the effects from such a small amount of supplemental chromium (1/30 of the daily requirement) from any other effects of the yeast is less than obvious.

Mossop [24] reported on the effects of daily administration of 600 μg Cr as $CrCl_3$ to 13 subjects while 13 others received placebos. No change was observed on fasting total cholesterol levels, but a decrease in fasting glucose levels and an increase in HDL levels were observed. Unfortunately, no statistical analysis was presented so that the significance of the potential changes cannot be determined, and no funding source was provided. Martinez *et al.* [25] reported results of a study involving diabetic subjects in 1985; however, the results from the diabetic group were not separated from those of the other subjects. The study was supported by Health and Welfare, Canada. Forty-three subjects were used in a

placebo-controlled, double-blind crossover study by Rabinowitz *et al*. [26], reported in 1983. The subjects received 150 μg Cr as $CrCl_3$ daily for 4 months in one test period; subjects also received Brewer's yeast for another period. The experiment lasted a total of 16 months. No effects from $CrCl_3$ were found for cholesterol, triglycerides, fasting insulin, or fasting glucose levels or glucose or insulin levels 2 h after a meal. The Veterans Administration and NIH funded the study.

Another double-blind, placebo-controlled crossover experiment was performed by Uusitupa *et al*. [27]. The study involved ten subjects who during the Cr administration period received 200 μg Cr as $CrCl_3$ daily for 6 weeks. No effects were observed on total: HDL, LDL (low-density lipoprotein), or VLDL (very low-density lipoprotein) cholesterol; triglycerides; or fasting insulin or glucose levels. Two hours after oral glucose challenges, no effects were observed on glucose or insulin levels. However, 1 h after the challenge, glucose levels were not affected but insulin levels were appreciably lower. The study was supported by the Foundation for Nutrition Research, Helsinki.

Evans [28] conducted a study of 11 subjects who received 200 μg Cr as $[Cr(pic)_3]$ daily for 6 weeks as part of a double-blind, placebo-controlled study reported in 1989; this is the same article that started the interest in $[Cr(pic)_3]$ as a dietary supplement for body mass loss and increased muscle mass. After being on Cr, subjects had lower glucose, total and HDL cholesterol and glycated haemoglobin levels; no funding source was provided. This study was one of three studies in this article, whose results were rapidly challenged [29–31], as discussed in Chapter 4, for the body composition changes claimed in this work. One interesting note about the study is whether the effects in the diabetic subject study are significant or not depends on how the statistical analysis is performed [32]. Evans had each subject serve as his or her own control (which is a valid procedure), and paired *t* values were calculated for each person. However, if one calculates the means and standard errors and compares the means (as generally performed), then the effects in this study are not significant at $P \leq 0.05$. The use of chromium(III) compounds, particularly $[Cr(pic)_3]$, to treat type 2 diabetes and related conditions drew considerable attention after this report.

Seven studies of relevance appeared in the 1990s, and the pace accelerated rapidly thereafter. The first report in the 1990s was by Abraham *et al*. in 1992 [33]. Thirteen subjects received 250 μg Cr as $CrCl_3$ daily, while 12 concurrently received placebos, for between 7 and 16 months. No effect was observed on serum glucose concentrations. No funding source was provided. Two years later Lee and Reasner [34] in a

double-blind, placebo-controlled crossover study reported the effects of administering 28 subjects 200 μg $Cr(pic)_3$ daily for 8 weeks. (The authors probably meant 200 μg Cr as $Cr(pic)_3$ daily, although experimental section does not read this way.) No effects were observed on glucose, HDL or LDL cholesterol, or glycated haemoglobin levels; however, triglycerides levels were significantly lower after Cr treatment. The study was supported by NIH.

Thomas and Gropper [35] administered 200 μg Cr as Cr nicotinate to five subjects in a placebo-controlled, crossover study lasting a total of 16 weeks, which was reported in 1996. No significant effects were found for plasma insulin; glucose; total, LDL and HDL cholesterol; triglycerides; and 90-min post-prandial glucose levels. The study was supported by InterHealth, Inc. In 1999, Bahadori *et al.* [36], in work described in a preliminary report, administered 16 patients 500 μg Cr as [$Cr(pic)_3$] daily for 4 months. No effects were observed on glycated haemoglobin, steady-state glucose, nor steady-state insulin levels or on steady-state insulin c peptide nor fasting insulin to c peptide ratios. However, lower fasting insulin concentrations were found after treatment. The study was not placebo controlled, and no funding source was indicated.

The largest study performed to date was by Anderson *et al.*, reported in 1997 and funded by Nutrition 21 and the Diabetes Action Foundation [37]. This paper is the foundation for claims that chromium supplementation, particularly [$Cr(pic)_3$] supplementation, has beneficial effects on type 2 diabetic subjects. The study involved 180 patients in China. Patients received a placebo or 200 or 1000 μg Cr daily as [$Cr(pic)_3$] for 4 months. Fasting serum glucose and glucose 2 h after a glucose challenge after 2 and 4 months of treatment were lower for the group receiving the larger, but not the smaller, amount of Cr. Fasting insulin levels and insulin levels after a glucose challenge were lower for both groups after 2 and 4 months of treatment. Glycated haemoglobin was lower for the group receiving 1 mg Cr after 2 months and for both groups after 4 months. Fasting serum cholesterol was only significantly lower for the 1 mg group after 4 months of treatment. All groups, including the placebo group, improved in terms of fasting glucose and insulin, insulin and glucose after a glucose challenge and glycated haemoglobin levels; the lower values for the Cr-receiving groups represent improvement beyond that of controls. This study has been thoroughly critiqued by Hellerstein [38]. A follow-up to this Chinese study has been reported [39]. Patients (833) on insulin or diabetes medications received 500 μg Cr as [$Cr(pic)_3$] for up to 10 months. While beneficial effects were

noted, the work was not placebo-controlled, and no source of funding was provided.

Ravina and co-workers reported two studies following the amounts of insulin, sulfonylurea, or metformin required by 138 and 114 type 2 diabetic subjects [40, 41] for 2 weeks or 10 days, respectively, while taking 200 μg Cr as $[Cr(pic)_3]$ or a placebo daily. In both studies, more than half the subjects receiving Cr required less medication while no effects were observed in subjects receiving placebos. Solgar Vitamin, Inc. provided the $[Cr(pic)_3]$.

Studies appeared rapidly after 1999 and are so numerous that only full papers will be considered further here. Anderson *et al.* reported on the effects of supplementation with 400 μg of Cr daily as chromium pidolate (tris(2-pyrrolidine-5-carboxylato)chromium(III)) for 6 months [42]. Subjects (110) were split into four groups, two of which are of interest here: a placebo group and a Cr-receiving group. No effects were observed on glycated haemoglobin, fasting glucose, insulin, total cholesterol, or HDL cholesterol. The study was supported by the Diabetes Action Foundation and Labcatal Pharmaceutical.

In an uncontrolled study using 100 mg Cr daily of 'Cr yeast' for 8 weeks, Trow *et al.* [43] observed no effects for their eight subjects in terms of: plasma glucose; total, HDL, or LDL cholesterol; triglycerides; or insulin concentrations in glucose tolerance tests. The study was supported by the UK Ministry of Agriculture, Fisheries and Food and Biotechnology and Biological Sciences Research Council. In another uncontrolled study, Morris *et al.* [44] reported that five subjects receiving 400 μg Cr as $[Cr(pic)_3]$ daily for 12 weeks had no changes in fasting glucose or glycated haemoglobin but after 6 and 12 weeks of supplementation had decreased insulin resistance in insulin tolerance tests. $[Cr(pic)_3]$ was provided by Nutrition 21. In a double-blind, placebo-controlled crossover study, Ghosh *et al.* [45] had 50 patients intake 400 μg Cr as $[Cr(pic)_3]$. After the Cr phases of the 32 week study, subjects had lower fasting serum glucose and insulin levels, lower glycated haemoglobin levels and lower post-prandial glucose levels. No effect was found on total, LDL and HDL cholesterol or triglycerides concentrations. No funding source was provided. Finally, Bahijri and co-workers [46, 47] have reported the results of double-blind, crossover studies using $CrCl_3$ and yeast. Seventy-eight subjects receiving 200 μg Cr as $CrCl_3$ for 8 weeks resulted in a decrease in fasting glucose and triglycerides and in glucose in a 2-h GTT. HDL cholesterol was found to increase. The King Abdul Aziz University research committee supported the study.

Rabinovitz *et al.* [48] in 2004 reported a double-blind study involving 112 subjects who received 400 μg Cr daily as $[Cr(pic)_3]$ for 3 weeks. Compared with the control group, the treatment group had a lowering of fasting glucose levels and total cholesterol levels. Solgar Vitamin and Herbal provided the supplement. Sharafetdinov *et al.* reported in 2004 that 50 μg chromium as chromium-spirula (blue–green algae/cyanobacterium) improved blood glucose but not cholesterol or triglycerides in type 2 diabetic subjects [49]; this material suffers from the same issues as chromium-enriched yeast. Also in 2004, Cheng *et al.* [50] examined the potential for Cr yeast to serve as an antioxidant in type 2 diabetic subjects. Subjects received 1000 μg Cr daily for 6 months or a placebo. Subjects with glycated haemoglobin (HbA_{1c}) values over 8.5% had decreased levels of TBARS (thiobarbituric acid reactive substances) while no effects were noted in placebos; the plasma total antioxidant status was increased. In contrast, when euglycemic subjects were examined ($HbA_{1c} < 6.0\%$), the opposite effects were observed. Thus, the Cr-enriched yeast appeared to protect against oxidative damage in diabetic subjects but serve as a prooxidant in euglycemic subjects [50]. No significant differences were found in antioxidant enzyme activities. The study was funded by the National Science Council of the Republic of China.

In 2005 Vrtovec *et al.* [51] in a double-blind, placebo-controlled crossover study examined the effects of 1000 μg Cr as $[Cr(pic)_3]$ on the QTc interval in 60 type 2 diabetic subjects. Chromium supplementation shortened the QTc interval during the chromium treatment period whether subjects received placebo or the treatment first, such that the QTc was shorter for all subjects at the end of the study. No effect from chromium was observed on body mass index, fasting glucose, insulin, total cholesterol, glycated haemoglobin levels, or blood pressure. No funding source was provided. Also in 2005, Vladeva *et al.* [52] gave 34 overweight subjects with type 2 diabetes 30 μg of chromium picolinate daily for 2 months. Thirty-five healthy volunteers matched by BMI (body mass index) were used as controls. No placebo control was utilized. Insulin levels were stated to decrease in diabetic subjects; however, no statistical analysis was presented.

Five studies of note appeared in 2006. Racek *et al.* reported on the effects of chromium-enhanced yeast (400 μg Cr/day) versus a placebo for 12 weeks in 36 subjects. Fasting serum glucose was decreased in the treatment group, although blood markers of oxidative stress decreased significantly in the control group and not the treatment group [53]. No statistically significant effects were observed on fasting serum insulin,

glycated haemoglobin, LDL or HDL cholesterol, or triglycerides. The yeast was provided by Pharma Nord. Kleefstra *et al.* [54] in a double-blind 6-month study provided 500 or 1000 μg Cr as [$Cr(pic)_3$] or a placebo to 46 'Western' subjects. The treatments had no effect on glycated haemoglobin; blood pressure, body mass index, or total, HDL or LDL cholesterol. However, a weak relationship was found between increasing serum chromium concentration and improvement of subjects' lipid profile. No funding source was provided. Pei *et al.* [55] uniquely provided milk powder or $CrCl_3$ mixed with milk powder (400 μg Cr daily) to 60 type 2 diabetic subjects for 16 weeks. The milk powder was provided by Maxluck Biotechnology, who also supported the study financially. Overall, fasting plasma glucose and insulin levels were lower in the Cr-receiving group; no change was observed in glycated haemoglobin, insulin resistance, or other variables examined. Male subjects on the Cr-containing milk powder had statistically lower insulin resistance and glycated haemoglobin than males on just milk powder, but comparable effects were not observed for female subjects. Given the lack of details on the milk powder preparation, determining whether this can be considered a well-defined source of chromium is not possible. Singer and Geohas examined the effects of a combination of chromium picolinate and biotin to type 2 diabetic subjects [56]; however, as the design does not allow the effects of chromium to be elucidated, the study will not be considered further. Martin *et al.* [57] examined the effects of [$Cr(pic)_3$] (1000 μg Cr daily) on 37 type 2 diabetics that had been treated previously for 3 months with sulfonylurea. For the next 6 months, subjects received either a combination of sulfonylurea and placebo or sulfonylurea and chromium. Subjects on the placebo had a significant body mass increase (2.2 kg) versus chromium treatment (0.9 kg) from baseline. The percentage body fat and total abdominal fat (measured by dual-energy X-ray absorptiometry) also increased in subjects on placebo. In addition, subjects on chromium had greater decreases in glycated haemoglobin and free fatty acids. The study was funded by NIH. One unusual aspect of these results has been eluded to by Mark [58]. The body mass loss from chromium administration compared with placebo all appears to have occurred in the first week after treatment was begun; after this, the body mass for placebo and treatment groups run parallel courses. How such a body mass loss tied to the loss of abdominal fat could occur in this manner is not readily explained [59].

Since 2006, Geohas and co-workers [60–62] followed up with three studies on the effects of the combination of biotin and [$Cr(pic)_3$], all lacking a group just receiving the chromium compound. Two studies

have examined the effects of chromium yeast. Lai [63] in a double-blind, placebo-controlled study examined the effects of 1000 μg Cr in yeast daily for 6 months in diabetic subjects with glycated haemoglobin levels equal to or greater than 8.5%. The effects of the Cr in addition to 1000 mg vitamin C and 800 units vitamin E were also examined. Unfortunately, only 30 subjects were examined, such that each group had only ten subjects. Chromium yeast alone was reported to lower fasting glucose, glycated haemoglobin and insulin resistance while lowering thiobarbituric acid reactive substances and increasing total antioxidative status. No funding source was indicated.

Kleefstra *et al*. [64] examined the effects of Cr yeast on a 'Western population' (subjects from a village in the northern Netherlands) to contrast the results with those of Anderson and co-workers in China. Fifty-seven subjects received either 400 μg Cr daily or placebo for 6 months. No effects were observed in fasting plasma glucose; glycated haemoglobin; blood pressure; percentage body fat; body mass index; total, HDL and LDL cholesterol; triglycerides; and insulin resistance. The research was supported by the Langerhans Foundation, the Medical Research Foundation and Pharma Nord.

Finally, Cefalu and co-workers [65, 66] in a preliminary and then a subsequent report may have found a relationship that could explain the different results between populations in the various studies. In a double-blind, placebo-controlled study 93 subjects with a fasting plasma glucose level of at least 6.94 mmol l^{-1} received 1000 μg Cr daily as $[Cr(pic)_3]$ or placebo for 24 weeks [66]. Comparison of the treatment and control groups found no effects on body mass, percentage body fat, free fat mass, or abdominal fat deposits, fasting glucose, glycated haemoglobin, or insulin sensitivity. However, effects at the end of the study were observed when the Cr-receiving subjects were divided into responders ($\geq$10% increase in insulin sensitivity from baseline) and non-responders. At baseline, responders had lower insulin sensitivity and higher fasting glucose and glycated haemoglobin levels than non-responders. Thus, Cefalu and co-workers may potentially have identified predictors for type 2 diabetic subjects that may preferentially respond to chromium treatment. These results will need to be carefully tested in additional studies where the 'responder' group is identified before the chromium administration. The study was supported by NIH, while the chromium supplement and placebo were provided by Nutrition 21.

Katz and co-workers recently reported in 2011 a study of 59 subjects with impaired fasting glucose, impaired glucose tolerance, or metabolic syndrome. In the double-blind, placebo-controlled study, no effect from

[$Cr(pic)_3$] supplementation (500 or 1000 μg daily) for 6 months was observed on serum insulin, fasting plasma glucose, body mass, glycated haemoglobin, triglycerides, 2-h glucose after GTT, or total, LDL or HDL cholesterol [67]. The study was supported by NIH.

To attempt to comprehend these studies, the focus will be on those that at least have a placebo control and use a chemically defined form of Cr (Table 5.1), which limits discussion to 19 of the studies described above (the two reports of Bahijri and co-workers. [46,47] cover the same data and are considered as a single study). Nine of the 19 reports saw no effects from supplementation; another may or may not have seen significant changes depending on how the statistical analysis is performed. Studies using 150–1000 μg Cr daily for 6 weeks to 16 months have reported no effects from Cr, while studies using 200–1000 μg for 10 days to 6 months have observed beneficial effects. Studies using over 100 subjects, which should have more power to distinguish potential differences, reported no effects in one case and beneficial effects from supplementation in the others. Several studies are quite small, lacking the statistical power to potentially observe effects.

Similarly no pattern exists in terms of particular symptoms. Fourteen studies examined fasting blood glucose levels. Five reported that levels dropped with supplementation while nine observed no effect. Four studies observed no effect on fasting insulin levels while levels were lower in three studies. Triglycerides levels were unaffected in four studies and lower in two studies. Glycated haemoglobin levels were reported to be lower in our studies, but no change was reported in five studies. Effects on cholesterol levels were slightly more consistent. Seven studies reported no lowering of total cholesterol while three noted decreases. For HDL, six studies reported no effect, while a single study reported an increase in levels; for LDL, five studies reported no effects, while only a single study reported a decrease. In response to some type of a glucose challenge, four studies observed no effects on glucose levels while three saw positive effects; in terms of insulin response, one study had mixed results depending on the time interval that Cr was administered, while another reported positive effects. Behaviour of the blood variables is simply too inconsistent to draw any firm conclusions. This inconsistent behaviour exists whether these studies are broken down by the compound used, the amount of Cr, the number of subjects, the length of the study, and so on.

Two thorough meta-analyses have been reported. Althius *et al.* [68] in 2002 performed a meta-analysis on studies with type 2 diabetic subjects under a contract from the Office of Dietary Supplements of NIH. Using

Table 5.1 Double-blind, placebo-controlled studies of chromium supplementation of type 2 diabetic subjects using well-defined forms of chromium.

Total No. subjects	Dose (μg Cr/day)	Form	Time on Cr	Crossover	Statistically significant effects	Ref.
7	150	$CrCl_3$	16 weeks	Yes	None	Sherman *et al.* [19]
43	150	$CrCl_3$	4 months	Yes	None	Rabinowitz *et al.* [26]
10	200	$CrCl_3$	6 weeks	Yes	None except decrease in 1-h insulin after GTT	Uusitupa *et al.* [27]
11	200	$[Cr(pic)_3]$	6 weeks	Yes	[a]—	Evans [28]
26	250	$CrCl_3$	7–16 months	No	None	Abraham *et al.* [33]
28	200	$[Cr(pic)_3]$	8 weeks	Yes	None	Lee and Reasner [34]
5	200	Cr nicotinate	8 weeks	Yes	None	Thomas and Gropper [35]
180	200	$[Cr(pic)_3]$	4 months	No	Lower fasting insulin and insulin in GTT	Anderson *et al.* [37]
180	1000	$[Cr(pic)_3]$	4 months	No	Lower fasting insulin, cholesterol, glucose and glycated hemoglobin and insulin and glucose in GTT	Anderson *et al.* [37]
138	200	$[Cr(pic)_3]$	2 weeks	No	Reduced insulin, sulfonylurea, or metformin requirement	Ravina and Slezack [40]
114	200	$[Cr(pic)_3]$	10 days	No	Reduced, insulin, sulfonylurea, or metformin requirement	Ravina *et al.* [41]

110	110	Cr pidolate	6 months	No	None	Anderson *et al.* [42]
50	200	$[Cr(pic)_3]$	4 weeks	Yes	Lower glucose and triglycerides and 2-h glucose in GTT; higher HDL	Ghosh *et al.* [45]
67	200	$CrCl_3$	8 weeks	Yes	Lower glucose, glycated hemoglobin and insulin and glucose in GTT	Bahijri *et al.* [46,47]
112	400	$[Cr(pic)_3]$	21 days	No	Lower fasting glucose and total cholesterol	Rabinovitz *et al.* [48]
60	1000	$[Cr(pic)_3]$	3 months	Yes	Shortened QTc interval	Vrtovec *et al.* [51]
46	500	$[Cr(pic)_3]$	6 months	No	None	Kleefstra *et al.* [54]
46	1000	$[Cr(pic)_3]$	6 months	No	None	Kleefstra *et al.* [54]
37	1000	$[Cr(pic)_3]$	6 months	No	Lower body mass, % body fat and total abdominal fat; lower glycated haemoglobin and free fatty acids	Martin *et al.* [57]
93	1000	$[Cr(pic)_3]$	6 months	No	None (before division into responders and non-responders)	Cefalu *et al.* [66]
60	500	$[Cr(pic)_3]$	6 months	No	None	Ali *et al.* [67]
60	1000	$[Cr(pic)_3]$	6 months	No	None	Ali *et al.* [67]

[a]Depends on how statistics calculated.

their criteria, the authors identified only four studies for analysis (Offenbacher and Pi-Sunyer [21], Uusitupa *et al.* [27], Abraham *et al.* [33], and Anderson *et al.* [37]); the combined data from the studies, except those from Anderson's group, showed no effect from chromium on glucose or insulin concentrations. They concluded that the data on diabetics were, thus, inconclusive. The authors also examined the effects of chromium supplements on healthy subjects or subjects with impaired glucose tolerance (but not type 2 diabetes) in 14 trials including 425 subjects; no associations between glucose or insulin concentrations were found. Another meta-analysis was reported by Balk *et al.* in 2007 [69]; this is the most thorough meta-analysis on chromium supplementation in terms of blood variables reported to date. Forty-one randomized controlled trials were identified that examined the effects of chromium supplementation on glucose metabolism and lipids in $\geq$10 non-pregnant adults (i.e. healthy and diabetic subjects) for $\geq$3 weeks; however, almost half were determined to be of poor quality. Nine studies were funded by the food or supplement industry, 18 were by non-industry sources and 14 did not indicate the funding source. Ten studies used Brewer's yeast, 15 studies used $CrCl_3$, 5 studies used chromium nicotinate and 15 studies used [$Cr(pic)_3$]; some studies compared multiple sources of chromium. No benefit from chromium supplementation was identified for healthy individuals [69]. This, along with the results of Althius *et al.* on non-diabetic subjects as well, reiterates the point from Chapter 4 that chromium supplementation has no benefit on healthy individuals, although Chapter 4 focused on body mass and composition, the same holds true for glucose metabolism and lipids.

Balk *et al.* [69] identified 18 studies examining type 2 diabetic subjects. Chromium supplementation was found to statistically improve glycemic control in type 2 diabetics. The effects were fairly small but significant overall. When broken down by chromium source, the effects were small but significant for subjects on yeast and [$Cr(pic)_3$] but not $CrCl_3$. Yet, the authors determined the results were not definitive because of the poor quality and heterogeneity of the studies. Overall chromium did not affect lipid levels, while [$Cr(pic)_3$] lowered glycated haemoglobin levels. However, lower glycated haemoglobin levels were only observed in three interventions out of 14, two of which came from a single, large study (that of reference [37]). Amongst fasting glucose studies, a trend was observed that industry-sponsored studies were more likely to observe beneficial effects. The authors also expressed concerns that the Brewer's yeast results suggested that another component in the yeast may be having an effect because effects were observed at lower doses of chromium.

As a bottom line the authors concluded that chromium supplementation 'may have a modest effect' on glucose metabolism in type 2 diabetics but that 'the large heterogeneity and the overall poor quality limit the strength of our conclusions' and that more randomized trials are required [69]. The study was supported by a contract from the Agency for Healthcare Research and Quality (US Department of Health and Human Services). Three studies meeting the appropriate criteria have appeared since the Balk *et al.* meta-analysis. These are the small study by Lai [63], the study with Cr yeast utilizing 57 subjects by Kleefstra *et al.* [64] that saw no effects and the study by Cefalu *et al.* [66] with 93 subjects that saw no effects with 1000 μg Cr. These studies, because of the participant size of the last two, would have significantly affected the results of the meta-analysis if they could have been included, making any effect of chromium on fasting glucose in type 2 diabetics even more questionable. One must also note that any meta-analysis is likely to be biased toward the positive as studies with negative results tend to be published less frequently than positive reports.

In a review in 1998, Anderson [70] broke the studies on chromium supplementation of type 2 diabetics into two groups: subjects receiving $\leq$200 μg Cr daily and subjects receiving >200 μg Cr daily. Using all the studies identified with diabetic subjects, it was suggested that >200 μg Cr were required for diabetic subjects to generate an observable effect. The effect appeared to be largest for chromium picolinate where it was the result of a single study (reference [37]). Subsequently, this requirement has commonly been cited. However, the study of Balk *et al.* [69] failed to find conclusive evidence of a dose effect as studies since 1998 clearly failed to follow the trend identified by Anderson.

Thus, what are the effects of chromium on humans with type 2 diabetes? The results are simply too inconsistent to draw a conclusion. When one considers the industry bias displayed in the studies of the effects of chromium on human body mass and composition, and the tendency toward the same bias in terms of observation of beneficial effects from chromium supplementation with type 2 diabetics, great caution must be exercised. While it is possible that a small improvement may occur in glucose concentrations, the magnitude of the effects would not merit the taking of chromium supplements. To state that chromium administration is beneficial would require high quality, independent trials that could demonstrate appreciable beneficial effects in glycated haemoglobins and other variables. These trials should use doses at least up to 1 mg chromium daily and include a population with the 'responder' conditions identified by Cefalu and co-workers. If one were to

be forced to draw a conclusion on Western populations, then one would have to say that an effect from chromium has not been demonstrated. One must wonder if the Chinese study, which dominates meta-analyses, is a statistical fluke or if the type 2 diabetes in the Chinese population tends to arise from a genetic propensity(ies) that is uniquely improved by chromium supplementation and not prevalent in Western populations. Why have no follow-up studies been performed in China in the last decade?

5.1.2 Subjects with Insulin Resistance or Glucose Intolerance

Several studies have examined the effects of chromium supplementation of individuals with a degree of insulin resistance or glucose intolerance while not having resistance great enough to be considered as type 2 diabetic (i.e. fasting glucose 5.6–7 mmol l^{-1} or 2-h post-load glucose 7.8–11.2 mmol l^{-1}) [69]. These studies [57, 71–79] have also been analysed by Balk *et al.* [69]. No effects were noted on glycated haemoglobin, fasting glucose, LDL or HDL cholesterol, or triglycerides as only one study reported a significant effect on one of these variables [69]. Thus, chromium has no effects on glucose or lipid metabolism on healthy or insulin resistant/glucose intolerant subjects without diabetes.

5.1.3 Other Forms of Diabetes

Additionally, one study has been reported on the effects of Cr on gestational diabetes [80]. Women (average body mass 82–84 kg) received 0, 4 or 8 μg Cr as $[Cr(pic)_3]$ per kg body mass daily; both Cr groups had significantly lower plasma insulin and glucose levels. If the results of this single study are reproduced by additional studies, this could have significant implications for the treatment of this condition.

Recently, studies on the effects of supplemental chromium on steroid-induced diabetes have generated interesting results [81, 82], including improvements in plasma glucose levels. These studies still need to be followed up by larger investigations, but they are also supported by the results of a recent study with rats treated with dexamethasone [83]. Dexamethasone-treated rats additionally receiving ~4 mg Cr (an extremely large dose) as $[Cr(pic)_3]$ daily had lower fasting serum insulin levels and lower insulin, triglycerides and glucose areas in glucose or insulin challenges.

5.1.4 Atypical Depression and Related Conditions

In 1999, McCleod *et al*. reported five case studies where [$Cr(pic)_3$] supplementation appeared to beneficially affect patients with dysthymic disorder [84]. This was followed in 2000 with a report of eight case studies of subjects with various types of mood disorders that also appeared to benefit from [$Cr(pic)_3$] supplementation [85]. In 2002, a placebo-controlled, double-blind study using 15 patients with atypical depression was reported by the same research group [86]; 78% of patients on chromium (400 μg [$Cr(pic)_3$] (probably meant to be Cr as the compound) for 2 weeks followed by 600 μg daily for 6 weeks) and none of the subjects on placebo met responder criteria. The work was funded by Nutrition 21, which licensed the patent of one of the authors for the use of [$Cr(pic)_3$] in the treatment of depression.

Another small double-blind, placebo-control trial on 113 subjects with atypical depression was reported in 2005 [87]. For 8 weeks, subjects received 600 μg Cr/day as [$Cr(pic)_3$] or placebo. No effects were reported from the supplement in the primary measures – 29-item Hamilton Depression Rating Scale or the Clinical Global Impressions Improvement Scale. The study was funded by Nutrition 21, and two co-authors were Nutrition 21 employees. In 2007, a two-year, open label study of treating treatment-resistant rapid cycling bipolar disorder was reported [88]. Subjects received 600–800 mg Cr daily as hypo-A Chrom (whose composition was not presented). Of the 30 subjects only seven completed at least a year of the study. Given the unidentified source of chromium, retention rate and lack of placebo control, this study will not be discussed further. Finally, in 2010 a related trial looking at cognitive function funded by Nutrition 21 was reported [89]. The trial involved 26 older subjects who, in a double-blind, placebo-controlled design, received either placebo or 1000 μg Cr as [$Cr(pic)_3$] daily for 12 weeks. Learning rates and retention were not improved, and also reduced semantic interference on learning, recall and recognition memory was observed. Frankly, these studies, because of lack of size or potential conflict of interest, require additional trials with better design and no potential associated conflict of interest before any conclusions can be made.

Some related studies have been reported utilizing rodents [90–94]. Piotrowska *et al*. [90] gave $CrCl_3$ (6, 12 or 32 mg/kg) by intraperitoneal injection to Albino Swiss mice; the mice were then examined in a forced swim test. At only the middle dose was immobility time reduced in the test. Antagonists of serotonin receptors could abolish the effect at the middle dose. Given the dose dependence and the magnitude of the dose

not given orally, the results are difficult to interpret. Khanam and Pillai [91] provided healthy rats and rats with streptozotocin-induced diabetes 226 and 561 μg Cr as [$Cr(pic)_3$] daily, respectively, in their drinking water. The compound had no effects on spontaneous alternation in a cross maze. It did, however, increase percentage preference to open arms in an elevated plus maze test. The same authors examined healthy rats and rats with streptozotocin-induced diabetes receiving 226 and 561 μg Cr as [$Cr(pic)_3$] daily, respectively, in their drinking water for 4 weeks in modified forced swimming tests [92]. The compound increased swimming with a subsequent decrease in immobility. None of these studies provided the funding source.

Attenburrow *et al.* [93] provided 100 mg/kg chromium picolinate (100 mg as chromium?) or placebo to Sprague–Dawley rats for 2 weeks. The rats were administered either 2,5-dimethoxy-4-iodophenyl-2-aminopropane or 5-hydroxytryptophan via intra-peritoneal injection. After 20 min, the animals were sacrificed, and blood samples were collected. Chromium picolinate receiving animals had higher levels of tryptophan and brain tryptophan and serotonin. In a related study, these researchers gave eight healthy human subjects 400 μg daily chromium picolinate for 7–9 days; patients received 5-hydroxytryptophan or placebo in a single-blind crossover fashion one day apart. The compound had no effect on plasma corticosterone [93]. The study was supported by the Medical Research Foundation. Anton *et al.* [94] fasted Sprague–Dawley rats for 24 h and then injected them intra-peritoneally with 0, 1, 10 or 50 μg/kg [$Cr(pic)_3$] (as chromium?); food intake was then monitored for 24 h. Then, rats were implanted with an indwelling third ventricular cannula; subsequently, the compound (0, 0.4, 4 or 40 μg/kg) was injected directly into the brain via the intracerebroventricular cannula. Spontaneous 24-h food intake was then measured. Food intake was decreased at the highest dose after intra-peritoneal administration. Food intake decreased in a dose-dependent fashion after administration into the brain. The study was supported by the Pennington Biomedical Research Center.

Picolinic acid/picolinate is a natural catabolite of the amino acid tryptophan, generated as an end product in the kynurenine pathway in the body [95]. Molecules generated along this pathway tend to have neurological effects because of their similarity to serotonin [96, 97]. In fact a recent report indicated that [$Cr(pic)_3$] modulates serotinergic properties [98]. This raises concerns about [$Cr(pic)_3$] in the body as the [$Cr(pic)_3$] should deliver and release three picolinates (or its degradation products) per Cr. Are the potential effects from chromium or are they from

picolinic acid or a degradation product of picolinic acid? Picolinic acid or its degradation products released from the chromium complex are absorbed significantly better than the chromium from the complex. ^{3}H from ^{3}H-labelled [$Cr(pic)_3$] when provided by gavage administration to Sprague–Dawley rats is absorbed with about 80% efficiency (J.B. Vincent *et al.*, unpublished results), in stark contrast to ~1% for the chromium. *This begs the obvious question as to why do none of these studies have picolinic acid or inorganic chromium salt controls!* Without these controls, any potential effects cannot be attributed to chromium. In fact, effects from picolinate would appear more likely.

5.1.5 HIV and PCOS

HIV-positive patients can share metabolic problems in glucose metabolism similar to subjects with type 2 diabetics; hence, a reasonable hypothesis can be formulated that if chromium administration can be beneficial to type 2 diabetic subjects then it might also be beneficial to HIV-positive patients. Two studies have looked into this hypothesis [99, 100], while another has examined chromium concentrations in the diet, plasma, urine and toenails of healthy and HIV-positive subjects (both receiving and not receiving antiviral therapy) [101]. In the last study, healthy and HIV-positive subjects with qualitatively similar diets in terms of chromium intake had differences in Cr content of some fluids and tissues. Plasma and toenail chromium were lower in HIV-positive subject whether on antiviral therapy or not. Urinary Cr loss was the same between healthy subjects and either HIV-positive subjects on or not on antiviral therapy [101]. In one study of the effects of chromium administration, eight HIV-positive subjects received 1000 μg/day [$Cr(pic)_3$] (probably meant to be 1000 μg Cr/day as pills were provided by Nutrition 21) for 8 weeks. The study was not placebo-controlled. No effect was observed on blood variables (glucose, insulin, triglycerides and HDL and LDL cholesterol) examined, although glucose disposal was found to increase in hyperinsulinaemic-euglycaemic insulin clamp studies. The study was funded by NIH [99]. In the only other study, a large subject set (46 subjects) was examined in a double-blind, placebo-controlled study [101]. The subjects received either placebo or 400 μg chromium nicotinate per day for 16 weeks. No effects were observed on fasting blood glucose, glycated haemoglobin or total, HDL or LDL cholesterol. Blood insulin, triglycerides, total body fat mass and trunk fat mass were lowered by Cr supplementation [101]. If the results of the better designed

study can be independently reproduced, then chromium treatment might prove interesting for improving insulin sensitivity in these patients.

Polycystic ovary syndrome (PCOS) is an endocrine disorder common in reproductive-age women and associated with insulin resistance. Two reports of effects of [$Cr(pic)_3$] administration to PCOS subjects have been reported [102, 103]. In the first study [102], only ten subjects were given either chromium (200 μg/day) or a placebo for 4 months. Chromium had no effect on serum insulin or other hormone levels examined or on fasting glucose, triglycerides or cholesterol levels. Plasma glucose levels 1 and 2 h after a glucose challenge were lower for the chromium treated group than for the controls, but the values were not different from baseline values for the chromium-receiving group. The second study involved six subjects. Subjects received 940 μg Cr daily for 2 months. Of several variables examined, only glucose disposal rate changed significantly (38% increase from baseline) [103]. The study was not placebo-controlled. Both studies were supported by NIH. Thus, given the size and design of the studies, any effects from chromium on PCOS are currently an open question.

5.2 RAT STUDIES

Several rat models of type 2 diabetes have been utilized to examine the effects of chromium(III) administration (Table 5.2). Three models have symptoms arising from similar mutations: the JCR:LA-cp, Zucker obese and Zucker diabetic fatty rats all have mutations of the leptin receptor. Leptin is a hormone produced by adipocytes that signals the brain that the appetite should be suppressed. Consequently, as the leptin signalling system is blocked at the receptor, the JCR:LA-cp and Zucker obese rats become markedly obese and insulin resistant and possess somewhat elevated blood glucose levels and elevated levels of blood insulin, triglycerides and cholesterols. The Zucker diabetic fatty (ZDF) rats have an additional, uncharacterized mutation that results in these rats developing symptoms very comparable to type 2 diabetes in humans, including elevated blood glucose levels, in addition to the high triglycerides and cholesterol levels. In contrast to the obese models, the ZDF rats have smaller body masses than healthy Zucker rats.

Three studies have appeared using the JCR:LA-cp rats, all by Russell and co-workers utilizing chromium picolinate [104–106]. Chromium administration (80 μg/kg body mass daily for from 8 weeks to 3 months) resulted in no changes in food intake or body mass. Observations of other effects were somewhat inconsistent. The first two studies reported

Table 5.2 Studies of chromium supplementation of model rats on selected variables: ADA = American Diabetes Association; CDNC = chromium dinicocysteinate; and Cr3 = $[Cr_3O(propionate)_6(H_2O)_3]^+$.

Rat model	Dose (μg Cr/kg body mass)	Form	Time on Cr	Results	Funding source	Ref.
JCR:LA-cp	80	$[Cr(pic)_3]$	8 weeks	No effect food intake or body mass; no effects fasting insulin or glucose or insulin or glucose after meal; decrease in non-esterified lipids but not cholesterol esters, total cholesterol, phospholipids, or triglycerides	Nutrition 21	Proctor *et al.* [104]
JCR:LA-cp	80	$[Cr(pic)_3]$	3 months	No effect food or water intake or body mass; lower fasting plasma insulin but not glucose; lower total cholesterol, total: HDL cholesterol, but not triglycerides; increased glucose disposal rate	Not provided	Wang *et al.* [105]
JCR:LA-cp	80	$[Cr(pic)_3]$	3 months	No effect food or water intake or body mass; lower fasting plasma insulin but not glucose; lower total and total: HDL cholesterol; higher HDL cholesterol; lower glucose and insulin areas under curve in GTT	Not provided	Cefalu *et al.* [106]

(*continued*)

Table 5.2 (*Continued*)

Rat model	Dose (μg Cr/kg body mass)	Form	Time on Cr	Results	Funding source	Ref.
Zucker diabetic fatty	400	$[Cr(pic)_3]$	8 weeks	No effect fasting plasma glucose; lower glycated haemoglobin	InterHealth	Jain *et al.* [107]
Zucker diabetic fatty	400	Cr nicotinate	8 weeks	No effect fasting plasma glucose; lower glycated haemoglobin	InterHealth	Jain *et al.* [107]
Zucker diabetic fatty	400	CDNC	8 weeks	Lower fasting plasma glucose and glycated haemoglobin	InterHealth	Jain *et al.* [107]
Zucker diabetic fatty	1000	Cr3	24 weeks	No effect food intake or body mass; lower fasting insulin but not glucose; lower total, LDL and HDL cholesterol while no effect total: HDL ratio; lower 2-h insulin but not glucose in GTT; lower glycated haemoglobin	NIH	Clodfelder *et al.* [108]
Zucker diabetic fatty (aged 70–75 weeks)	40/80[a]	Cr nicotinate	6 weeks	Lost less body mass and consumed more food but not water; no effect fasting blood glucose	InterHealth	Talpur *et al.* [109]
Zucker obese	5000	$[Cr(pic)_3]$	20 weeks	No effect food or water intake or body mass; no effect on plasma glucose or insulin; no effect glycated haemoglobin	NIH	Mozaffari *et al.* [110]
Zucker obese	10000	$[Cr(pic)_3]$	20 weeks	No effect food or water intake or body mass; no effect on plasma glucose or insulin; no effect glycated haemoglobin	NIH	Mozaffari *et al.* [110]

Zucker obese	1000	Cr3	24 weeks	No effect food intake but increased body mass after day 100; lower fasting insulin but not glucose; lower total, LDL and HDL cholesterol while lower total: HDL ratio; lower 2-h insulin but not glucose in GTT; lower glycated haemoglobin	NIH	Clodfelder *et al.* [108]
Zucker obese	20	Cr3	24 weeks	No effect food intake or body mass; lower fasting plasma insulin but not glucose; lower total, HDL, and LDL cholesterol and triglycerides while lower total: HDL ratio; lower 2-h insulin but not glucose in GTT	ADA	Sun *et al.* [111]
Zucker obese	—[b]	Cr nicotinate	300 days	No effect on body mass or food intake; lower blood glucose; no effect GTT; longer life span	InterHealth	Preuss *et al.* [112]
Streptozotocin (Sprague Dawley)	20	Cr3	24 weeks	No effect food intake or body mass; lower fasting plasma insulin but not glucose; no effect on total and HDL cholesterol and triglycerides; no effect 2-h insulin and glucose	ADA	Sun *et al.* [111]
Steptozotocin (Wistar, fat-fed)	8	Cr histidinate	10 weeks	Increase in body mass while no change in feed intake; lower serum glucose	Nutrition 21	Dogukan *et al.* [113]
Streptozotocin (Sprague Dawley)	400	Cr nicotinate	30 days	No effect blood glucose	InterHealth	Penumathsa *et al.* [114]

(*continued*)

Table 5.2 (*Continued*)

Rat model	Dose (μg Cr/kg body mass)	Form	Time on Cr	Results	Funding source	Ref.
Streptozotocin (Sprague–Dawley)	400	Cr nicotinate	7 weeks	No effect body mass; lower glycated haemoglobin, total cholesterol, and total: HDL cholesterol; higher HDL cholesterol; no effect fasting glucose	NIH and InterHealth	Jain *et al.* [115]
Streptozotocin (Sprague–Dawley)	400	$[Cr(pic)_3]$	7 weeks	No effect body mass, glycated haemoglobin, total cholesterol, total: HDL cholesterol, HDL cholesterol, fasting glucose	NIH and InterHealth	Jain *et al.* [115]
Streptozotocin (Sprague–Dawley, fat fed)	80	$[Cr(pic)_3]$	10 weeks	Increase in body mass; lower fasting glucose and higher insulin; lower total cholesterol, free fatty acids, and triglycerides	Nutrition 21	Sahin *et al.* [116]
Streptozotocin (Wistar)	600	Cr yeast	4 weeks	Lower fasting blood glucose, LDL cholesterol; no effect HDL cholesterol and triglycerides; no effect body mass or food or water intake	Not provided	Lai *et al.* [117]
Spretozotocin (Wistar)	125	$[Cr(pic)_3]$	4 weeks	Lower plasma glucose, water intake, and food intake; eliminated body mass loss; increased glucose uptake by liver	University Grants Commission, New Delhi	Sundaram *et al.* [118]

Alloxan (no strain provided)	40	$[Cr(pic)_3]$	30 days	Lower fasting glucose; no effect on glucose in GTT or after meal	Not provided	Ou *et al.* [119]
Streptozotocin (Sprague Dawley)	42	$[Cr(pic)_3]$	3 weeks	No effect fasting blood glucose; increased body mass; no effect on food intake	Not provided	Machalinski *et al.* [120]
Streptozotocin (Sprague Dawley)	42	CRC454	3 weeks	Lower fasting blood glucose; increased body mass and food intake	Not provided	Machalinski, *et al.* [120]
Streptozotocin (Wistar)	10.79	$[Cr(pic)_3]$	4 weeks	No effect body mass, or food or water intake; lower blood glucose but not insulin	Not provided	Khanam and Pillai [121]
Streptozotocin (Wistar, adult)	11.19	$[Cr(pic)_3]$	6 weeks	No effect body mass or food or water intake; lower serum glucose, total cholesterol, and triglycerides; lower area under curve for glucose but not insulin in GTT; no effect serum insulin	All India Council of Technical Education	Shinde *et al.* [122]
Streptozotocin (Wistar, 2 day old neonates)	3.57	$[Cr(pic)_3]$	6 weeks	No effect body mass or food or water intake; no effect serum glucose or serum insulin; lower total cholesterol and triglycerides; lower area under curve for glucose but not insulin in GTT	All India Council of Technical Education	Shinde *et al.* [122]
Streptozotocin (Wistar, adult)	20.78	$CrCl_3$	6 weeks	No effect body mass or food or water intake; no effect on serum glucose or insulin but lower cholesterol and triglycerides; lower area under curve for glucose but not insulin in GTT	All India Council of Technical Education	Shinde *et al.* [123]

(*continued*)

Table 5.2 (*Continued*)

Rat model	Dose (μg Cr/kg body mass)	Form	Time on Cr	Results	Funding source	Ref.
Streptozotocin (Wistar, 2 day old neonate)	8.41	$CrCl_3$	6 weeks	No effect body mass or food or water intake; lower serum glucose but not insulin; lower cholesterol and triglycerides; lower area under curve for glucose but not insulin in GTT	All India Council of Technical Education	Shinde *et al.* [123]
Streptozotocin (Wistar, adult)	—[c]	$[Cr(pic)_3]$	6 weeks	No effect on serum glucose or insulin; no effect body mass or food or water intake lower area under curve for glucose but not insulin in GTT	All India Council of Technical Education	Shinde and Goyal [124]
Streptozotocin (Wistar, 2 day old Neonate)	—[c]	$[Cr(pic)_3]$	6 weeks	Lower serum glucose but not insulin; lower area under curve for glucose but not insulin in GTT; no effect body mass or food or water intake	All India Council of Technical Education	Shinde and Goyal [124]
Streptozotocin (Wistar)	20	$CrCl_3$	4 weeks	No effect on body mass; lower area under glucose and insulin curve in GTT	Not provided	Yoshimoto *et al.* [125]
Streptozotocin (spontaneously hypertensive stroke-prone)	20	$CrCl_3$	4 weeks	No effect on body mass; lower area under glucose but not insulin curve in GTT	Not provided	Yoshimoto *et al.* [125]

Alloxan (ICR)	750	Cr citrate	3 weeks	Reduced blood glucose, triglycerides, and total cholesterol	Higher Education of China	Li *et al.* [126]
Goto–Kakizaki	1000[d]	$[Cr(pic)_3]$	32 weeks	No effect on body mass or fasting blood glucose or insulin; no effect area under curve for glucose in GTT at 6 weeks or 32 weeks	New York Medical College Endowment Fund	Abdourahman and Edwards [127]
Goto–Kakizaki	10000[d]	$[Cr(pic)_3]$	32 weeks	No effect on body mass or fasting blood glucose or insulin; no effect area under curve for glucose in GTT at 16 weeks	New York Medical College Endowment Fund	Abdourahman and Edwards [127]
Goto–Kakizaki	100000[d]	$[Cr(pic)_3]$	4 weeks	Increased body mass; no effect in area under curve for glucose and insulin in GTT lower glucose levels in insulin sensitivity test	Not provided	Kim *et al.* [128]

[a]40 μg Cr during weeks 1–3; 80 μg Cr during weeks 4–6.
[b]2 mg/kg diet.
[c]8 μg/ml concentration in drinking water.
[d]Cannot be certain if amount of $[Cr(pic)_3]$ or Cr.

no effect on fasting blood glucose levels but a reduction in fasting insulin levels [105,106]. Also both studies reported that total cholesterol and the total: HDL cholesterol ratio were reduced. Triglycerides were examined in one of the studies; levels were reduced [105]; however, despite the magnitude of the reduction, the change was not statistically significant. HDL levels were increased in one study, while areas under the insulin and glucose curves in GTT were reduced [104]. In the most recent and shortest study, $[Cr(pic)_3]$ had no effect on fasting glucose or insulin in addition to body mass and food intake. No effect was observed on total cholesterol or triglycerides. Funding, when indicated, was provided by Nutrition 21.

Three groups have examined the use of ZDF rats. Jain *et al.* gave the rats 400 mg Cr/kg body mass daily for 8 weeks as $[Cr(pic)_3]$, Cr nicotinate and chromium dinicocysteinate (CDNC) [107]. All three chromium(III) compounds lowered glycated haemoglobin levels, while only CDNC significantly lowered fasting plasma glucose levels. Clodfelder *et al.* used Cr3 (i.e. $[Cr_3O(propionate)_6(H_2O)_3]^+$), at a daily dose of 1000 μg Cr/kg for 24 weeks [108]. No effects on body mass or food intake were observed. Fasting plasma insulin, but not glucose, was lower. Additionally, total, LDL and HDL cholesterol were lower while the total: HDL cholesterol ratio was unaffected. Given the extremely high HDL levels in these rats, the lowering of HDL levels with no increase in total: HDL cholesterol level may actually be beneficial. Glycated haemoglobin levels were also lower at the end of the study. Also, Talpur *et al.* [109] have utilized ZDF rats but started with rats 70–75 weeks of age; they provided 40 μg Cr/kg for 3 weeks and then 80 μg/kg for another 3 weeks; the form of chromium was Cr nicotinate. Rats on chromium lost less body mass and consumed more food but not water; no effect was observed on fasting blood glucose. Funding for the studies, except for that by Clodfelder *et al.*, was provided by InterHealth Nutraceuticals; the other was funded by NIH.

Three studies, two by one group and one by another, have also examined the effects of chromium on Zucker obese rats. While all the research was performed with federal funding, the groups get quite different results. Vincent and co-workers [108, 111] provided 20 or 1000 μg Cr/kg daily as Cr3 for 24 weeks, while Mozaffari *et al.* [110] provided 5000 and 10 000 μg/kg as $[Cr(pic)_3]$ for 20 weeks. While both groups observed no effects on food intake or body mass, Mozaffari *et al.* observed no effects on plasma glucose or insulin or glycated haemoglobin. In contrast, Vincent and co-workers [108, 111] observed lower fasting insulin but not glucose, lower total, LDL, HDL and total: HDL cholesterol and

triglycerides and also lower glycated haemoglobin. The differences cannot be attributed to the length of the study as Vincent and co-workers examined several of the variables every 4 weeks during the studies. Curiously, the glycated haemoglobin levels in the study by Mozaffari *et al.* were the same for both the Zucker obese and control Zucker lean rats; thus, they failed to observe the expected increase for the Zucker obese rats and consequently might not be expected to observe any effects from chromium administration.

Some general statements can be made. In these genetically similar rats, when chromium is administered at a young age to these diabetes models, it has no effect on body mass and food intake. Chromium administration generally appears to have no effect on fasting blood glucose levels but to lower glycated haemoglobin levels. (This might be explained by the data of Vincent and co-workers, which show that while glucose levels tend to be lower in chromium-treated animals at several instances during the administration period, this effect is not significant; however, glycated haemoglobin levels, which serve as a window to the average exposure of red blood cells to glucose over 60–90 days, can reflect a beneficial effect on blood glucose over this time.) Chromium appears generally to be beneficial to lipid metabolism, lowering total cholesterol levels; however, effects on other lipid variables are inconsistent. Thus, in these rat models of diabetes and obesity-related insulin resistance, chromium appears to have beneficial effects on insulin resistance, marginally beneficial effects on blood glucose and effects on the grossly elevated plasma lipid levels. Unfortunately, only a tiny percentage of human type 2 diabetes cases are the result of mutations in leptin or its receptor.

For comparison, mouse models of diabetes with mutations to the genes for leptin, the ob/ob mouse, and leptin receptor, the db/db mouse, have also been studied. Both these models display obvious obesity. Unfortunately, not all the studies have used well-defined forms of chromium [129,130]. For the db/db mouse, Mannering *et al.* [131] gave mice water containing 130 mg l^{-1} $KCr(SO_4)_2 \cdot 12H_2O$ for 4–5 weeks. Chromium had no effect on glycated haemoglobin, lowered fasting glucose and lowered glucose 30 min (but not 60 min) after a glucose challenge. The research was funded by KALSEC, Inc. Rink [132] and co-workers provided these mice daily doses of 10 mg/kg chromium nicotinate (meant to be mg Cr?) for 6 weeks. No effect was observed on fasting glucose, but the chromium nicotinate lowered total cholesterol, total: HDL cholesterol ratio and triglycerides. Blood glucose was lower between 1 and 2 h after a glucose challenge. The study was supported by NIH. Beneficial effects on renal function of db/db mice on $[Cr(pic)_3]$ have been reported. However, the

doses were extremely large (100 mg/kg diet calculated to be equivalent to 9.7 mg Cr/kg body mass) [133, 134]. The work was funded by NIH.

Seaborn and Stoecker [135] fed ob/ob mice diets containing an add 1 mg Cr as $CrCl_3$/kg diet for 26 days. The chromium had no effect on body mass or the mass of several organs and tissues. Most of the research with the ob/ob mice has been performed by Sreejayan and co-workers [136–139] using Cr(D-phenylalanine)$_3$, Cr(D-phe)$_3$, as the chromium source. In their first study [136], mice were provided with 150 μg of the complex per kg body mass daily for 6 weeks; the treatment resulted in lower area under the curve for glucose in GTT. The same treatment [137] has also been shown to have no effect on body mass or the mass of several organs. Glucose levels in response to an insulin challenge were lower for chromium-treated animals. Total cholesterol and total: HDL cholesterol were lower in treated mice while triglyceride levels were raised. In their next study, mice received 3.8 μg Cr/kg daily as Cr(D-phe)$_3$ or chromium picolinate for 6 months [138]. The treatments had no effect on body mass or organ or tissues masses. Blood insulin concentrations were not affected by chromium treatment. Cr(D-phe)$_3$ resulted in lower glucose levels in a subsequent GTT, but the effect was not statistically significant for [Cr(pic)$_3$]. Both compounds were reported to lower the glucose area under the curve; however, for [Cr(pic)$_3$] where the glucose was not lower at any given point, this must result from an error in statistical treatment. In their final study, ob/ob mice were administered 3.8 μg Cr as Cr(D-phe)$_3$/kg body mass daily for 6 months [139]. Again no effects were observed on body or tissue masses or on serum levels of insulin, triglycerides or cholesterol. Lower glucose concentrations resulted from the treatment in GTT and insulin challenge tests. The conflicting results by the same group on cholesterol and triglyceride levels make interpretation difficult. The research was funded by the American Diabetes Association, American Heart Association and NIH.

The Goto–Kakizaki rat is a non-obese model of type 2 diabetes; the origins of the diabetes at a molecular level are not known. Two studies have examined the effects of [Cr(pic)$_3$] (1000–100 000 μg/kg daily, i.e. 1–100 mg/kg daily) for 4 or 32 weeks [127, 128]. Unfortunately the reports do not allow the reader to determine if the dose is of Cr as the compound or of the compound (in which case ~12.5% of the dose would be Cr). No effects were observed on body mass, fasting blood glucose or insulin levels, or glucose or insulin areas under the curve in GTT. For this model, chromium appears to have no appreciable effect. No funding source was provided for one source, while the other was funded by an academic source.

The chemical streptozotocin when administered intravenously or intra-peritoneally relatively selectively kills the beta cells of the pancreas, destroying nearly all the body's ability to produce insulin. Thus, rats treated with the chemical serve as an excellent model of type 1 diabetes (not type 2 diabetes) [113–125]. The chemical alloxan produces a similar effect. To generate a better model for type 2 diabetes studies, the addition of a high-fat diet was been utilized. Additionally, the treatment of new born rats, rather than adult rats, with streptozotocin has been used to attempt to yield a model better applied to type 2 diabetes. Because of the popularity of the streptozotocin-treated rat model, studies using this model and the alloxan model have been included in Table 5.2. Two studies have looked at fat-fed, streptozotocin-induced diabetic rats [113, 116]. Both studies were funded by industrial sources. Dogukan *et al.* observed that 8 μg/kg daily of chromium as chromium histidine for 10 weeks resulted in an increase in body mass without a change in food intake and lower serum glucose levels [113]. Sahin *et al.* also found increased body mass along with lower fasting glucose and total cholesterol, free fatty acids and triglycerides and higher fasting insulin using [$Cr(pic)_3$] (80 μg Cr/kg for 10 weeks) [116]. Both studies on neonates were performed by the same laboratory but using different forms of chromium. Chromium as $CrCl_3$ or [$Cr(pic)_3$] was placed in the drinking water. Rats on $CrCl_3$ received 8.41 mg/kg daily for 6 weeks [123]; insufficient information was presented for the [$Cr(pic)_3$] to determine the dose of Cr per day for the 6-week period [124]. For the $CrCl_3$ no effects were observed on body mass, food or water intake, or serum glucose or insulin; in contrast, levels of serum cholesterol and triglycerides and area under the curve for glucose (but not insulin) in GTT were lower. For [$Cr(pic)_3$], the results were similar except serum glucose was lower. Funding for the studies was provided by an academic source. Note that in all four studies on these models lower fasting glucose concentrations were generated while total cholesterol and triglycerides were lowered. One other study deserves notice here. Yoshimoto *et al.* [125] have treated spontaneously hypertensive stroke-prone rats with streptozotocin and administered 20 mg Cr/kg daily for 4 weeks as $CrCl_3$. They observed no effect on body mass but observed lower glucose (but not insulin) area under the curve in GTT. (The design and effects on the type 1 streptozotocin-treated rat model are too heterogeneous to warrant discussion.)

Thus, the results of the modified streptozotocin treatments (lower fasting glucose but not insulin and effects on lipids) are different from those of the Goto–Kazizaki rats (no effects) that are in turn different from the

results from the leptin-receptor mutation models (lower fasting insulin but not glucose and effects on lipids). No great dependence appears on dose (all the doses in Table 5.2 are supranutritional), length of time of chromium administration or form of chromium. The origin of the diabetes appears to make a significant difference on the potential benefits of chromium administration.

5.3 CONCLUSION

The author believes that this survey suggests that:

(1) Researchers studying the effects of chromium on type 2 diabetes should restrict themselves initially to a single type of rodent model that is likely to have beneficial effects from the treatments. Given the variability in response of rats to streptozotocin treatment, resulting in larger numbers of rats to achieve suitable power to resolve effects, models based on mutations of leptin receptor would appear optimal, with the caveat that this model may not reflect the human disease well in general. Then, attention can be focused on molecular level effects in this model. Work to address the mechanism of these effects is desperately needed. Only this will determine whether the results can be translated to human studies.
(2) The use of different supplements and doses should be minimized to reduce the heterogeneity in the studies. The use of $CrCl_3$ or Cr3 is suggested by the author. These substances are free from potential ligand effects as could occur with picolinate or nicotinate [140]. Additionally, Cr3 is absorbed with much greater efficiency (>40–60% versus ~1% for $CrCl_3$) probably making it the ideal form of chromium(III) (see Chapter 6).
(3) Human studies need to be performed with sufficient power to be able to realistically observe effects, on subjects whose baseline characteristics are well established, and for periods of time of at least 4–6 months. Knowing baseline characteristics is particularly important, given the possibility at the current dosages that only subjects with the highest degrees of insulin resistance may be responsive to chromium.
(4) Human studies also need to be performed with larger doses of chromium(III). Studies using JCR:LA-cp or ZDF rats utilized 80–1000 μg Cr/kg daily corresponding to approximately 10–130 mg daily for a human (based on body mass). If corrected for the

increased metabolic rate of rats, this still correspond to ~2–26 mg daily. Based on safety recommendations (see Chapter 9), studies are needed using 5–7 mg Cr(III) daily for 4–6 months or longer.

(5) Human subjects need to be carefully monitored for any deleterious effects, especially when using the higher doses of chromium(III).

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6

Biochemical Mechanisms

Since the early 1960s, chromium has been associated with insulin (see Chapter 3). While originally chromium was proposed to have an essential function in amplifying insulin signalling, the proposal has not shown to be sustainable, as discussed in the earlier chapters. In other words, if chromium cannot be conclusively shown to be an essential element, then it cannot be said to have an essential role in any particular function. However, physiological doses of chromium(III) in some rat models of insulin resistance and diabetes and possibly in humans have effects on insulin sensitivity and on glucose and lipid metabolism. Chromium must interact with some biomolecules(s) to facilitate this action. The thrust of this chapter will be efforts to identify this biomolecule(s). Unfortunately, again the result will be a review of inconclusive and contradictory results that will end with an appeal for better designed and focused studies. The author has recently discussed the search for a bioactive chromium biomolecule as the 'holy grail' of chromium biochemistry [1].

6.1 THE INSULIN SIGNALLING PATHWAY

From the appearance of insulin in the bloodstream, the insulin signalling pathway begins with the binding of insulin to the extracellular α-subunits of the transmembrane protein insulin receptor (Figure 6.1). Insulin receptor consists of two extracellular α-subunits and two transmembrane β-subunits. This binding of insulin turns the receptor into an autokinase, phosphorylating itself at three tyrosine residues (1158, 1162 and 1663 following the human sequence) of the β-subunit. This conversion

The Bioinorganic Chemistry of Chromium, First Edition. John B. Vincent.

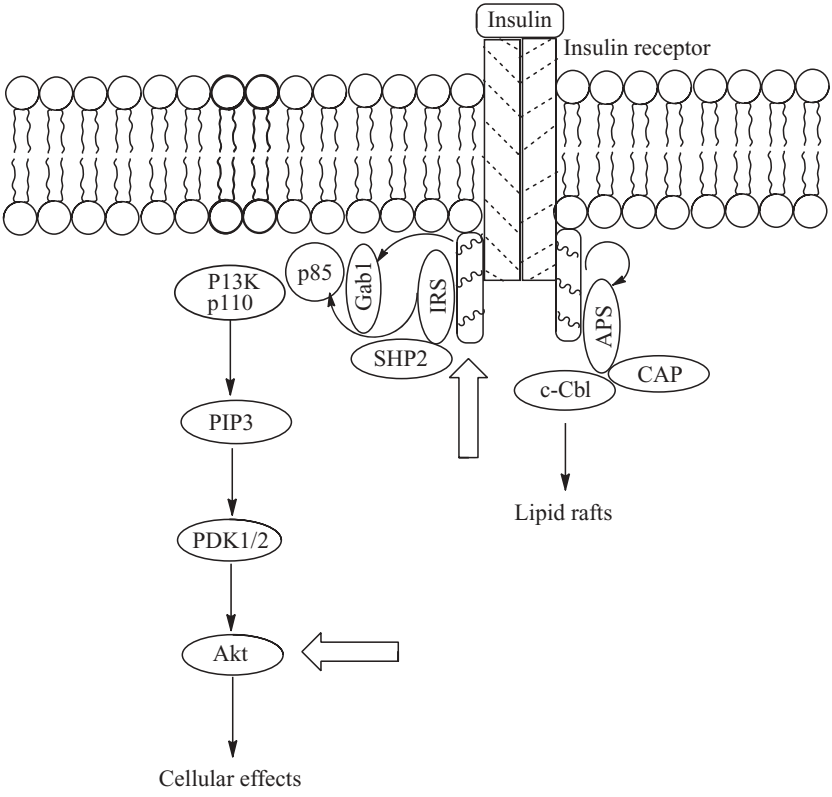


Figure 6.1 Insulin signalling pathway with proposed sites of chromium action indicated by arrows. The binding of insulin to insulin receptor results in the autophosphorylation of the beta (intracellular) subunit of the receptor, converting the receptor into an active tyrosine kinase. Substrates for the active receptor include insulin receptor substrate (IRS)-1, Gab-1 and adaptor protein with a pleckstrip homology (APS). Insulin receptor substrates bind other proteins with SH2 domains, forming signalling centres. These adaptor molecules include PI3K (phosphatidylinositol 3-kinase). Association of the p85 subunit of PI3K with IRS-1 or Gab-1 activates the catalytic p110 subunit. Activated PI3K phosphorylates serine and threonine residues of Akt (protein kinase B) and other members of the signalling cascade, activating these kinases. Continued propagation of this signal results in cellular effects. c-Cbl couples to the insulin receptor, where c-Cbl ubiquitinates the receptor.

turns the receptor into an active kinase catalytically phosphorylating tyrosine residues of several substrate proteins. Known substrates include the insulin receptor substrate proteins (IRS), Shc, Gab-1 and others. These proteins in turn recruit other proteins inside with cell which possess phosphotyrosine-binding domains (SH2 and PTB domains), forming signalling centres. These adaptor molecules include PI3K (phosphatidylinositol 3-kinase) and Grb2. Association of the p85 subunit of PI3K with

IRS-1 or Gab activates the catalytic p110 subunit. In turn PI3K phosphorylates serines and threonine residues of Akt (protein kinase B), activating this kinase. Further propagation along this pathway leads to the major cellular effects associated with insulin action, including glucose uptake and metabolism. Thus, enhancing the binding of insulin to its receptor or the activation of any of the kinases along the cascade could enhance insulin signalling.

Alternatively, preventing the signalling system from being deactivated, for example the inhibition of phosphatase enzymes, could also enhance insulin action. Notably, phosphotyrosine protein phosphatase 1B (PTP1B) has been implicated in the dephosphorylation of insulin receptor. For reviews see references [3] and [4].

6.2 CHROMIUM TRANSPORT AND EXCRETION

As discussed in Chapter 2, chromium absorbed from the gastrointestinal tract quickly passes into the bloodstream where transferrin is responsible for maintaining chromium supplies in the bloodstream and transporting chromium to the tissue. This chapter will look at the movement of chromium from transferrin to the tissues and ultimately to the urine.

The most detailed studies of chromium–transferrin movement have been reported in the last 5 years using ^{51}Cr-labelled transferrin administered intravenously to rats [2]. Injection of ^{51}Cr-labelled transferrin into the bloodstream resulted in a rapid and insulin-sensitive movement of chromium into the tissues as Cr–transferrin; greater than 50% of the chromium is transported to the tissues within 30 min. Tissue levels of chromium were maximal 30 min after injection; decreases in tissue chromium with time were mirrored by increases in urine chromium.

Approximately 50% of the ^{51}Cr appeared in the urine within 360 min of injection in the absence of added insulin; insulin treatment concurrent with injection of ^{51}Cr-labelled transferrin results in approximately 80% of the label appearing in the urine within 180 min. The removal of ^{51}Cr from the blood was faster than the appearance of ^{51}Cr in the urine; the lag in time indicates that the Cr–transferrin in the blood and chromium in the urine are not in direct equilibrium and that intermediates in the transport of chromium must be involved. Separation of the urine components by G-25 size-exclusion chromatography revealed that chromium occurred in the urine as apparently a single low molecular weight species, assumed to be low molecular weight chromium-binding substance (LMWCr, also called chromodulin) (see below) (Figure 6.2). LMWCr when added to the urine co-migrated with the urine chromium [2].

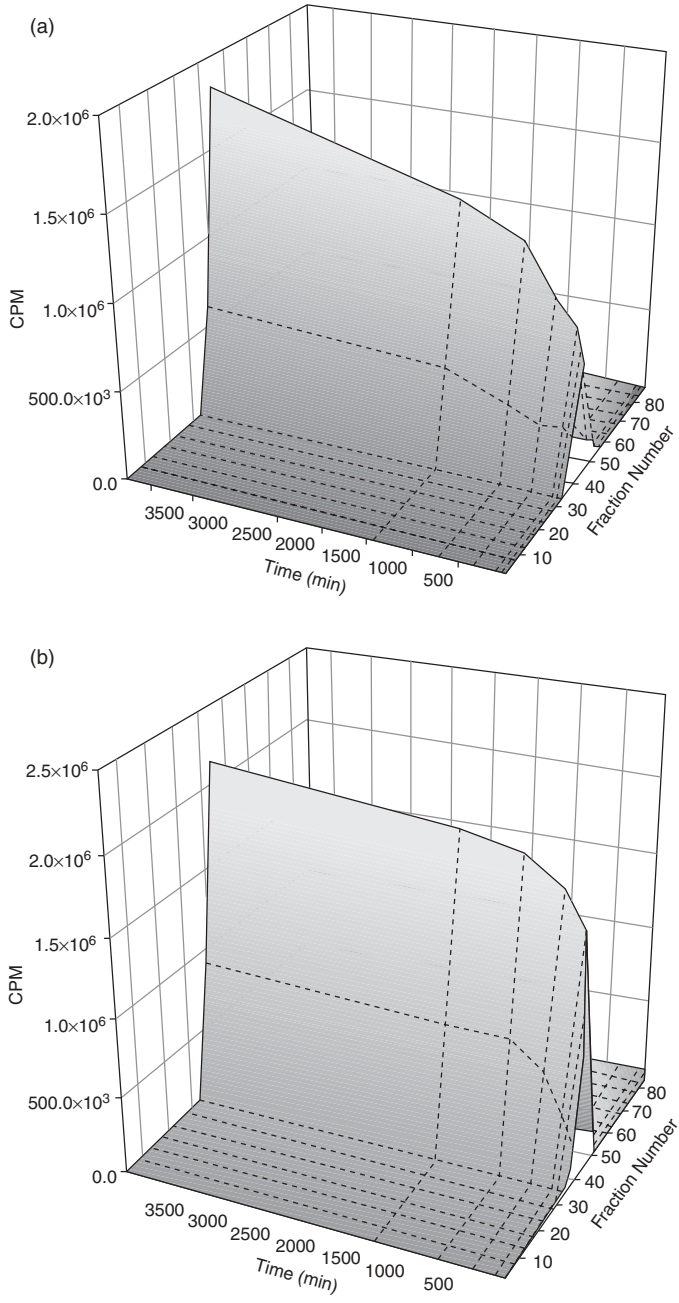


Figure 6.2 Elution profiles of ^{51}Cr in urine from adult rats from a G-25 column as a function of time. (a) Rats not receiving insulin; (b) rats receiving insulin. Reproduced from [4] with kind permission from Springer Science and Business Media.

When the species of chromium in the blood plasma as a function of time were examined by S-200 size-exclusion chromatography, two primary features were observed (Figure 6.3). The first was Cr–transferrin, which disappeared quickly from the blood stream. With time a low molecular weight species, also proposed to be LMWCr, appeared. (Two species of intermediate molecular weight are also observable in Figure 6.3 but they account for $<10\%$ of the applied chromium and probably were degradation products of transferrin [2].)

This work established a clear pathway for the transport of chromium starting from transport by transferrin from the bloodstream into the tissues, followed by release and processing in the tissues to form a low molecular weight chromium-binding species, excretion into the bloodstream, rapid clearance of the low molecular weight species or a similar species into the urine and ultimately excretion as this species. Insulin stimulates the processing of chromium in the tissues. The rates of chromium movement were estimated, and on the basis of these results, a kinetic model for the movement of chromium from transferrin in the blood to LMWCr-like species in the urine was proposed (Scheme 6.1). The model assumes the presence of five major types of chromium: Cr–transferrin in the blood plasma (A), chromium in the tissues (B), LMWCr-like species in the plasma (C), the smaller unidentified species in the plasma (F), the larger unidentified species in the plasma (E) and LMWCr-like species in the urine (D). Two pathways were required to fit the movement of Cr from transferrin to the urine and the Cr-binding species in the bloodstream. The first involves the transport of chromium by transferrin to the tissues (k_1), followed by the release of chromium and production and release of LMWCr-like species into the blood (k_2) and the movement of chromium as LMWCr-like species from the blood to the urine (k_3). The presence of LMWCr-like species in the tissues and in the urine necessitates the presence of LMWCr-like species in the blood plasma, although its presence appears to be masked by the smaller unidentified species. Hence, the LMWCr-like species is assumed to be kept at low steady-state levels in the blood, and k_3 is assumed to be much larger than k_1 and k_2. The second pathway includes the appearance of the larger unidentified species (k_4), which in turn is either metabolized to generate the smaller unidentified species or gives up its Cr to the smaller species (k_5). This kinetic model fits the experimental data well at the early time points but failed to simulate the gradual loss of chromium from the tissues to the urine. This failure arose because the model did not incorporate the processing and loss of chromium from the various tissues occurring at different rates. This manifested

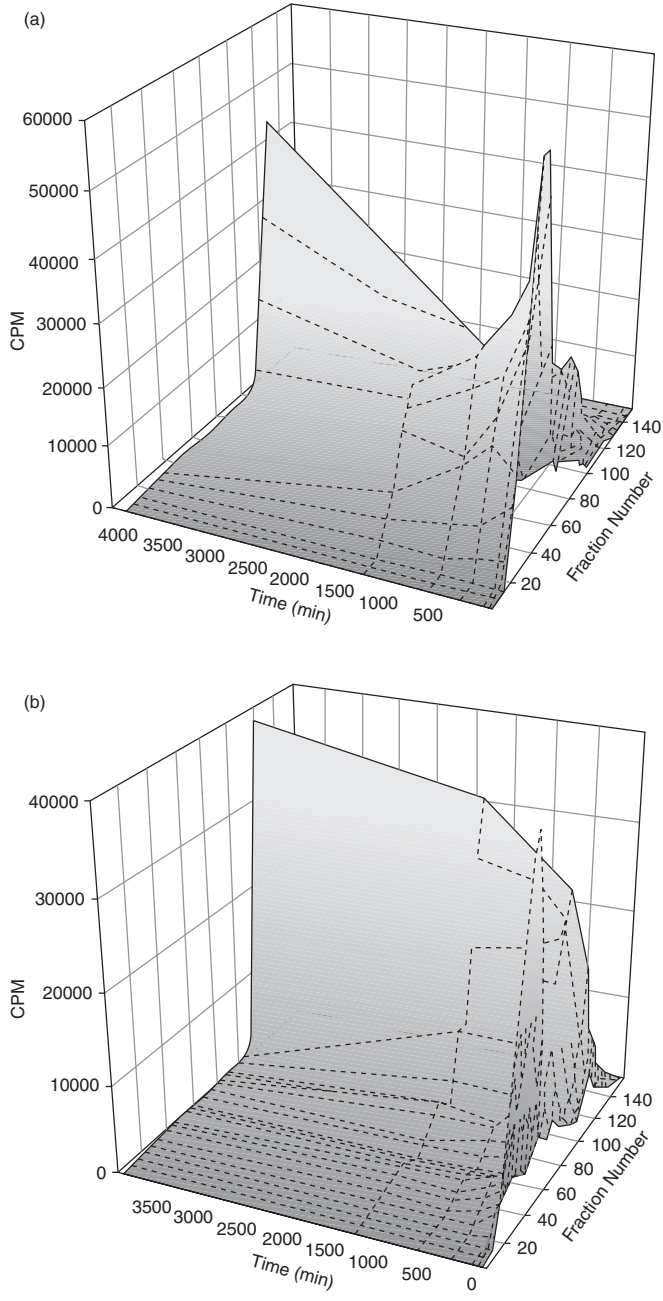
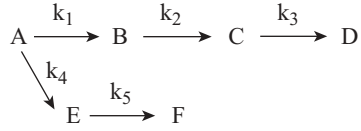


Figure 6.3 Elution profiles of ^{51}Cr in blood plasma from adult rats from an S-200 column as a function of time. (a) Rats not receiving insulin; (b) rats receiving insulin. Reproduced from [4] with kind permission from Springer Science and Business Media.



Scheme 6.1 Proposed mechanism of distribution of chromium from Cr–transferrin. Reproduced from [4] with kind permission from Springer Science and Business Media.

itself, for example, in a requirement for at least a biphasic function to fit to the appearance of LMWCr-like species in the urine as a function of time [2].

Little is known beyond this about the handling of chromium. Only minor amounts of chromium are lost through the bile (for example, references [5] and [6]). In bile, Cr^{3+} occurs as part of a low molecular weight organic complex [7]; the molecular weight of this species was not determined. The authors postulated that this complex might be involved in the passage of chromium from the liver to the bile.

Exactly how chromium is handled by the kidneys is difficult to determine from the literature. Some useful tracer studies with ^{51}Cr have been reported (for example, see references [8–10]), yet much of the literature looking at absolute chromium levels dates before 1980 and, thus, suffers from analytical problems. The tracer studies appear to be influenced by the form of chromium used and perhaps by the species of animal utilized.

Similar problems with chromium form are associated with studies examining chromium distribution in mammals. Differences exist between studies that give chromium orally or intravenously as different chromium(III) species are introduced into the blood (see below). Similarly tracer studies may not accurately reflect the fate of dietary chromium; for example, inorganic chromium added to a diet may not bind to the same chelates and other ligands binding to the chromium naturally in the diet. Yet, some consensus does appear. In tracer studies ^{51}Cr accumulates in the bone, kidney, spleen and liver (for example, references [5, 10–17]). A three-compartment model has been proposed to examine the kinetics of chromium tissue exchange and distribution for studies with rats and humans [11, 14, 15]. Plasma chromium is in equilibrium with the three pools: a small pool with rapid exchange ($T_{1/2} < 1$ day), a medium pool with a medium rate of exchange (days) and a large, slowly-exchanging pool (months). Jain *et al.* have suggested from these studies that chromium has specific transporters that regulate its movement [14]. Ageing appears to affect chromium distribution and transport [17].

6.3 LMWCr/CHROMODULIN

Two biomolecules are known to bind chromium *in vivo*: transferrin and low molecular weight chromium-binding substance (LMWCr), also termed chromodulin. The role(s) of LMWCr is unclear. LMWCr has been reviewed twice in recent years [1, 18]; these form the starting point for this section of this chapter.

LMWCr was first reported by the toxicology group of Osamu Wada [19] in 1981. A low molecular weight chromium compound was identified by size-exclusion chromatography of the cytosol of liver cells of male mice injected with a single dose of potassium chromate. A similar low molecular weight compound was found in the faeces and urine and 2 h after injection in the plasma. These researchers suggested that a low molecular weight chromium-binding substance (LMWCr) was formed in the liver, which participates in retention and excretion of chromium in the body. The material from the livers of rabbits treated similarly with chromate was partially purified and found to apparently be an anionic organic–chromium complex containing amino acids. This same year, Wu and Wada reported additional studies on LMWCr from urine [20]. LMWCr was found to occur in urine normally, although the amounts were greatly increased after rats were injected with chromate. Normal human and rat urine LMWCr was found not be saturated with chromium. The LMWCr was believed to be similar to that of the liver and other organs of rabbits and dogs and to be involved in removing excess chromium from the body.

Follow-up studies were performed looking at the effects of inhalation exposure of $CrCl_3$. Chromium as LMWCr in the lungs (of rats exposed to the Cr-containing aerosol) slowly decreased while levels in the liver increased. Thus, LMWCr was proposed to be in equilibrium with Cr in the rest of the body; the long half-life of chromium in the lungs was proposed to be the result of low LMWCr levels or a slow rate of synthesis of the LMWCr [21]. Wada and co-workers have also examined the distribution of LMWCr [22]. LMWCr was found in liver, kidney, spleen, intestine, testicle, brain and blood plasma, with the greatest amount in the liver followed by kidney. The organs were obtained from mice 2 h after injection with potassium dichromate. Supernatants of homogenates of the organs were found to possess more chromium bound to LMWCr when dichromate was added to the homogenate than when the mice were injected with dichromate. The time course of chromium binding to LMWCr after injection of dichromate was also examined [22]. Chromium was found to be associated with liver and kidney LMWCr only 2 min after

injection and reached a maximum 1–2 h after treatment. In these studies, LMWCr was again identified by its elution behaviour in size-exclusion chromatography and its Cr-binding ability.

Efforts have continued to isolate and characterize LMWCr. To date LMWCr has been isolated and purified from rabbit liver [23], bovine liver [24], porcine kidney [25] and porcine kidney powder [25], and partially purified from dog [26] and mouse liver [22]. Inclusion of protease inhibitors in buffers during the isolation of bovine liver LMWCr does not affect the amount of oligopeptide isolated [24], suggesting it is not a proteolytic artefact generated during the isolation procedure. The materials from rabbit and dog liver were loaded with Cr by injection of the animal with chromate (or Cr(III), which provides lower yields). For the materials from bovine liver and porcine kidney and kidney powder, chromate was added to the homogenized liver or kidney or suspended kidney powder. Cr(III) could also be added to the bovine liver homogenate to load LMWCr with chromium, but the loading was not as efficient as when chromate was utilized [24]. A Cr-loading procedure is required so that the material can be followed by its chromium content during the isolation and purification procedures [23, 24]. The isolation procedures are similar involving an ethanol precipitation, anion-exchange chromatography and finally size-exclusion chromatography.

Thus, LMWCr appears to be a naturally occurring oligopeptide composed of glycine, cysteine, aspartate and glutamate with the carboxylates comprising more than half of the total amino acid residues (Table 6.1). The amino acid composition data for the rabbit liver LMWCr (injected chromate) and bovine liver (chromate added to homogenate) are extremely similar, indicating that the type of Cr loading procedure utilized is probably not critical to the composition of the isolated material. Until very recently (see below), no amino acid sequence data had

Table 6.1 Amino acid composition data for isolated LMWCrs.

Source	Glycine	Glutamic acid	Aspartic acid	Cysteine	Ref.
Rabbit liver	3.22	3.91	1.98	1.75	[23]
Dog liver[a]	2.71	4.3	1.00*	2.23	[26]
Bovine liver	2.47	4.47	2.15	2.19	[24]
Bovine colostrum	1.98	5.0	4.12	0.93	[27]
Porcine kidney	1.45	4.05	2.31	0.622	[25]

[a]For the dog liver material (only partially purified), ratios are based on the assumption of only one aspartic acid residue per oligopeptide; amino acid analysis indicated the presence of significant amounts of threonine, serine and leucine.

appeared, despite attempts at sequencing by Edman degradation, NMR and mass spectrometry. The lack of additional characterization of the organic components of the materials was a notable matter of concern. Additionally, note that the material from urine had not been isolated and characterized until very recently (see below); its assumed identity with the material from liver had been based solely on their similar apparent molecular weight from size-exclusion and similar chromatographies and their chromium-binding potential. Unfortunately to date, LMWCr has not proven to be antigenic, preventing its presence to be detected using immunological techniques.

The amino acid composition of LMWCr has been used to search the human genome for protein possessing fragments comprised of these amino acids. Two candidate sequences, EDGEECDCGE and DGEECDCGEE, from the beginning of the disintregrin domain of the protein ADAM 19 have been identified [28, 29]. ADAM 19 is a multidomain membrane protein with disintegrin and metalloproteinase domains [30]. The ten amino-acid sequences are conserved in rat, mouse and human ADAM 19, the only sources for which the sequence is known. Only preliminary results of chromium binding to a peptide with the former sequence have been reported [29]. Yet, subsequent sequencing of a heptapeptide fragment of LMWCr indicates that LMWCr does not derive from ADAM19 (see below).

Despite its small size (approximately 1500 molecular weight; 1438 by MALDI-TOF (matrix-assisted laser desorption-ionisation time-of-flight) mass spectrometry for bovine liver LMWCr) [31], the molecule from rabbit and bovine liver tightly binds four equivalents of chromic ions. The binding is fairly tight ($K_a \sim 10^{21}$ M^{-4} for bovine liver) and highly cooperative (Hill coefficient, $n = 3.47$) [32]; thus, essentially only apoLMWCr and holoLMWCr (Cr_4-LMWCr) co-exist in solution. ApoLMWCr can accept chromic ions from biological molecules including Cr_2–transferrin [22, 32]. Spectroscopic studies suggest that the chromic ions comprise an anion-bridged multinuclear assembly supported by carboxylates from the oligopeptide [24, 33]. Electronic studies clearly indicate that the Cr bound to LMWCr exists in the trivalent oxidation state; for the bovine liver material, 10Dq and the Racah parameter B were found to be 1.74×10^3 and 847 cm^{-1}, respectively [24], an indication of predominately oxygen-based coordination. In the ultraviolet region, the spectra of LMWCrs possess a maximum or shoulder at approximately 260 nm; this feature may arise from a disulfide linkage [34]. Paramagnetic ^1H NMR spectroscopy of the bovine liver reveals a downfield-shifted resonance at approximately $+45$ ppm, suggestive of the protons

of a methylene carbon bound adjacent to a carboxylate bridging two chromic centres [24]. The presence of a bridging ligand suggests the existence of a multinuclear assembly.

X-ray absorption spectroscopic studies on the bovine liver LMWCr have shown that the chromium atoms are surrounded by six oxygen atoms at an average distance of 1.98 Å and are consistent with a lack of sulfur-based ligands [33]. A long Cr . . . Cr interaction at ~3.79 Å is present, and another such interaction may be present at 2.79 Å. This is also consistent with the presence of a multinuclear chromium assembly. As holoLMWCr can be prepared simply by addition of chromic ions to solutions of apoLMWCr, anionic bridges to the chromium assembly are probably hydroxide ions; [24, 35], the X-ray absorption studies failed to detect any short Cr–oxo interactions [33]. The nature of the assembly has been narrowed to a few possibilities from electron spin resonance (ESR) spectroscopy and variable temperature magnetic susceptibility measurements [33]. X-band ESR studies indicate that at least three chromic ions are coupled to give a species with an $S = 1/2$ ground state giving rise to a broad signal at $g \sim 2$; this signal appears to be broadened by interaction with another chromium species giving rise to a complex ESR signal centred about $g \sim 5$. The $g \sim 2$ ESR signal sharpens as the temperature is raised from 5 to 30 K, suggesting that dipolar coupling exists between the two chromium species giving rise to the ESR signal.

Finally magnetic susceptibility studies are consistent with the presence of a mononuclear chromic centre and an unsymmetric trinuclear chromic assembly [33]. Put together the spectroscopic and magnetic data on bovine LMWCr suggest the occurrence of a Cr_4 assembly in LMWCr. The chromium environment is mostly if not exclusively composed of O atoms, and the assembly is comprised of a single chromic ion and a trinuclear unit. Additionally, the sulfur atoms of the two cysteine residues of LMWCr appear to be involved in a disulfide linkage and not be involved in binding chromium. Similarly, the *N*-terminal amine group can be derivatized, suggesting it is not coordinated to chromium. Thus, oligopeptide-provided ligands appear to be limited to carboxylates from the side chains of the aspartate and glutamate residues and possibly the carboxy terminus. One laboratory has reported not being able to isolate bovine liver LMWCr while instead isolating a material they identified as containing Cr(IV) or Cr(V) [36]. Much of the work in this paper has been refuted [37], and the existence of such a Cr(IV) or Cr(V) species with the stability necessary to be isolated under these conditions is difficult to fathom. The Vincent group has infrequently observed a similar species. The greenish-brown species that elutes from the size-exclusion

columns of the LMWCr purification scheme overlaps the elution band of LMWCr; the two are very difficult to separate. The Vincent group has not attempted to characterize this material (Vincent, unpublished results). Works and co-workers [140] have also attempted to isolation chromium-containing peptides or proteins from liver homogenates to which chromate has been added. They observed at least four species that bound chromium and isolated an ~15.6 kDa protein. As would only be expected by adding chromate to a liver homogenate, these workers suggested that chromium binding was nonspecific. The other three Cr-containing species were not characterized.

A related chromium-containing oligopeptide from bovine colostrum (M-LMWCr) is comprised of the same amino acids but in distinctly different ratios and also stimulates insulin-dependent glucose metabolism in rat adipocytes [27, 38]. Whether the oligopeptide is present in other forms of milk is unknown. The significance of these differences between the liver and colostrum oligopeptides is essentially unexplored. The existence of multiple forms of LMWCr in cows raises concerns about identifying LMWCr in tissues or body fluids from only its apparent molecular weight and chromium-binding ability. For example, are blood and urine LMWCr fractions comprised of one or more of these oligopeptides?

Curiously, the oligopeptide is maintained in the soluble portion [22] and the nucleus [31] of liver cells in the apo form. As noted above, the oligopeptide is isolated as the holo-oligopeptide (so that it may be followed in the purification schemes by its chromium content), which means that an *in vivo* or *in vitro* chromium-loading step is required [23, 24]. This observation has resulted in the suggestion that LMWCr may play a role in chromium detoxification; however, injection of chromic ions or chromate into mice does not stimulate the production of LMWCr [22]. Thus, while LMWCr does carry chromium into the urine after intake of large dosages of Cr(III) or Cr(VI) [26], the suggested detoxification role of LMWCr is unlikely to be its primary function.

Insulin dose response studies using rat adipocytes have indicated a potential intrinsic biological function for LMWCr. Isolated rat adipocytes in the presence of LMWCr and insulin display an increased ability to metabolize glucose to produce carbon dioxide or total lipids; this increase occurs without a change in the insulin concentration required for half-maximal stimulation [27, 38, 39]. This lack of change in half-maximal insulin concentration suggests a role for LMWCr inside the insulin-sensitive cells after insulin binds externally to the insulin receptor [39]. The stimulation of glucose metabolism by LMWCr is proportional to the chromium content of the oligopeptide [40].

While insulin dose response studies with LMWCr suggested a role inside the cells and because the primary events between insulin binding to its receptor and glucose transport are signal transduction events, that is phosphorylation–dephosphorylation of protein residues, a role for LMWCr in these events has been probed. LMWCr has been shown to activate the tyrosine kinase activity of insulin-activated insulin receptor [35] and to activate a membrane phosphotyrosine phosphatase in adipocyte membranes [41]. For example, the addition of bovine liver LMWCr to rat adipocytic membranes in the presence of 100 nM insulin results in a concentration dependent stimulation of insulin-dependent protein tyrosine kinase activity (up to an eightfold stimulation), while no activation of kinase activity is observed in the absence of insulin [35]. The dependence of the kinase activation on the concentration of LMWCr can be fit to a hyperbolic curve to give dissociation constants (K_m) of approximately 875 pM, indicating extremely tight binding. Blocking the insulin-binding site on the external β-subunit with antibodies whose epitope lies in this region results in the loss of the ability to activate insulin receptor kinase activity [35]. Examining the potential activation of isolated rat insulin receptor by bovine liver LMWCr in the presence of insulin indicates that LMWCr can amplify the isolated receptor protein tyrosine kinase activity by approximately sevenfold, conclusively demonstrating that the receptor is the site of interaction with LMWCr. Fitting the activation curve to a hyperbolic function gives a dissociation constant of approximately 250 pM [35]. The site of LMWCr binding on insulin receptor can be further refined. Studies with a catalytically active fragment (residues 941–1343) of the β-subunit of human insulin receptor (which does not require insulin for kinase activity) reveal that LMWCr can stimulate kinase activity threefold with a dissociation constant of 133 pM. Thus, LMWCr binds at or near the kinase active site [42].

Chromium plays a crucial role in the *in vitro* activation of insulin receptor kinase activity by LMWCr [35]. ApoLMWCr displays little ability to activate insulin-dependent tyrosine kinase activity in the rat adipocyte membranes, with the small amount of activity readily attributable to residual chromium. However, titration of apoLMWCr with chromic ions results in the total restoration of the ability to activate kinase activity; approximately four chromic ions per oligopeptide are required for maximal activity. This is consistent with the number of chromiums (four per oligopeptide) reported to be bound to holoLMWCr from liver sources. The activity of LMWCr is rapidly restored upon the addition of chromium, consistent with binding studies which indicate that Cr

binding is highly cooperative, such that holoLMWCr forms rapidly to the exclusion of complexes of LMWCr without its full complement of chromium. This reconstitution of the activation potential of LMWCr is specific to chromium. Transition metal ions other than chromium that are commonly associated with biological systems (V, Mn, Fe, Co, Ni, Cu, Zn and Mo) are ineffective in potentiating the ability of apoLMWCr to activate kinase activity. In fact, all the ions except Cr^{3+} resulted in loss of activation potential relative to apoLMWCr. Similarly, the metal ions themselves (in the absence of apoLMWCr) are ineffective in activating the insulin-dependent kinase activity. Thus, the ability of LMWCr to potentiate the effects of insulin in stimulating the insulin-dependent protein tyrosine kinase activity of insulin receptor is specific to chromium and is directly dependent on the chromium content of LMWCr.

The activation of a membrane-associated phosphotyrosine protein phosphatase (PTP) by LMWCr has been little explored. Studies with isolated LAR and PTP1B have shown that chromodulin has no effect on these phosphatases [41, 43]. One group [44] has reported that the results of references [41] and [43] and on human PTPs are contradictory; however, both studies are in agreement in that no activation of isolated PTPs was observed [41, 43].

Based on these results and additional studies (see below), LMWCr has been proposed to function as part of a unique autoamplification system for insulin signalling (Figure 6.4) and the new (and shorter) name, chromodulin, has been put forward [45,46]. In this mechanism, apoLMWCr is stored in insulin-sensitive cells. In response to increases in blood insulin concentrations (as would result from increasing blood sugar concentrations after a meal), insulin binds to its receptor bringing about a conformation change, which results in the autophosphorylation of tyrosine residues on the internal side of the receptor. This transforms the receptor into an active tyrosine kinase and transmits the signal from insulin into the cell. In response to insulin, chromium is moved from the blood to insulin-sensitive cells. Here, the chromium flux results in the loading of apoLMWCr with chromium. The holoLMWCr then binds to the receptor, presumably assisting in maintaining the receptor in its active conformation, amplifying its kinase activity. When the signalling is to be turned off, a drop in blood insulin levels facilitates relaxation of the conformation of the receptor, and the holoLMWCr is excreted from the cell into the blood. Ultimately, LMWCr is efficiently excreted in the urine. The basis of the alternative name chromodulin is the similarity of the proposed mechanism of action to that of the calcium-binding protein calmodulin [47]. Both bind four equivalents of metal ions in response

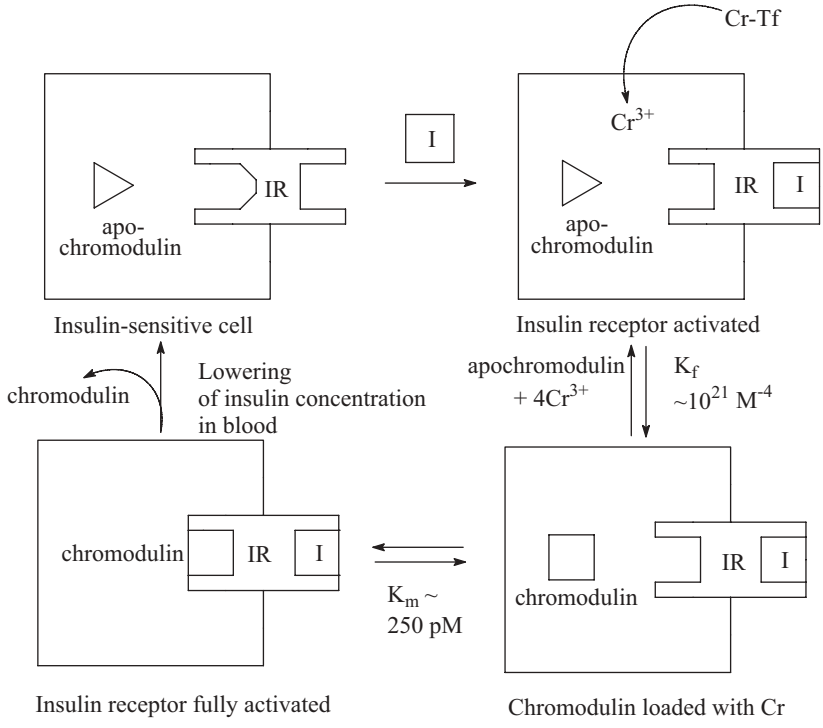


Figure 6.4 Proposed mechanism for the activation of insulin receptor kinase activity by chromodulin in response to insulin. The inactive form of the insulin receptor (IR) is converted into the active form by binding insulin (I). This triggers a movement of chromium (presumably in the form of chromium transferrin, Cr–Tf) from the blood into insulin dependent cells, which in turn results in a binding of chromium to apochromodulin (apoLMWCr) (triangle). Finally, the holochromodulin (square) binds to the insulin receptor, further activating the receptor kinase activity. Apochromodulin is unable to bind to the insulin receptor and activate kinase activity. When the insulin concentration drops, holochromodulin is released from the cell to relieve its effects. Reproduced from [37] © 2000 ASN Journals.

to a metal ion flux; however, the four calcium ions that bind to the larger protein calmodulin rest in mononuclear sites. Both holoproteins selectively bind to kinases and phosphatases stimulating their activity.

The model is also based on the results of studies in humans. Most notably, in euglycemic hyperinsulinemic clamp studies, Morris *et al.* have shown that increases in blood insulin concentrations following an oral glucose load result in significant decreases in plasma chromium levels; a subsequent infusion of insulin led to further chromium losses [49]. Within 90 min after the increases of blood insulin concentrations,

blood chromium levels started to recover. Patients also showed increased urinary chromium losses during the course of the experiments, with the amount of chromium lost roughly corresponding to the amount of chromium estimated to be lost from the intravascular space [49]. Numerous other studies have demonstrated that chromium is released in urine within 90 min of a dietary stress such as high sugar intake [50–55]. As glucose tolerance as a result of repeated application of carbohydrate stress decreases, the mobilization of chromium and resulting chromium loss have been shown to decrease [52]. These homoeostasis and urinary output studies suggest that chromium stored or maintained in the blood is mobilized in response to increases in blood insulin concentrations where ultimately it appears in the urine.

Recently, a proposal for chromium transport from the bloodstream to the urine has been presented (Figure 6.5) [48]. As described above, transferrin has been shown to be responsible for the transport of Cr from the bloodstream to the tissues, and the transport is insulin-sensitive [56, 57]. While these data on chromium transport have been incorporated into the proposed mechanism of enhancement of insulin signalling by LMWCr, they will need to be incorporated into any complete mechanism of *in vivo* chromium action.

Noting that all the studies on LMWCr and insulin receptor are *in vitro* studies is important. This proposed mechanism of action of chromium needs to be supported by *in vivo* studies. Does LMWCr bind to insulin receptor *in vivo* and is insulin receptor kinase activity affected *in vivo* by interaction directly with LMWCr *in vivo*? The only reported *in vivo* studies with LMWCr are of a quite different nature. LD_{50} [22] and mean tubular reabsorption rates [26] have been measured as noted above. Additionally, intravenous treatment of rats with 20 μg of chromium as LMWCr per day for 12 weeks has been shown to have little if any effect on rats [58]. Thus in terms of its use as a dietary supplement or therapeutic compound, LMWCr appears to be recognized and readily excreted. Injection of LMWCr into rabbits has been shown to lead to rapid excretion of chromium, especially compared with the use of other forms of chromium; this is reflected in the mean tubular reabsorption rate for LMWCr of 23.5% in contrast to rates of 85.7 and 92.5% for chromate and chromium chloride, respectively [26]. This is probably also responsible for the extremely high LD_{50} for LMWCr injected into mice of 135 mg/kg body mass [22]. These studies looking at effects of injection of LMWCr need to be followed with studies designed to probe the fate and potential action of holoLMWCr generated in the tissues.

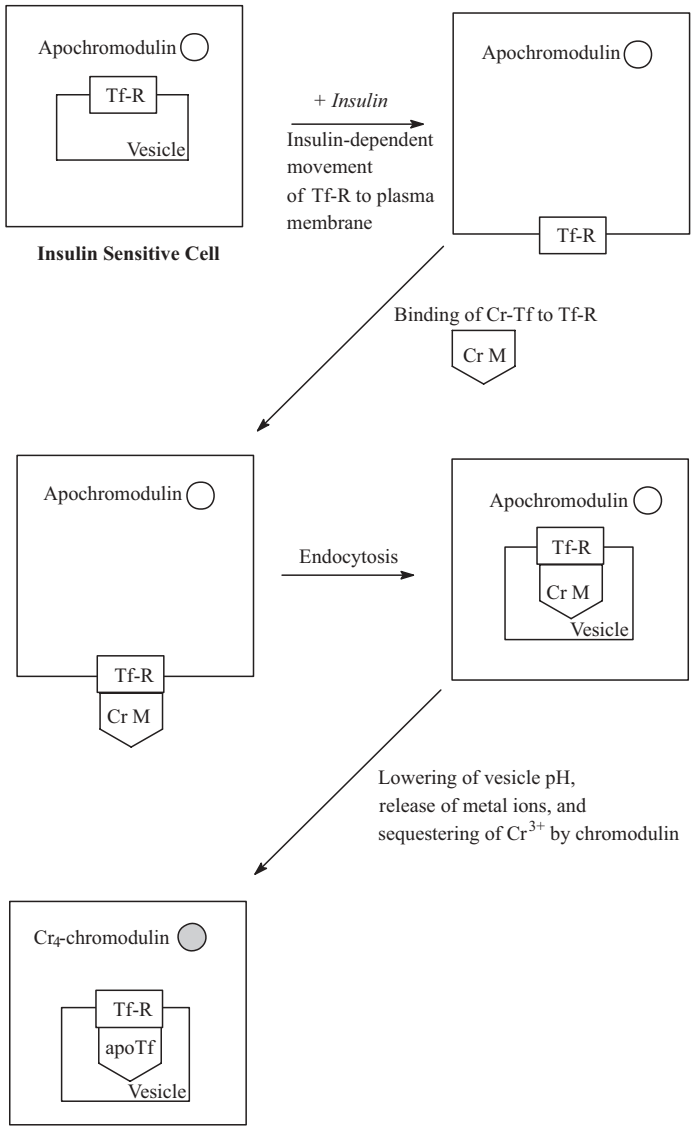


Figure 6.5 Proposed mechanism for the movement of chromium from blood to chromodulin (LMWCr). In response to increases in plasma insulin concentrations, transferrin receptor (Tf-R) in insulin-sensitive cells migrates from vesicles to the plasma membrane. Transferrin (pentagon), which contains two bound metal ions (in this case one chromic ion and one other metal cation (M)), binds to the receptor and is internalized by endocytosis. The pH of the resulting vesicle is reduced by ATP-driven proton pumps, resulting in release of the metal ions from transferrin. Chromium released from multiple transferrin molecules is sequestered by apochromodulin (open circle) to produce chromium-loaded chromodulin (dark circle). Reproduced from [37] © 2000 ASN Journals.

Another proposal for chromium-activating insulin receptor related to the LMWCr mechanism has been proposed by Van Horne and co-workers [28]. Noting that the amino acid composition of LMWCr was approximately 4 Glu : 2 Asp : 2 Cys : 2 Gly or for the smallest whole number ratio 2 Glu : 1 Asp : 1 Cys : 1 Gly, these workers postulated that LMWCr could be comprised of two linked pentameric sequences and searched the sequences of a set of proteins related to insulin signalling for segments with the composition 2 Glu : 1 Asp : 1 Cys : 1 Gly. They identified an acidic region on the α-subunit of insulin receptor with the sequence EECGD (residues 175–184), and proposed a structure for LMWCr where the four metal centres are bound to two equivalents of the pentamer, which are held together by a disulfide linkage. A synthetic peptide derived from this sequence was shown to form a Cr(III) complex with a proposed formula of Cr_3O [28]. The basis for the formula was not presented.

Based on these results, Van Horn and co-workers have proposed multinuclear chromium assemblies binding to the α-subunit of insulin receptor in conjunction with insulin binding activating the receptor's kinase activity. The binding of chromium would be to these pentameric sequences in the α-subunit. After insulin is degraded, the chromium assembly or Cr-assembly–peptide complex is released by a yet to be determined mechanism to return the receptor to its inactive form. This proposal is not consistent with the amino acid sequence of the fragment of LMWCr (see below) and will not be considered further.

Overall, these studies show that chromium in tissues is transferred to a low molecular weight species and is subsequently lost in the urine as a low-molecular-weight species. These species are similar, if not identical. An isolated species from liver and urine treated with chromium has been characterized and called LMWCr or chromodulin. While it is logical and likely that this species that tightly binds chromium is the same species that occurs naturally in tissues and urine, the chromium-loading process required to isolate the biomolecule does not allow this to be determined with certainty as it could represent an artefact generated during chromium loading. As chromium is probably not an essential element, LMWCr (if this is the natural chromium-binding biomolecule) should not have a role in insulin signalling during normal conditions. The activity observed in test-tube studies could reflect a mechanism for altering carbohydrate and lipid metabolism when excess chromium is present, as when rats are treated with high doses of chromium(III) supplements. However, this will require *in vivo* evidence.

The sequence of a heptapeptide comprising most of the organic portion of LMWCr has very recently been reported [59]. Because Cr^{3+} ions bind

so tightly to LMWCr so that only harsh methods had previously been successful in removing Cr from LMWCr, a gentler method to remove the Cr^{3+} ions was investigated. The reaction

$$Cr\text{–}O_2CR + HO_2CR' \rightarrow HO_2CR + Cr\text{–}O_2CR' \qquad (6.1)$$

occurs if HO_2CR' is more acidic than HO_2CR, where the symbols R and R′ denote attached hydrocarbon side-chains and has been used previously to exchange carboxylate ligands in multinuclear chromium assemblies [60]. This process was hoped be more gentle and to lead to more recoverable material.

Trifluoroacetic acid (TFA) was chosen as it is fairly acidic and water soluble. TFA was used to remove chromium from LMWCrs from mammalian (bovine), avian (chicken) and reptilian (American alligator) liver and human urine, revealing that the LMWCrs are apparently all composed of an heptapeptide of an identical molecular weight and apparently identical composition, Glu_4Asp_2Gly as indicated by amino acid analysis of the bovine liver material and mass spectrometry (see below). Unfortunately, the procedure resulted in the cyclization of the *N*-terminal glutamate residue to pyroglutamate. The procedure also resulted in the separation of the contiguous heptapeptide from additional glycine and cysteine amino acids, although previous NMR data suggest these additional amino acids may be connected to the heptapeptide through a non-standard linkage. The ability of the heptapeptide component of LMWCr to be identified in the urine and/or tissue of insulin-utilizing animals of three different phyla (mammals, aves and reptiles) increases the likelihood that LMWCr is not an artefact of its isolation procedure.

The peptide sequence of the heptapeptide fragment was analysed by mass spectrometry (MS) and tandem MS (MS/MS and MS/MS/MS) using collision-induced dissociation (CID) and post-source decay (PSD) [59]. Two candidate sequences, pEEEEGDD and pEEEGEDD (where pE is pyroglutamate), were identified from the MS/MS experiments; additional tandem mass spectrometry suggested the sequence is pEEEEGDD. The *N*-terminal glutamate residues explain the inability to sequence LMWCr by the Edman method, as they would readily cyclize under the Edman conditions. Langmuir isotherms and Hill plots were used to analyse the binding constants of chromic ions to synthetic peptides similar in composition to apoLMWCr. The sequence pEEEEGDD was found to bind four chromic ions per peptide with nearly identical cooperativity and binding constants to those of apoLMWCr [59]. Studies to examine the environment of the chromium centres are ongoing in the Vincent laboratory.

In the body, peptides, including numerous peptides with bioactivity, originate from the processing of proteins. Thus, the heptapeptide isolated from LMWCr should have, at a point in its history, been be part of a larger protein. A genomic search against the databases of the National Center for Biotechnology Information (NCBI) using the sequence EEEEGDD was performed to identify proteins containing this sequence motif [59]. Multiple 100% hits were found due to the short sequence and low complexity: seven sequences in *Homo sapiens*; two in *Bos taurus*; two in *Gallus gallus*; one in *Mus musculus*. Unfortunately, very little of the American alligator genome has been sequenced. None of the hits contain glycine and cysteine residues flanking the EEEEGDD sequence, suggesting that these residues are not part of a contiguous peptide and are attached to the heptapeptide in a non-standard fashion. None of these hits correspond to a responsive gene in chromium treatment of mice (isolated adipocytes) or mouse testis (TM4 Sertoli-like) cells [61, 62]. This is an area worthy of continued investigation. Antibodies raised against the sequence CEEEEGDD conjugated via the cysteine to limpet haemocyanin react with holoLMWCr; unfortunately, while this represents a first step towards developing techniques to examine the intracellular distribution of LMWCr or measure *in vivo* concentration of LMWCr, the recognition of holoLMWCr by the antibodies is not sufficient for use in Western blotting and other techniques [59].

Most importantly the heptapeptide has been examined for biological activity (N. Sreejayan and J.B. Vincent, unpublished results). The peptide in the presence of added Cr^{3+} increases insulin stimulated glucose uptake in mouse C2C12 skeletal muscle cells, even in cells exposed to high glucose so that they are not responsive to insulin. The peptide in the presence of chromium increases Akt phosphorylation in these cells. *In vivo* studies with healthy mice indicate that the peptide given intravenously can lower glucose concentrations and glucose concentrations in glucose tolerance tests. *If the results of such studies continue to be positive, then the sequencing of this peptide could represent a major breakthrough in the field with significant implications in the treatment of insulin resistant conditions.*

6.4 SYNTHETIC MODELS OF LMWCr

The recent research on LMWCr has inspired synthetic efforts to prepare new chromium-carboxylate assemblies. Known assemblies with nuclearity greater than two (but less than eight) possess four types of cores:

symmetric [63] and unsymmetric [64] Cr_3O, $Cr_3(OH)_2$ [65] and Cr_4O_2 [66–69]. Numerous examples of the type containing the symmetric Cr_3O core have been well characterized, and interest in these complexes date back to the late nineteenth century [63]. The other cores have only been prepared during the last decade using the symmetric trinuclear complexes as starting materials.

Given the novel role in the autoamplification of insulin signal transduction for LMWCr and its rather simple composition (carboxylate-rich oligopeptide binding four chromic ions), attempts have been made to identify a functional model for chromodulin. Such a biomimetic would be required to be soluble and stable in aqueous solution. Few of the known trinuclear and tetranuclear Cr(III) oxo(hydroxo)-bridged carboxylate assemblies are soluble in water. On the basis of these requirements, two assemblies have been examined: $[Cr_3O(O_2CCH_3)_6(H_2O)_3]^+$ and $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ [42]. Both possess the symmetric basic carboxylate-type structure comprised of a planar triangle of chromic ions with a central μ_3-oxide. The acetate complex does not activate the tyrosine protein kinase activity of the active site fragment of insulin receptor or of adipocytic membrane fragments in the presence of insulin and actually inhibits the activity. In stark contrast, the propionate analogue activates the kinase activities in a fashion very similar to LMWCr. The kinase activity of the isolated receptor fragment, for example, is stimulated approximately threefold with a dissociation constant of 1.00 nM [42]. (One laboratory has reported not detecting stimulation of insulin receptor kinase activity by this complex [34]). The complex appears to be a functional biomimetic for LMWCr, and this was previously used as support for the existence of a multinuclear chromic assembly in LMWCr. However, the recent spectroscopic and magnetic studies on LMWCr clearly indicate that LMWCr does not possess a Cr_3–μ_3–O^{2-} core as does the synthetic compound. Additionally, LMWCr is anionic while the synthetic species is cationic. Thus, while the two species possess anion-bridged multinuclear Cr(III) assemblies bridged by carboxylate ligands, they possesses distinctly different structures and charges such that any functional similarities must be considered coincidental.

The propionate biomimetic has been found to have striking *in vivo* effects, lowering plasma triglycerides, total cholesterol, LDL cholesterol and HDL cholesterol levels after 12 weeks of supplementation in rats at a level of 20 μg Cr/kg body mass daily and potentially lowering body mass and fat content [58]. The *in vivo* effects of administration of the synthetic cation $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ to healthy and type 1 and type 2 diabetic model rats has been examined. (The synthetic cation

was initially given intravenously to avoid potential differences in absorption between the healthy and diabetic model rats.) After 24 weeks of intravenous administration (0–20 μg Cr/kg body mass) to healthy male rats, the cation resulted in a concentration dependent lowering of levels of fasting blood plasma LDL cholesterol, total cholesterol, triglycerides and insulin and of 2-h plasma insulin and glucose levels after a glucose challenge. These results confirmed the result of a previous 12-week study examining the effect of the synthetic cation on healthy rats [58] and are in stark contrast to those from administration of other forms of Cr(III) to healthy rats, which have no effect on these parameters. The cation had little, if any, effect on rats with streptozotocin-induced (type 1 model) diabetes. This may result from the increased spread of the values of the blood variables in these rats compared with those of controls, such that insufficient power existed to observe any potential effects. However, Zucker obese rats (an early stage type 2 diabetes model) after 24 weeks of supplementation (20 μg/kg have lower fasting plasma total cholesterol, triglyceride, insulin and LDL and HDL cholesterol levels and lower 2-h plasma insulin levels after a glucose challenge. The lowering of plasma insulin concentrations with little effect on glucose concentrations suggests that the supplement increases insulin sensitivity. No acute toxic effects were observed for supplementation with the compound, and it does not give rise to DNA damage in *in vitro* studies as observed with chromium picolinate [70].

The effects of oral (gavage) administration of the complex have been examined [71]. At levels of 250, 500, or 1000 μg Cr/kg body mass, the treatment at all concentrations lowered fasting plasma insulin, triglycerides, total cholesterol and LDL cholesterol levels of healthy rats while having no effect on plasma glucose or HDL cholesterol. These levels were lower after 4 weeks of treatment and remained lower for the next 20 weeks of treatment. The maintenance of glucose levels with less insulin indicates increased insulin sensitivity. Both plasma glucose and insulin levels were lowered in 2-h glucose tolerance tests. Previously, Anderson *et al*. have shown that $CrCl_3$ and Cr picolinate when given orally up to 100 mg Cr/kg food (equivalent to ~8 mg Cr/kg body mass, eight times the largest amount administered in this study and, after correcting for absorption differences, approximately equivalent to the highest dose in this study) had no effect on any of these plasma variables [70]. Effects on healthy rats appear to be unique to the biomimetic complex. In Zucker obese rats, the early stage type 2 diabetes model, receiving 1000 μg Cr/kg body mass, the results were similar to those from intravenous administration. In this study, the effects of the biomimetic cation

on ZDF rats, a genetic model for type 2 diabetes, were also examined using 1000 μg Cr/kg body mass. Again fasting plasma insulin, triglycerides, total cholesterol and LDL cholesterol levels were all lower while glucose concentrations were consistently but not statistically lower. HDL levels were lowered from their very high levels; 2-h plasma insulin levels were also lowered.

Plasma glycated haemoglobin levels, a measure of longer term blood glucose status, were examined in the healthy, Zucker obese and ZDF rats after 4, 12 and 24 weeks of treatment. No effect was seen for the healthy rats; however, significant effects were noted for the diabetic models. For the ZDF rats, glycated haemoglobin was lower after 12 and 24 weeks of treatment, reaching almost a 22% drop compared with ZDF controls by week 24; for the Zucker obese rats, glycated haemoglobin was 27% lower at week 24. Notably, Cr was not found to accumulate in the liver or kidney at these doses of Cr3. Control studies using an intravenous injection containing an amount of propionate equivalent to that received in the largest dose used above have not observed similar effects [71].

The effects of the cation on healthy and model diabetic rats have also been examined by Krejpcio and co-workers. Male Wistar rats were provided a control diet or a diet containing 5 mg Cr/kg diet as the cation for 10 weeks [72]. Blood plasma insulin levels were lowered 15.6% by the Cr-containing diet, while glucose transport by red blood cells was increased 9.6%. In another study, this group utilized male Wistar rats with streptozotocin-induced diabetes. Using similar diets for 5 weeks, the rats that had the Cr diet had lower blood serum glucose levels (26%) and increased HDL levels (14%) [73]. Follow-up studies showed similar effects [74, 75]. At a dose of Cr3 of 5 mg Cr/kg body mass (but not 1 mg Cr/kg), these researchers found that Cr accumulated in the kidney. Thus, much higher doses of Cr3 are required for chromium accumulation compared with $CrCl_3$ or $[Cr(pic)_3]$ [70]. This group has also shown that Cr3 given in the diet at 100 mg Cr/kg body mass to female Wistar rats for 4 weeks leads to no genotoxicity as measured by Comet assays [76]. They have also demonstrated that the LD_{50} of Cr3 is greater than 2000 mg/kg when given orally to male and female Wistar rats [77].

During the first 24 h after intravenous injection, the fate of the ^{51}Cr-labelled complex in tissues, blood, urine and faeces has been followed [78]. Remarkably, the complex is readily incorporated into tissues and cells. The complex rapidly disappears from the blood (<30 min) as radiolabeled Cr from the cation appears in tissues. In hepatocytes, the intact cation is efficiently transported into microsomes where its concentration reaches a maximum in approximately 2 h (and corresponds to >90% of

Cr in the cells from the injected complex); this suggests that the cation is actively transported into cells via endocytosis; identification of the protein(s) responsible is needed. As the complex is degraded in hepatocytes and the levels in microsomes rapidly decrease, Cr appears in the urine as chromodulin (or a similar molecular weight chromium binding species (see above)). The synthetic complex is degraded before or during its disappearance from the microsomes. During the initial periods when the blood concentration of the complex is high, some of the complex passes into the urine intact [78]. The rats have also been given the ^{51}Cr- or ^{14}C-labelled complex by intravenous injection daily for 2 weeks [79], and 30% of the injected Cr is lost daily as chromodulin or a similar species; only very small amounts are lost in the faeces. The tissue and subcellular hepatocyte distribution of chromium after 2 weeks was examined; no intact complex could be detected. Degradation of the complex resulted in accumulation of Cr in the liver and kidney as observed with other Cr sources. Only ~3% of the propionate from each injection of the complex was lost daily; the tissue and cellular distribution of derivatized propionate after 2 weeks varied greatly from that of Cr. Thus, the active transport of the biomimetic is different from the transport of all other synthetic forms of Cr proposed as dietary supplements; these proposed supplements appear to enter cells passively by diffusion. Hence, the biomimetic complex has a significantly greater ability to enter cells than other Cr supplements and can bring about positive changes in carbohydrate and lipid metabolism unlike other chromium supplements.

At a nutritionally relevant level (3 μg Cr/kg body mass) and a pharmacologically relevant level (3 mg/kg), at least 60 and 40% of the compound, respectively, are absorbed in 24 h [80]. The compound was absorbed rapidly. Most of the absorbed chromium appeared to be absorbed by the stomach in less than 30 min. This represents an ~10-fold increase over the absorptions for Cr picolinate (marginally soluble in water, 0.6 mM), $CrCl_3$ (which oligimerizes in water) and Cr nicotinate ('$Cr(nic)_2(OH)$', insoluble in water). The solubility of the biomimetic cation and its stability, thus, allow a unique amount of the material to enter the circulatory system and tissues.

Lay and co-workers have examined the stability of Cr3 and other Cr complexes in: simulated gastric juice and intestinal fluid; whole blood, blood plasma, or blood components; cell culture media; and rat L6 skeletal muscle cells [81]. No significant change occurred in the X-ray absorption near edge spectroscopy (XANES) spectrum of Cr3 after 24 h in the artificial gastric fluid, but noticeable changes occurred after 24 h in the artificial intestinal fluid. Similarly, 24 h exposure to cultured cells and

blood plasma resulted in changes in the XANES spectra [81]. How these results can be compared with the *in vivo* data where Cr3 appears in cells by 2 h and quickly disappears is difficult to determine. These workers have also shown that exposure of Cr3 to albumin-depleted bovine serum for 2 h at 37 °C leads to chromium binding to high-molecular weight species [82]. Interpretation is again difficult.

6.5 PROPOSED MECHANISMS OF CHROMIUM ACTION

Numerous pathways by which a Cr biomolecule could manifest its effects have been proposed. However, research results in *in vitro* and *in vivo* systems are contradictory, such that the state of the field is not immediately clear (Table 6.2). Attention has been focused on two sites of action in the insulin signalling cascade as the potential sites of Cr action, insulin receptor and Akt (Figure 6.1), and on cholesterol synthesis and metabolism.

6.5.1 Direct Chromium Binding to Insulin Receptor

Chromium as $CrCl_3$, chromium picolinate or $[Cr(pic)_3]$ and Cr–peptide complexes have been reported to improve glucose uptake and up-regulate mRNA levels of insulin receptor, glucose 4 transporter (GLUT4), glycogen synthase and uncoupling protein-3 in skeletal muscle cells [83]. However, $[Cr(pic)_3]$ has been reported to increase glucose uptake and metabolism and GLUT4 translocation to the plasma membrane in control and insulin resistant 3T3-L1 adipocytes, while having no effect on mRNA levels of insulin receptor (IR), Akt and other insulin signalling cascade components [84].

Brautigan and co-workers [85] reported that pre-incubation of Chinese hamster ovary cells overexpressing insulin receptor with chromium picolinate, chromium histidine, or $[Cr_3O(propionate)_6(H_2O)_3]^+$ activated insulin receptor tyrosine kinase activity in the cells at low doses of insulin. While the concentration dependence was only examined for the histidine complex, the effect was concentration dependent. Neither insulin binding to the cells nor insulin receptor number were affected. Also, the addition of chromium did not inhibit dephosphorylation of the insulin receptor by endogenous phosphatases or added PTP1B. Additionally, chromium apparently did not alter redox regulation of PTP1B

Table 6.2 Selected studies of effects of chromium administration on insulin signalling pathway.

Cell or organism	Chromium compound	Effect	Ref.
Skeletal muscle	$CrCl_3$, $[Cr(pic)_3]$, Cr peptide complexes	Up-regulation of insulin receptor mRNA levels	[83]
Insulin-resistant 3T3-L1 adipocytes	$[Cr(pic)_3]$	No effect on insulin receptor and Akt mRNA levels	[84]
Chinese hamster ovary cells	$[Cr(pic)_3]$, Cr3, Cr histidine	Activated insulin receptor kinase activity	[85]
Type 2 diabetic humans	$[Cr(pic)_3]$	Increased Akt phosphorylation but not insulin receptor or IRS-1	[86]
JCR:LA rat	$[Cr(pic)_3]$	Increased insulin receptor, IRS-1, and Akt phosphorylation and increased PI-3 kinase activity	[87]
3T3-L1 adipocytes	$Cr(D\text{-}phe)_3$	Increased phosphorylation of Akt but not insulin receptor	[88]
3T3-L1 adipocytes	$[Cr(pic)_3]$	No effect on phosphorylation of insulin receptor, IRS-1, or Akt	[89]
KK/HIJ mice skeletal muscle	Milk powder enriched with trivalent Cr	Increased IRS-1 tyrosine phosphorylation, increased Akt activity, and decreased IRS1 serine-307 phosphorylation	[90]
3T3-L1 adipocytes	Chromium histidine	Increased insulin-stimulated glucose uptake and insulin-stimulated tyrosine phosphorylation of IR	[91]
C2C12 skeletal muscle cells	Chromium oligomannuronate	Enhanced phosphorylation of IR, PI3K, and Akt and AMPK	[92]

(i.e. by trapping the oxidized inactive form or by preventing its reduction and reactivation). $CrCl_3$ and chromium histidine were found not to activate the kinase activity of a recombinant fragment of insulin receptor. The authors concluded that chromium inside the cell modified the receptor in some manner, activating its kinase activity [85]. Subsequently,

Brautigan *et al*. [91] demonstrated that a Cr–histidine complex (actually a complex mixture of numerous Cr–histidine complexes) stimulated tyrosine phosphorylation of IR in 3T3-L1 adipocytes in the presence of insulin but not of MAPK (mitogen-activated protein kinase) or 4E-BP1, markers for activation of transcription and translation, respectively, in the presence of insulin; glucose uptake in the presence of insulin was also stimulated by the Cr complex. The effects of the Cr compound were also examined in competition with those of vanadate [91]; the results were interpreted in terms of Cr having an action involving IR activation and potentially in another action beyond IR activation that increases GLUT4 transport.

Krejpcio and co-workers [93] have examined the effects of Cr3 and its acetate, glycinate and lactate analogues, $[Cr_3O(O_2CR)_6(H_2O)_3]^+$, along with Cr–histidinate and $CrCl_3$, on mouse C2C12 myoblasts and 3T3-L1 adipocytes. Cr3 and its acetate and glycinate analogues decreased IRS-1 phosphorylation at serine. None of the compounds affected insulin-stimulated IRS-1 tyrosine phosphorylation, although the glycinate and acetate analogues increased the tyrosine phosphorylation in the absence of insulin [93]. None of the chromium compounds affected insulin binding to the cell membranes.

6.5.2 Akt

Cefalu *et al*. [86] in a preliminary report has indicated that chromium picolinate may act by increasing the activation of Akt phosphorylation. In this study, eight type 2 diabetic subjects received 1 mg Cr as chromium picolinate while eight subjects received placebos; the diabetic subjects receiving Cr after insulin treatment had increased Akt phosphorylation but not IR or IRS-1 phosphorylation. Increased Akt phosphorylation was suggested to be the mechanism of chromium action. Yet, at a CADRE Research summit in 2003, Cefalu [87] reported results of a similar study using JCR:LA-corpulent rats, a model of insulin-resistant cardiovascular disease. (Cefalu *et al*. had previously reported that daily oral administration of chromium picolinate (18 μg Cr/kg body mass) to the rats for 12 weeks resulted in lower plasma total cholesterol and high HDL cholesterol and lower fasting insulin levels) [94]. No effects were observed with healthy controls.) Chromium picolinate treatment resulted in increased IR, IRS-1 and Akt phosphorylation and increased PI-3 kinase activity after insulin administration. These results are actually consistent with Cr acting at insulin-stimulated insulin receptor, leading to the other effects downstream in the signal cascade.

Sreejayan and co-workers [95] using a different Cr complex have generated additional evidence for an association between Cr and Akt. $Cr(phenylalanine)_3$ (5 or 25 μM for 10 days) was found to increase insulin-stimulated glucose uptake by cultured mouse 3T3-adipocytes. Treatment of the cells with 5 μM Cr for 0.5–4 h or 0.1–100 μM Cr for 2 h did not increase insulin-stimulated phosphorylation of insulin receptor (Tyr 1146) significantly, while under similar conditions insulin-stimulated Akt phosphorylation (Thr 308) was increased significantly.

6.5.3 Cholesterol

Yao and co-workers [84, 96] determined that $[Cr(pic)_3]$ increased glucose uptake and metabolism and GLUT4 transport in 3T3-L1 adipocytes; the effects were independent of insulin. Chromium (60 nM) had no effect on IR or Akt phosphorylation but was found to activate MAPK (mitogen-activated protein kinase) independent of its effect on GLUT4 translocation. Elmendorf and co-workers have also examined the effects of $CrCl_3$ and $[Cr(pic)_3]$ on 3T3-L1 adipocytes [89, 90, 97]. In their first report [89], $CrCl_3$ and $[Cr(pic)_3]$ were shown to increase GLUT4 transport to the plasma membrane in the presence of insulin. Chromium treatment did not affect IR, IRS-1, PI3K, or Akt regulation but decreased plasma membrane cholesterol. Subsequently, the effects of $[Cr(pic)_3]$ were shown to be dependent on the glucose concentration of the media with the effects being observed at 25 mM, but not 5.5 mM [97]. In contrast, Yao and co-workers also looked at the effects of Cr at both 25 and 5.5 mM glucose in their studies described above; similar results were observed at both glucose concentrations [84, 96]. Elmendorf and co-workers have postulated that Cr manifested its effects via affecting cholesterol homoeostasis [89, 90, 97].

6.5.4 Chromate

Lay and co-workers [44, 98] have proposed that chromate generated enzymatically (i.e. from hydrogen peroxide or other species generated by enzymes) from Cr(III) in the body could act as a phosphotyrosine phosphatase (PTP) inhibitor, in a similar manner to vanadate, and that the site of action of Cr is at the PTPs. Chromate has been shown in cell studies to have insulin-mimetic effects similar to vanadate [99–102]. However, some studies have shown that streptozotocin-treated rats (which

are insulin deficient) do not show benefit from chromium administration [103, 104] (although others have (see Chapter 5)), while numerous studies have shown vanadium can serve as an insulin substitute in these rats [103, 104]. Despite the nature of the vanadium compound utilized, the active species in the inhibition appears to be 'naked' vanadate [105]. Vanadate increases basal levels of phosphorylation of IR and IRS-1 and activity of PI3-kinase, but these levels are not further stimulated by insulin treatment [106]. Additionally, the increased phosphorylation of Akt and IR in response to vanadate display different vanadate concentration dependence [107], as might be expected if different PTPs are involved in inactivation. Consequently, if chromate is responsible for the *in vivo* effects of chromium, then examining the effects of Cr on the insulin cascade system *in vivo* both before and during insulin stimulation should be distinctly different (i.e. vanadate-like) than that if Cr acts by stimulating insulin receptor kinase activity.

The proposal that chromate could be involved in chromium action *in vivo* is based on the ability of hydrogen peroxide to oxidize Cr(III) compounds to chromate [44]. To demonstrate this, Lay and co-workers exposed chromium picolinate, $CrCl_3$ and the basic chromium carboxylate cation $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ to 0.10–1 mM hydrogen peroxide for 1–6 h in 0.10 M HEPES buffer, pH 7.4. This resulted in the formation of chromate in efficiencies of from 1% (chromium picolinate for 6 h with 1 mM H_2O_2) to 33% (the cation for 6 h with 1 mM H_2O_2). The cation could also be oxidized with hypochloride or glucose oxidase or xanthine oxidase (enzymes that produce H_2O_2).

To put the potential for chromate to be the biological active form of chromium in perspective, some simple calculations are in order. Over-the-counter chromium supplements and chromium supplements used in human studies generally provide from 200 μg to 1 mg of Cr daily. Using the best-selling and most studied supplement chromium picolinate as an example, absorption is estimated to be about 2% [108, 109]. If one assumes a 1 mg dose with 5% absorption, a human would have 50 μg Cr enter the bloodstream. Using the atomic mass of Cr of 52 g mol^{-1}, this represents approximately 1 μmole Cr. Assuming an average human body mass of 65 kg giving a volume of approximately 65 l, the average Cr concentration in the body should be approximately 15 nM. Using 1 mM hydrogen peroxide and letting chromate accumulate for 6 h, Lay and co-workers observed at best about 30% conversion of any of the Cr(III) compounds examined into chromate. Assuming 30% conversion gives an average chromate concentration of approximately 5 nM. While Cr does accumulate better in some organs than in others,

such that Cr concentrations locally can be higher than the average, the 5 nM concentration (calculated otherwise assuming the best of all conditions for chromate production) is roughly 15 000 times smaller than the K_i values reported for phosphate inhibition by chromate. When one remembers that the typical peroxide concentration in the body is from 10^{-7} to 10^{-8} M (rather than mM) and instances where locally higher concentrations of hydrogen peroxide are unlikely to remain for prolonged periods of time, the probability that chromate is responsible for any effects on insulin sensitivity in human studies is essentially nill. (When similar calculations are performed for vanadium supplements being tested for anti-diabetic effects, the calculations are consistent with vanadate potentially being a phosphatase inhibitor, that is, increased size of the dose, greater percentage absorption and much smaller inhibition constants.)

This is not to rule out the possible significance of Cr(IV) or Cr(V) species from the oxidation of chromium(III) supplements in the effects of chromium supplements on insulin sensitivity and related effects. These species as noted by Lay and co-workers [44] could serve to inactivate tyrosine protein phosphatases through oxidation of the active site cysteine thiol. Indeed, insulin action triggers the production of reactive oxygen species including hydrogen peroxide and oxidants can facilitate or mimic insulin action; reactive oxygen species have even been proposed as second messengers in insulin signal transduction, including through inhibition of tyrosine protein phosphatases via thiol oxidation [110]. Yet again, reviewing the effects of *oral* administration of chromium(III) supplements on oxidative damage associated with the development of diabetes and related conditions has been shown to lead to reduction in markers of oxidative stress [111–118]. The significance of the oral administration will be discussed in Chapter 9.

6.5.5 Cytokines

Jain and Kannan have shown that monocytes exposed to high glucose concentrations have lower levels of the cytokine TNF-α (tumour necrosis factor-α) in the presence of 100 μM $CrCl_3$ for 24 h at 37 °C [119]. Treatment with $CrCl_3$ also inhibited stimulation of TNF-α secretion in these cells by 50 μM H_2O_2. Lipid peroxidation and protein oxidation in the presence of H_2O_2 was also inhibited by $CrCl_3$. As increased TNF-α secretion may be associated with insulin resistance, Jain has proposed that increased insulin sensitivity arising from chromium administration may be mediated by lowering of TNF-α levels (as discussed in reference [120]). In a follow-up study, this group found that

$CrCl_3$ in combination with oestrogen lowered lipid peroxidation in high glucose-treated monocytes [121]. The combination was also found to decrease interleukin-6 (IL-6) secretion. Chromium was proposed to potentiate the effects of oestrogen [121]. Curiously, another group has shown that Cr(III) treatment (350– 500 ppm) results in increased TNF-α production by macrophages (in the absence of high glucose concentrations) [122]. This activation by chromium ($CrCl_3$) may be regulated by tyrosine kinases [123]. The results in the presence of high glucose could also point to an association between reactive oxygen species and chromium, but these studies must be considered extremely preliminary. Studies examining the levels of TNF-α, IL-6, oestrogen and related cytokines and hormones in humans administered chromium are required before further conclusion can be drawn. Additionally, the fate of the chromium in these cell culture studies needs to be examined. $CrCl_3$ (actually *trans*-$[CrCl_2(H_2O)_4]Cl{\cdot}xH_2O$) readily hydrolyses in solution to form hydroxide-bridged oligomers, while Cr(III) can bind to components of the media. Thus, the form of chromium the cells are exposed to may or may not resemble physiologically relevant forms, influencing the results. As with all cell culture studies using chromium compounds, the fate of the chromium introduced needs to be carefully elucidated (see below).

6.5.6 Insulin Receptor Number

Insulin receptor number has often been claimed as a potential indicator of Cr deficiency in humans (e.g. reference [124]). Despite the number of times this claim has been made, it is based on purely a single study using only seven hypoglycaemia subjects; IR number per cell (red blood cell) was increased significantly after 6 weeks but not 12 weeks of Cr treatment [125]. Other studies, including rat studies, have failed to observe any effects on IR number (e.g. reference [126] but see reference [127] on statistical analysis of the data).

6.6 COMPARISON OF CELL CULTURE STUDIES BY CELL TYPE

6.6.1 Skeletal Muscle

Cell culture studies with rat L6 myoblasts or mouse C2C12 cells suggest an effect for chromium in the insulin signalling system. Qiao *et al.*

[83] found that adding $CrCl_3$ to L6 cells led to increases in insulin-stimulated glucose uptake and increased levels of mRNA of insulin receptor, GLUT4, glycogen synthase and uncoupling protein-3; however, the concentrations of these proteins were not determined. Further studies [128] also found $CrCl_3$ and $Cr(pic)_3$ treatment increased basal and insulin-stimulated protein deposition and increased mRNA levels of insulin-like growth factor and insulin-like growth factor receptor. Insulin-dependent glucose uptake has also been also observed in C2C12 cells when treated with $CrCl_3$ [129] and $[Cr(pic)_3]$ [130]. Earlier studies with C2C12 which used chromium-depleted media [131] must be treated with caution as Cr was removed by Chelex-100 treatment, which also removed other cations. Enhanced phosphorylation of insulin receptor, Akt, and PI3K has been reported for addition of a chromium oligosaccharide complex to C2C12 cells [92].

6.6.2 Hepatocytes

While HepG2 cells have been used in several toxicological studies examining the effects of chromate, only two studies have examined the effects of trivalent Cr that had conflicting results. Sripurkpong and Na-Bangchang [132] found treatment with 3 mM $CrCl_3$ generated increased levels of mRNA of apolipoprotein A-1 and ATP-binding cassette transporter A1; however, the concentrations of the proteins were not measured. Cholesterol levels were not affected. In contrast, Hass *et al.* found no effects from $CrCl_3$ on levels of apolipoprotein A-1 levels or levels of the corresponding mRNA [133].

6.6.3 Adipocytes

As reflected in Table 6.2, the most commonly utilized cultured cells in Cr(III) studies are 3T3-L1 adipocytes; yet, the results of studies using these cells are also the most contradictory. Despite the discussion above, these studies need to be elucidated in more detail. In 2004, 3T3-L1 cells were reported to accumulate intracellular triglycerides in the presence of insulin and $CrCl_3$ or $[Cr(pic)_3]$ in a dose-dependent fashion requiring a Cr concentration of 0.4 μM for half-maximal effect [129, 130]. Subsequently, Sreejayan and co-workers [134] found Cr(D-phenylalanine)$_3$ increased insulin-stimulated glucose uptake. At a molecular level, the Cr compound was found to enhance insulin-stimulated phosphorylation

of Akt without affecting the extent of insulin receptor phosphorylation [134]. In contrast, Brautigan *et al.* [91] demonstrated that a Cr–histidine complex (actually a complex mixture of numerous Cr–histidine complexes) stimulated tyrosine phosphorylation of IR in 3T3-L1 adipocytes in the presence of insulin but not of MAPK (mitogen-activated protein kinase) or 4E-BP1, markers for activation of transcription and translation, respectively, in the presence of insulin; glucose uptake in the presence of insulin was also stimulated by the Cr complex. These results were consistent with their previous results using CHO cells overexpressing IR [85].

The effects of the Cr compound were also examined in competition with those of vanadate [91]; the results were interpreted in terms of Cr having an action involving IR activation and potentially in another action beyond IR activation that increases GLUT4 transport. Yao and co-workers [84, 96] determined that $[Cr(pic)_3]$ increased glucose uptake and metabolism and GLUT4 transport in 3T3-L1 adipocytes; the effects were independent of insulin. Cr (60 nM) had no effect on IR or Akt phosphorylation but was found to activate MAPK independent of its effect on GLUT4 translocation.

Finally, Elmendorf and co-workers have also examined the effects of $CrCl_3$ and $[Cr(pic)_3]$ on 3T3-L1 adipocytes [89, 90, 97]. In their first report [89], $CrCl_3$ and $[Cr(pic)_3]$ were shown to increase GLUT4 transport to the plasma membrane in the presence of insulin. Cr treatment did not affect IR, IRS-1, PI3K, or Akt regulation but decreased plasma membrane cholesterol. Subsequently, the effects of $[Cr(pic)_3]$ were shown to be dependent on the glucose concentration of the media with the effects being observed at 25 mM, but not 5.5 mM [97]. In contrast, Yao and co-workers also looked at the effects of Cr at both 25 and 5.5 mM glucose in their studies described above; similar results were observed at both glucose concentrations [84, 96].

The potential toxicity of Cr picolinate, $[Cr(pic)_3]$, the most popular form of Cr supplement, has been an area of intense debate, but consensus has probably recently been reached (see Chapter 9 for an intense discussion). In mammalian cell culture studies and studies in which the complex is given intravenously, $[Cr(pic)_3]$ is clearly toxic and mutagenic, unlike other commercial forms of Cr(III). However, when given orally to mammals, the complex does not appear to be toxic nor appear to be a mutagen or carcinogen. An NIH study of the effects of up to 5% of the diet (by mass) of rats and mice for up to 2 years found no harmful effects on female rats or mice and at most ambiguous data for one type of carcinogenicity in male rats (along with no changes in body mass in

either sex of rats or mice) [135]. Despite numerous claims that $[Cr(pic)_3]$ is absorbed better than inorganic forms of Cr used to model dietary Cr, $CrCl_3$, Cr nicotinate (the second most popular form of Cr sold as a nutritional supplement), and $[Cr(pic)_3]$ are absorbed to a similar degree in rats [108, 109]. Only 1% of absorbed Cr from the supplement is found in the bloodstream as $[Cr(pic)_3]$, suggesting that little of the intact molecule is absorbed [136]. When ingested, the complex probably hydrolyses near the stomach lining, releasing the Cr, which is subsequently absorbed. The picolinate ligands also alter the redox properties of the Cr centre such that it is more susceptible to undergoing redox chemistry in the body than hexaaquoCr(III) [137]. The hydrolysis of the complex in the stomach is probably fortuitous, releasing the chromium before the intact complex can be absorbed to an appreciable level and potentially enter into redox chemistry, in contrast to the cell studies where the very stable, neutral complex could be absorbed intact. The message of these conflicting results is that *applying solutions of Cr(III) compounds to cultured cells in general does not present Cr(III) to the cells in a comparable fashion to that in which Cr(III) is presented to cells in the body*; *the difference may be crucial to the results and interpretation of the study*.

To determine why the cells study lead to such heterogeneous results, the complexes need to be studied under uniform conditions – the same cells treated in the same manner for the same period of time with the same chromium complex at the same concentrations. Actually the compounds need to be examined over a range of concentrations over varying periods of time with each of the cell types. The stability of the complexes in the culture media needs to be established as begun by Lay and co-workers [81, 82] and by Tabrizian and co-workers [138, 139]. Only in this manner will the actual chromium species in contact with the cells be established. Similarly the distribution, concentration and form of the chromium in the cells need to be determined. Control experiments using just the ligands need to be performed to determine if any effects arise from just the ligands. Without this type comprehensive treatment, progress in interpreting the body of cell culture experiments is going to be difficult if not impossible.

6.7 CONCLUSION

If chromium is an essential element, the mechanism by which it acts *in vivo* has not yet been elucidated, although several proposed mechanism of actions based on varying amount of *in vitro* data exist. As evidence

mounts that Cr(III) may have positive pharmacological effects, understanding the mechanism of this action (which may or may not be related to a physiological mechanism) will become increasingly important. A consensus is emerging in the cell studies that chromium(III) appears to stimulate glucose transport, by changes in distribution of GLUT4, and affects insulin sensitivity, although the effect may be via a direct effect of the insulin signalling a cascade or be completely independent of this cascade. Devising experiments to test the proposals presented herein or to establish that Cr acts by a completely different mechanism will be a non-trivial enterprise, 'as is the search for any grail' [1].

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7

Menagerie of Chromium Supplements

More than 10 years after the latest National Academy of Sciences recommendation for an AI for Cr, several nutritional supplement companies in their advertising materials still use the National Academies' outdated ESADDI (Estimated Safe and Adequate Daily Dietary Intake) of 50–200 μg Cr, which was replaced by the AI value of 2001 [1]. The ESADDI was originally established in 1980 when almost no accurate analytical data on Cr content of food existed and remained unchanged in 1989 [2, 3]. A recent perusal of one of our local nutrition supplement vendors revealed supplements containing 200, 400 and 500 μg Cr per pill or capsule, with the 500 μg Cr/pill supplement suggesting taking one or two pills per day. Two pills per day would provide 1000 μg Cr or 40 times the AI for women, hardly a nutritionally relevant dose. Given that humans are receiving these pharmacological doses of chromium–organic complexes, the nature of these species needs to be examined.

7.1 CHROMIUM PICOLINATE

Chromium picolinate has already been discussed in detail in Chapters 4 and 5. The leading supplier of chromium picolinate is Nutrition 21, which sells the supplement under the name Chromax and as a combination with biotin under the name Diachrome. Nutrition 21 declared bankruptcy in 2012, and the company's assets were sold at auction. The assets, including the Nutrition 21 name, were purchased by a

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limited liability corporation that continues to sell chromium picolinate. The toxicology of [Cr(pic)$_3$] will be discussed in detail in Chapter 9.

7.2 CHROMIUM NICOTINATE (OR CHROMIUM POLYNICOTINATE)

The proposed identification of nicotinate (3-carboxypyridine) in the postulated GTF sparked an interest in the synthesis of chromic–nicotinate or –nicotinic acid ester complexes starting in 1981 (reviewed in reference [4]). However, the inability of the GTF to be characterized or its biological activity to be reproduced led to a rapid decline in studies after circa 1985. In well-characterized complexes, nicotinate coordinates in a variety of manners to Cr(III): bridging through the carboxylate (μ-O,O′), monodentate through a carboxylate oxygen, or monodentate through the pyridine nitrogen. Legg and co-workers developed a clever method for determining whether nicotinate (either as the anion or zwitterion) is bound through the oxygen(s) or nitrogen using ^{2}H nuclear magnetic resonance (NMR) spectroscopy [5,6]. The ^{2}H NMR signal of 2-d-nicotinate compounds is shifted to approximately –70 to –75 ppm if the ligand is bound through the nitrogen and to +6 to +10 ppm if bound through the oxygen. Recently all the nicotinate proton or deuteron resonances of a number of chromium(III)–nicotinate complexes have been assigned [7]. The assignment for Cr(III)–nicotinate complexes described by Anderson and co-workers [8] are incorrect as no paramagnetically-broadened resonances were observed [7].

A number of studies have examined a material characterized as 'Cr(nicotinate)$_2$(OH)(H$_2$O)$_3$' (subsequently to be referred to as chromium nicotinate or chromium polynicotinate in the nutrition literature), which is the product of the reaction of two or three equivalents of nicotinic acid with chromic ions in aqueous solution at elevated temperatures (reviewed in reference [4]). The complex has not yet been crystallized, and NMR studies suggest it is more complex than the proposed formula would indicate [7]. Recently, Rhodes *et al.* have undertaken a thorough examination of chromium nicotinate produced by the different methods in the literature by mass spectrometry, NMR and EPR spectroscopy [9]. These researchers found that addition of a chromium(III) salt to an aqueous solution of sodium nicotinate generates purple solutions from which grey–purple polymers of the general formula [Cr(nic)$_2$(H$_2$O)$_x$(OH)]$_n$ precipitate along with some [nicH]X, which can be removed by subsequent extraction; this includes the patented

process [10] for the synthesis of chromium nicotinate. The addition of a chromium(III) salt to a hot aqueous solution of nicotinic acid yields blue solutions, which contain a mixture of species with 1 : 1 and 1 : 2 Cr to nicotinate ratios; increasing the pH yields a polymer with a 1 : 1 Cr to nicotinate ratio [9]. Dissolution of the polymers in mineral acid results in the formation of soluble species with an overall purple colour and a 1 : 1 Cr to nicotinate ratio [9]. Thus, studies using the second method produce a material differing somewhat from the patented material, and their results should be considered carefully (e.g. references [11] and [12]).

Because this chromium–nicotinate material was reportedly absorbed better than dietary chromium by mammals (although the absorption is equivalent to $CrCl_3$) [13,14], it has gained substantial use as a nutritional supplement, especially before the rise in the popularity of chromium picolinate. Chromium nicotinate is marketed primarily by InterHealth Nutraceutical under the name ChromeMate. This choice of name is surprising given that it is pronounced similarly to name of the toxic and carcinogenic chromium(VI) species chromate, $[CrO_4]^{2-}$. While the choice of nicotinate as an organic ligand would appear obvious from the history of GTF and that nicotinic acid (i.e. niacin) is a vitamin, the choice of nicotinate is actually problematic. Firstly, chromium nicotinate is an intractable polymer with virtually no solubility in water. This lack of solubility limits its absorption so that its absorption (in terms of chromium) is no better than that of $CrCl_3$. Secondly, nicotinic acid actually can generate insulin resistance [15], which could oppose any potential beneficial effects from the chromium; in terms of identifying the effect of chromium, the organic component can potentially produce other physiological effects that cannot readily be separated from potential effects from the chromium [16,17].

InterHealth has funded or participated in a substantial number of studies of the effects of chromium nicotinate in rodents and humans [18–46]. The conclusions of this research would be strengthened by reproduction by researchers without potential conflict of interest. One must be careful in reviewing the literature that chromium nicotinate is being used rather than a combination of a chromium source and nicotinic acid as has been utilized in a few cases (e.g. references [47–56]).

Cell culture studies reported in 1995 by Stearns *et al.* [57] demonstrated that exposure of Chinese hamster ovary cells to chromium nicotinate at concentrations up to 1 mM (non-toxic doses) did not cause appreciable clastogenicity. Bagchi and co-workers found that chromium nicotinate and chromium picolinate lead to low levels of oxidative stress in cultured macrophages, although generally less damage was observed

with chromium nicotinate [39, 58]. Very recently, InterHealth reported a series of toxicology studies on chromium nicotinate [34]. The compound did not induce mutagenic effects in bacterial reverse mutation tests in five strains of *Salmonella typhimurium* or in L5178Y mouse lymphoma cells. Similarly, a variety of acute toxic effects were not observed in rats. In a 2002 case study, one patient who received a chromium nicotinate supplement for 5 months (200 μg Cr/day) in combination with various vegetable extracts was diagnosed with necrosis of the liver; the disorder was attributed to toxic hepatitis as a result of the chromium supplementation [59]. Two components of the vegetable extracts contained appreciable amounts of Cr^{3+} as well; the subject had a dry liver chromium mass (in a biopsy fragment) of 917 μg Cr/kg. An isolated case study is impossible to interpret, especially when multiple supplements are involved.

Curiously, InterHealth recently supported a study of the toxicity of chromium(III) dinicotinate cysteinate or 'chromium(III) dinicocysteinate' [60]. One must wonder if this signals a potential replacement for ChromeMate given the expiration date of the initial chromium nicotinate patent and a recent patent application on 'chromium(III) dinicocysteinate' [61]. This compound(s) is the product of the reaction of $CrCl_3$ with two equivalents of nicotinic acid and one equivalent of cysteine in warm water [61]; it currently is not well characterized.

7.3 CHROMIUM HISTIDINE

Recently Nutrition 21 licensed a patent from the USDA for a mixture of chromium histidine complexes. Chromium histidine is made by adding a threefold molar excess of histidine to an aqueous solution of a chromium(III) salt at 80 °C and adjusting the pH to 5–5.5 with aqueous hydroxide. The product after freeze-drying is referred to as 'chromium histidinate' [62]. Based on similar synthetic procedures, the product of this synthesis is probably a complex mixture of numerous products and needs to be elucidated, particularly so that any especially well absorbed or bioactive components could be identified. Hoggard [63] found that a similar reaction produced a mixture from which the isomers *trans*-(imidazole)-$[Cr(\text{L-histidine})_2]^+$ and *trans*-(carboxylate)-$[Cr(\text{L-histidine})_2]^+$ crystallized; also the dimer $[(\text{L-histidine})_2Cr(OH)_2Cr(\text{L-histidine})_2]$ could be isolated. The third possible isomer of the formula $[Cr(\text{L-histidine})_2]^+$ where the amine group are trans was not isolated. Numerous possible isomers of the dimer could be present; no attempt was made to separate them. The *trans*-imidazole isomer has been

characterized by X-ray crystallography [64]. Both mononuclear isomers and the dimer (isomers not separated) have also been characterized by variable temperature magnetic susceptibility and electron paramagnetic resonance (EPR) spectroscopy [65]. Positively charged $[Cr(L\text{-histidine})_2]^+$ has been shown to weakly bind to isolated DNA [66]. *Trans*-(imidazole)-$[Cr(L\text{-histidine})_2]^+$ has been found not to lead to appreciable nicking of DNA *in vitro* [67]. A possibly similar mixture of Cr(III)–histidine complexes has been shown not to have significant DNA damaging capacity when added to the culture medium of *Saccharomyces cerevisiae* [68].

Anderson *et al*. reported that chromium from the mixture was better absorbed than other forms of chromium on the market at the time, including a 75% increase over chromium picolinate [69]. This study examined only urine chromium and not retention in the tissues (an issue discussed in Chapter 3) plus a 75% increase over ~1% absorbance is not particularly significant. At a level of 2 mg Cr/kg diet, chromium histidine has very recently been reported to lower triglycerides and HDL cholesterol in spontaneously hypertensive rats fed a high sucrose diet [70]. Additional studies using chromium histidine continue to appear in the literature [71–75].

7.4 CHROMIUM454

FutureCeuticals has recently begun marketing chromium454. The material is advertised as a mixture resulting from a combination of chromium(III) with water-soluble small molecules from biological extracts [76]; hopefully, greater details will be available in the future. Chromium454 has been given in drinking water (42 mg Cr/kg (body mass?)) to male Sprague–Dawley rats with streptozotocin-induced diabetes for 3 weeks [77]. CRC454 resulted in a 38% reduction in blood glucose levels compared with controls and improvements in body mass.

7.5 CHROMIUM NANOPARTICLES

Chromium nanoparticles, under the name CrNano, in the size range 40–70 nm have been examined as nutritional supplements [78]. Male Sprague–Dawley rats maintained at 30 °C have been provided a diet containing 150, 300 and 450 *m*g Cr/kg diet as these nanoparticles for 8 weeks. The nanoparticles had no effect on body mass or food intake.

Serum insulin concentrations were lowered at all doses of chromium. No funding source was provided. Readers should expect to see more publications resulting from use the of chromium nanoparticles.

7.6 CHROMIUM SMALL PEPTIDE COMPLEXES (CrSP)

Zhou and co-workers [79, 80] have reported the effects of $CrCl_3$, $[Cr(pic)_3]$ and CrSP (chromium small peptide complexes) on certain mRNA levels in rat L6 myoblasts. CrSP is a poorly characterized mixture of complexes of chromium(III) with small dipeptides and tripeptides with an overall Cr : peptide ratio of 1 : 3 where the peptides were generated by protein hydrolysis. The cells were exposed to 0.5 μM solutions of the chromium species for 72 h. Nothing was done to examine the stability of the chromium species in the media over this period of time. The chromium compounds were found to increase glucose uptake and increase levels of mRNA of insulin receptor, GLUT4, glycogen synthase, uncoupling protein-3, insulin-like growth factor 1 (IGF-1) and IGF 1 receptor in these skeletal muscle cells in the presence of 100 nM insulin. Unfortunately, the levels of the corresponding proteins were not examined so the significance of these changes in mRNA levels cannot be determined. The research was supported by the Program for Changjiang Scholars and Innovative Research Team in University, China.

7.7 DINAKROME

Dinakrome is an organic chromium preparation (300 ppm Cr) consisting of chromium yeast, Yucca shidigera extract, silica and magnesium mica marketed by Dinatec, Gainesville, GA, USA, for use in farm animal feeds [81]. Reports of its use are appearing in the literature [82]; however, distinguishing the effects of chromium from other components of the yeast and yucca extract complicate any interpretation of the results.

7.8 CHROMIUM(D-PHENYLALANINE)$_3$

In the last few years, several publications on the use of Cr(D-phenylalanine)$_3$ as a potential nutritional supplement or pharmaceutical agent have appeared [83–89]. The non-natural D-isomer of the amino acid limits its utilization in the body. The compound is made by

mixing aqueous solutions of $CrCl_3$ and D-phenylalanine and refluxing for 4 h; freeze-drying produces a greenish–violet solid with the formula Cr(D-phenylalanine)$_3$·3HCl·2H$_2$O. Data on the elemental composition, the electronic spectrum in methanol solution and the infrared spectrum of the compound have been reported, while electrospray mass spectroscopy indicated that the compound [Cr(D-phenylalanine)$_3$] and free ligand were present [83]. An X-ray structure and further characterization are desirable to determine which isomer(s) (e.g. facile or meridianal) is present and other details. Because the characterization was performed on the products of freeze-drying the bulk, products other than [Cr(D-phenylalanine)$_3$] could also be present. Studies to examine the extent of absorption of the compound when taken orally and its stability are needed. The cost of D-phenylalanine probably prevents the use of this compound as a nutraceutical; however, if it can be shown to have any unique effectiveness as a pharmaceutical agent, then it could prove cost effective. Note that this statement holds for any of the forms of chromium used as nutritional or pharmacological agents compared with $CrCl_3$. The compound is not commercially available.

7.9 CHROMIUM NICOTINATE GLYCINATE (OR CHROMIUM DINICOTINATE GLYCINATE)

This compound is assumed to have the formula Cr(nicotinate)$_2$(glycine), although no characterization could be found in the literature. It appears to also be sold as Chromium Chelavite by Albion; however, the definition of Chelavite is broad enough to contain other amino acids. The absorption of this complex by humans has been examined, although only by using urinary chromium excretion [90]. Chromium chelavite was one of six commercial chromium(III) compounds used in a comparative study in rats [25]. It has also been used in a study in sheep [91]. Chromium dinicotinate glycinate has been used in a mixture with several other supplements in a trial examining reward deficiency syndrome [92].

7.10 CHROMIUM PIDOLATE

Pidolate is pyroglutamate or 2-pyrrodine-5-gluconate. Chromium pidolate is the tris chelate of this ligand and is provided by Labcatal Pharmaceutical. No characterization of the compound could be found in the

literature. The absorption of this complex by humans has been examined, although only by using urinary chromium excretion [69]. Supplementation of type 2 diabetic subjects with 400 mg Cr/day as chromium pidolate for 6 months had no effect on body mass, fasting glucose, glycated haemoglobin, insulin or total or HDL cholesterol but lowered plasma TBARS (thiobarbituric acid reactive substances) [93].

7.11 CHROMIUM METHIONINE OR CHROMIUM METHIONINE CHELATE

The hydrogen chloride of chromium(III) tris(L-methionine), [Cr(L-methionine)$_3$]·HCl, is marketed as AvilaCr and MicroPlex1000 by ZinPro. While approved as a supplement of porcine and bovine feed in the United States, it was recently rejected for use in Europe in a decision by the European Food Authority [94]. The EFSA determined that chromium dietary deficiency had not been established in animals; thus, any risk from the supplement would outweigh benefits. Hence, trivalent chromium is not authorized as a feed additive in the European Union [94]. Chromium methionine, along with Kemtrace, chromium yeast and chromium picolinate, have been approved as feed additives for swine in the United States. Studies of the effects of chromium methionine on beef cattle, dairy cattle and pigs have appeared in the literature [95–102].

Chromium(methionine)$_3$ has been characterized to some degree. Details of its infrared and visible spectra and the stability constants for the binding of each equivalent of methionine have been reported [103].

7.12 Cr3/KEMTRACE

The compound $[Cr_3O(propionate)_6(H_2O)_3]^+$ was identified independently by two different groups, both of which have patented the compound for use as a nutritional supplement and pharmaceutical. The Vincent group, in preparing a series of anion-bridged Cr(III)–carboxylate complexes to generate a library of spectra for comparison against the electronic, NMR, EPR, and so on, spectra of the oligopeptide low molecular weight chromium-binding substance (LMWCr) (see Chapter 6), also examined the ability of the synthetic complexes to activate insulin-stimulated insulin receptor kinase activity. One of the compounds, $[Cr_3O(propionate)_6(H_2O)_3]^+$ or Cr3, was found to activate the receptor in a similar fashion to LMWCr [104]. Cr3 is very

water soluble and can be recrystallized from dilute mineral acids so that it should be reasonably stable in the stomach. At a nutritionally relevant level (3 μg Cr/kg body mass) and a pharmacologically relevant level (3 mg/kg), at least 60 and 40% of the compound, respectively, are absorbed in 24 h [105]. This represents a greater than 10-fold increase over those of Cr picolinate (marginally soluble in water, 0.6 mM), $CrCl_3$ (which oligimerizes in water) and Cr nicotinate ('$Cr(nic)_2(OH)$,' insoluble in water). The solubility of Cr3 and its stability, thus, allows a unique amount of the material to enter the circulatory system and tissues.

During the first 24 h after intravenous injection, the fate of the ^{51}Cr-labelled complex in tissues, blood, urine and faeces has been followed [106]. The complex is readily incorporated into tissues and cells. The complex rapidly disappears from the blood (<30 min) as radiolabeled Cr from Cr3 appears in tissues. In hepatocytes, the intact Cr3 is efficiently transported into microsomes where its concentration reaches a maximum in approximately 2 h (and corresponds to >90% of Cr in the cells from the injected complex); this suggests that Cr3 is actively transported into cells via endocytosis; identification of the protein(s) responsible is needed. As the complex is degraded in hepatocytes and the levels in microsomes rapidly decrease, Cr appears in the urine as LMWCr (or a similar molecular weight chromium binding species). The synthetic complex is degraded before or during its disappearance from the microsomes. During the initial periods when the blood concentration of the complex is high, some of the complex apparently passes into the urine intact [106].

The University of Alabama owns five patents on the use of Cr3; the author of this book is inventor or co-inventor on all the patents. The effects of Cr3 on healthy rats and rat models of insulin resistance, colorectal cancer and type 1 and type 2 diabetes have been examined where it was found to have beneficial effects on insulin sensitivity, the incidence of colorectal cancer and, in most cases, lipid parameters [107–115]. The compound has been tested for toxic effects *in vivo* [116–120] and *in vitro* [121, 122], and activity assays have been performed in cell culture [74, 104, 123, 124]. The compound was found to be non-toxic in the *in vivo assays* and to not damage DNA *in vitro*, although the compound could be oxidized by biological oxidants in the absolute absence of reducing agents. When given orally to rats, the LD_{50} of the cation was estimated to be greater than 2 g/kg body mass [119].

Kemin Americas patented the product of the reaction of chromium(VI) with a reductant in propionic acid, which they indicated was anhydrous $Cr(propionate)_3$ [125, 126]. This product is known commercially

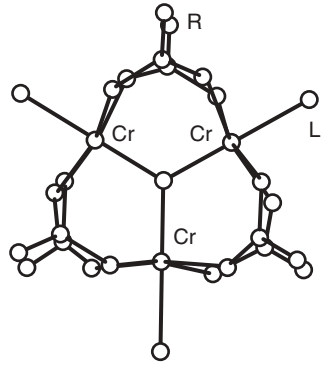

Figure 7.1 Structure of the $[Cr_3O(O_2CR)_6]^+$ core. For Cr3, R = ethyl, and $L = H_2O$.

as Kemtrace or Kemtrace chromium and is referred to in the scientific literature as chromium propionate. The product has been approved for swine and cattle diets in the United States (the only chromium supplement approved in the latter), and its effects have been tested in cattle, swine and poultry [127–137]. The reaction of a chromium(III) source, water and a carboxylic acid yields a variety of products including dinuclear species, trinuclear species with the $[Cr_3O(O_2CR)_6]^+$ core (such as Cr3) (Figure 7.1), and larger multinuclear species [138–141]. As the trinuclear cation has the largest carboxylate to Cr ratio (2 : 1), the greater the excess of carboxylic acid in the reaction mixture, the greater percentage of the product is the trinuclear cation.

In the Kemin syntheses, the reduction of CrO_3, chromate, or dichromate yields 3–4 equivalents of water per chromium; consequently, the system is far from anhydrous, making the synthesis of $Cr(propionate)_3$ impossible; with a large excess of propionic acid present, the major Cr-containing product is Cr3/Kemtrace chromium. The conditions required for the synthesis of $Cr(propionate)_3$ have been reported and include the reduction of CrO_3 in propionate anhydride [142] and an electrochemical synthesis in rigorously dried solvents (which still required recrystallization to remove waters of hydration from the product) [143]. That Kemtrace chromium is the trinuclear cation, Cr3, has recently been confirmed by growing crystals of the cation from Kemtrace chromium and characterizing the compound by X-ray crystallography [144]. The complex has recently been shown to increase insulin sensitivity [145], in a very similar effect to previous studies in healthy rodents and rodent models of diabetes. Increases of acute phase response in cattle have also recently been reported [146].

7.13 CLOSING THOUGHTS

This menagerie of chromium supplements has led to heterogeneity in scientific investigations, which complicates interpretation. From an inorganic chemistry point of view, only two of the supplements, chromium picolinate and Cr3, are well characterized – known three-dimensional structure and variety of magnetic and spectroscopic properties. Along with $CrCl_3$, these two supplements are the only ones whose absorption and distribution in the body (in terms of chromium and the organic ligand) have been elucidated in any appreciable detail. Definitive scientific evidence does not indicate that any of these supplements have any action in the body other than as a source of chromium. The limited solubility of $[Cr(pic)_3]$ adds a variable to interpretation of studies with this compound as it has been used as a solution, a solid and a suspension. The extent of absorption of the compound differs somewhat depending on whether it is given orally as a solid or solution (Vincent and co-workers, unpublished results), probably because the compound can break down more readily in solution. As all the supplements, with the exception of Cr3, that have been examined to date have similar extents of absorption to $CrCl_3$, use of $CrCl_3$ would appear to be cost effective (if chromium supplementation should prove to be beneficial); thus, the author in terms of interpretation of the science would recommend that $CrCl_3$ be used in future studies. Other than $CrCl_3$, the use of Cr3 might also prove valuable. It is highly soluble in water (solubility greater than 1 M), well characterized and is absorbed on the order of at least 50 times better than $CrCl_3$; it also appears to be able to be absorbed intact. Consequently, Cr3 should allow for the ready incorporation of chromium into tissues in higher concentrations than are possible for the other supplements, including $CrCl_3$, which could prove beneficial in attempting to establish whether chromium can have a role as a pharmacological agent in the treatment of conditions such as type 2 diabetes. Additionally, the propionate ligand is entirely innocuous in the body at the amounts that would be released from the use of Cr3; hence, interpretation of studies would not be complicated by concerns over possible effects from the ligands as with $[Cr(pic)_3]$ or chromium nicotinate.

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8

Potential Use of Chromium in the Farm Livestock Industry

As urinary chromium loss has been shown to increase during periods of stress, leading to proposals that stress could lead to chromium deficiency, and chromium was previously claimed to lead to beneficial changes in body composition, extension of the use of chromium supplementation to animal farm feed is logical. The effects of chromium supplementation have been examined most commonly with cattle, pigs and chicken and occasionally with horses, goats, sheep, rabbits, other birds and fish.

8.1 PREVIOUS REVIEWS

Studies of chromium supplementation have been thoroughly reviewed. Most notable are the first evaluation in the mid-1990s by the Committee on Animal Research, Board on Agriculture of the National Research Council [1] and the most recent review by Lindemann in 2007 [2]. The Lindemann review is particularly useful for those not familiar with farm livestock research as he carefully defines several terms used in regards to livestock for the reader. As these two sources comprehensively review the literature, an in depth discussion of the literature with a long list of references is not required, especially given the results are consistent with previous discussions of studies using rodents and humans.

Looking at the conclusions of the review by Committee on Animal Research, despite its age, it is most informative, as little really has changed since the panel presented its conclusions. In general the available data

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were insufficient for conclusions to be drawn [1]. Thus, no conclusions were drawn about the need for supplemental chromium in the diets of fish, rats, rabbits, sheep and horses. Specific recommendations could not be made about the diets of poultry, swine and cattle, although chromium was determined possibly to have a beneficial effect for cattle under stress and to maybe improve swine carcass leanness and reproductive efficiency [1]. Determining whether chromium actually had beneficial effects would require establishing the required chromium concentrations and factors that 'effect the efficacy of supplemental chromium' [1]. Chromium was, however, found to be safe as a food additive. Additional research was noted as being necessary to determine the symptoms of chromium deficiency in animals and to establish the bioavailability of chromium from dietary supplements [1].

A meta-analysis of effects of dietary chromium supplementation on parameters of growing–finishing swine has recently appeared [3]. Thirty-one studies (including abstracts) were included that utilized chromium methione, chromium nanocomposite, chromium nicotinate, chromium propionate, chromium picolinate and chromium-enhanced yeast. Studies with chromium chloride were not included because of its poor bioavailability; this is surprising as its bioavailability is probably similar to that of at least chromium nicotinate and chromium picolinate based on rodent studies. Additionally, the inclusion of effects from chromium-enhanced yeast is also potentially problematic as yeast delivers several compounds that could affect characteristics examined in the study. The inclusion of non-peer reviewed abstracts is also of concern. Given these caveats, no effects were found on parameters related to meat quality (colour, drip loss, cook loss and shear force). The results indicated that chromium supplementation decreased fat and increased lean deposition in the carcass [3], results contradictory to studies in healthy rodents and humans. Average daily gain and gain-to-feed ratio were also improved with supplementation, but results were subject to publication and small-study bias (in other words, the statistical treatment suggested that studies with negative results were under reported in the literature and that studies with small samples sizes overly affected the meta-analysis). Clearly, this is not the last word on the effects in growing–finishing swine.

The updated review of Lindemann, focusing on research published since the mid-1992, reveals that chromium supplementation has no uniform effects in weanling pigs or growing pigs, although studies on growing pigs had a tendency to report positive results on feed efficiency and improved muscling [2]. Litter size of reproducing pigs also demonstrated a tendency to be larger as a result of chromium supplementation,

although just under half of the studies observed no statistically significant effects at $P < 0.05$. Effects of chromium supplementation of cattle, regardless of age, were inconsistent [2]. Chromium supplementation of poultry had no consistent effects on young animals, with some tendency for improved carcass characteristics in older growing birds. Chromium studies on other animals are still limited in scope [2].

8.2 APPROVED USE OF CHROMIUM SUPPLEMENTS

The approved use of chromium supplements in animal feed varies greatly between the United States, Canada and Europe. Chromium is not authorized as a feed additive in the European Union. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in 2009 found avoiding 'any additional exposure of consumers resulting from the use of supplementary Cr in animal nutrition' to be prudent [4]. Similarly, the panel found that 'any occupational exposure to Cr(III) in the feed industry should be kept to a minimum' [4]. Consistent with the conclusions of Chapter 1, the panel also determined that chromium deficiency in farm animals had never conclusively been observed such that 'no evidence of the essentiality of Cr(III) as a trace element in animal nutrition' exists [4].

In the United States, three forms of chromium supplements are allowed by the Food and Drug Administration (FDA) to be used in swine feed: chromium picolinate (up to 200 ppb Cr in the diet), chromium propionate (Cr3) (200 ppb Cr) and chromium methionine (400 ppb Cr) [2]. In 2009, the FDA approved chromium propionate for use in cattle feed up to 500 ppb, making it the only form of chromium approved for use in cattle in the United States [5]. The Canadian Food Inspection Agency has allowed chromium yeast (400 ppb Cr) to be provided to first lactation dairy heifers in Canada [4].

8.3 SAFETY

One uniform result of studies of the effects of chromium supplementation on farm livestock is a lack of observation of toxic effects [1, 2]. Chromium supplementation leads to chromium accumulation, particularly in the liver and kidneys, in species of livestock examined to date (reviewed in reference [6]). At the chromium levels generally used in animal feed, chromium accumulated in the muscle is generally minimal

or negligible, while chromium does not appear to accumulate in cows' milk [6].

8.4 CONCLUSIONS

Overall, chromium nutritional studies with farm livestock display the same heterogeneity of design that plagues similar studies with humans and rodents. As a result, attempting to summarize the studies is most difficult. As a whole, however, chromium supplementation, regardless of species, does not appear to result in changes in body mass or body composition, in agreement with results on rodents and humans. Supplementation has been reported to affect blood insulin, glucose and lipid levels, but the results are not consistent, strongly reminiscent of the results of studies on rodents and humans. The potential effects on reproductive success and unique stresses associated with shipping livestock require more study. Studies associated with shipping are particularly heterogeneous in design. Most of the animal studies have been funded by commercial interests; given the potential bias presented earlier associated with body mass loss and body composition, diabetes and toxicology studies, one must approach this with a sense of caution. Most notable is the lack of demonstration of symptoms of chromium deficiency in livestock or significant improvements in establishing the bioavailability of chromium from chromium supplementation, since the Committee on Animal Research, Board on Agriculture of the National Research Council called for such studies almost 15 years ago.

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9

Toxicology of Chromium(III)

The requirement for large, pharmacological doses of chromium to potentially observe beneficial effects from chromium(III) supplementation obviously raises questions about the safety of introducing such large quantities of a transition metal. Unfortunately, the outcomes of studies on the safety of chromium(III) supplements at first glance are about as confusing as the results of other studies with chromium. Most toxicology studies on chromium supplements have been performed on chromium picolinate, which will be the focus of this chapter. Studies on chromium nicotinate will also be described briefly.

A potential bias of studies on the toxicology of chromium picolinate depending on the relationship of the studies to the supplements supplier has been noted [1] (Table 9.1). The first column lists studies by Nutrition 21 or Richard Anderson, who was an advisor to the Chromium Information Bureau. The Chromium Information Bureau was self-described as a 'non-profit, industry-supported (primarily Nutrition 21) organization created to provide information to nutrition researchers, healthcare providers, the media and consumers on the function, actions and clinical effects of dietary chromium' and was in operation in the latter part of the 1990s. According to the Berkeley Wellness Letter [38], the Bureau was established as part of the response to the first report of potential toxic effects from chromium picolinate by Stearns *et al.* in 1995 [12]. The second column of Table 9.1 includes studies supported by InterHealth Nutraceutical, the provider of the second best-selling chromium supplement ChromeMate and the third column presents other studies. Studies in which detrimental effects from chromium picolinate are observed are italicized. (Note: The author of this chapter has several

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Table 9.1 Investigations of the potential toxicity of chromium picolinate separated by researcher or sponsorship. Papers reporting harmful effects are italicized.

In vitro Studies:		
Nutrition 21	InterHealth	Other
—	—	*Speetjens* et al. [2] *DNA cleavage*
—	—	*Sun* et al. [3] *Generation of ROS*
—	—	*Chaudhary* et al. [4] *DNA cleavage*
—	—	*Yang* et al. [5] *DNA cleavage and ROS generation*
—	—	*Nguyen* et al. [6] *Converted to Cr(V) in blood serum by H_2O_2*
—	—	*Mulyani* et al. [7] *Converted to Cr(V) by 1 mM H_2O_2*
Bacterial Cell Culture Studies:		
Nutrition 21[a]	InterHealth	Other
Esber *et al.* [8] [abstract] Negative in Ames assay	—	Whittaker *et al.* [9] Negative in Ames assay
Mammalian Cell Culture Studies:		
Nutrition 21	InterHealth	Other
Slesinki *et al.* [10] No mutagenicity at hprt locus in CHO (used DMSO as solvent)	*Bagchi et al.* [11] *DNA fragmentation in macrophages*	*Stearns* et al. [12] *Clastogenic damage in CHO*
Gudi *et al.* [13] No chromosomal aberrations in CHO (used DMSO as solvent)	*Bagchi et al.* [14] *DNA fragmentation in macrophages*	*Stearns* et al. [15] *Mutagenic at hprt locus in CHO*
Hininger *et al.* [16] No DNA damage in HaCaT cells	—	*Manygoats* et al. [17] *Mitochondrial damage and apoptosis in CHO*
—	—	*Whittaker* et al. [9] *Mutagenic in mouse lymphoma assay*

Table 9.1 (*Continued*)

Mammalian Cell Culture Studies: Nutrition 21	InterHealth	Other
—	—	*Coryell and Stearnsz* [18] *Mutagenic at hprt locus in CHO*
—	—	*Jana* et al. [19] *Apoptosis in human lymphocytes*
—	—	*Jana et al.* [20] *Autoschizis of human T-cells*
—	—	*Andersson* et al. [21] *DNA damage in human lymphocytes (Integrity of [Cr(pic)3] questioned)*
—	—	*Shrivastava* et al. [22] *Cytotoxicity in human dermal fibroblasts*
—	—	Witmer *et al.* [23] No ROS in A549 cells (used methanol as solvent)
Drosophila Studies:		
Nutrition 21	**InterHealth**	**Other**
—	—	*Hepburn* et al. [24] *Heritable mutations and sterility in* Drosophila
—	—	*Stallings* et al. [25] *Chromosomal aberrations and impeded progeny development*
Mammalian Studies (Intravenous or Intraperitoneal):		
Nutrition 21	**InterHealth**	**Other**
—	—	*Hepburn* et al. [26] *8-OHdG and lipid peroxidation in rats*
—	—	Andersson *et al.* [21] No increase in micronucleus frequency and in DNA damage in comet assay tests in mice (Integrity of [$Cr(pic)_3$] questioned)

(*continued*)

Table 9.1 (*Continued*)

Mammalian Studies (Oral): Nutrition 21	InterHealth	Other
Preuss *et al.* [27] Lower TBARS or no effect in rats	—	Rhodes *et al.* [28] No acute toxicity in rats or mice for 3 months
Anderson *et al.* [29] No acute toxicity in rats	—	*Bailey* et al. [30] *Skeletal defects in offspring of pregnant mice consuming chromium picolinate*
Komoroski *et al.* [31] No chromosome aberrations	—	Kato *et al.* [32] No effect on urine 5-OHuracil levels in humans
—	—	Mozaffari *et al.* [33] Lower urinary 8-OHdG in Zucker obese rats
—	—	Stout *et al.* [34] No effects on survival or neo-plastic lesions in rats or mice for 2 years
—	—	Bailey *et al.* [35] No increased incidence of skeletal defects in offspring of pregnant mice consuming chromium picolinate
—	—	Bailey *et al.* [36] No effects on neurological development of offspring of mice
—	—	*Mahmoud* et al. [37] *Corneal damage in rats*

[a]Published abstracts are included only for Nutrition 21.

studies in the third column. Given that the author is an inventor or co-inventor of patents on potential chromium supplements, one could move these publications to a fourth column; however, this does not change the overall appearance of the table.)

9.1 CHROMIUM PICOLINATE

9.1.1 Ames Assays

Ames assays using chromium picolinate have uniformly been negative [8, 9]. Chromium picolinate is unlikely to penetrate the bacterial

cell membrane in Ames assays, making positive results unlikely even if chromium picolinate were toxic, mutagenic, or carcinogenic.

9.1.2 Cultured Mammalian Cells

In contrast to the Ames assays, studies using cultured mammalian cells have had mixed results, although these can be reconciled. Cell culture and *in vitro* assays of the genotoxicity has been reviewed recently by Stearns [39]; this review excellently describes the chemistry and biology behind the various cell culture and *in vitro* assays and should be consulted for more details in these areas.

The first study to raise concerns about potential toxic effects, by Stearns and co-workers [12], demonstrated, using Chinese hamster ovary (CHO) cells, that chromium picolinate as a solid suspension in acetone or the mother liquor from the synthesis of chromium picolinate (before the compound precipitates from solution) caused chromosomal aberrations. Equivalent concentrations of picolinic acid had no effect. Subsequent studies by this group have shown that chromium picolinate is mutagenic at the *hprt* locus in CHO cells [15] and generates mitochondrial damage and apoptosis [17]. Recent studies in CHO cells by Slesinski and co-workers failed to observe chromosomal aberrations or mutagenicity at the *hprt* locus [10, 13]. These workers used DMSO (dimethylsulfoxide) rather than acetone (as did Stearns and co-workers) as a solvent for $[Cr(pic)_3]$ (as the compound has a solubility of only 600 μM in water). These apparently contradictory experiments are actually a case of apples and oranges. The use of the DMSO solvent as opposed to acetone explains the discrepancies; DMSO is a radical scavenger, which could quench reactive oxygen-based species (ROS) (see below). Coryell and Stearns [18] have demonstrated this quenching by DMSO. They also found that substitutions comprised 33% of $[Cr(pic)_3]$-derived DNA mutations, with transversions being predominant; 62% were deletions with one-exon deletions predominating. Insertions of 1–4 base pairs comprised 5% [18]. $[Cr(pic)_3]$ is clearly mutagenic in CHO cells.

In murine macrophages, Bagchi and co-workers have observed increases in cytochrome c reduction and DNA fragmentation from chromium picolinate, although the increases were not much larger than when using chromium nicotinate [11, 14]. A study by the Food and Drug Administration and National Cancer Institute [9] found chromium picolinate to be mutagenic in murine lymphoma. Jana and co-workers found $[Cr(pic)_3]$ to give rise to apoptosis in human lymphocytes [19] and

autoschizis in human T-cells [20]. The apoptosis apparently resulted from the generation of ROS and mitochondrial damage [19]; events accompanying the autoschizis included an increase in intracellular ROS and loss of mitochondrial membrane potential [20]. The damage was concentration dependent. Andersson *et al.* in 2007 observed concentration dependent DNA damage from the supplement in human lymphocytes using Comet assays [21]. No effect was observed if the $[Cr(pic)_3]$ was dissolved in DMSO in either human lymphocytes or L5178Y mouse lymphoma cells, effectively confirming the results of Stearns and co-workers. Curiously, using serum to dissolve the $[Cr(pic)_3]$ also eliminated the effects on the lymphocytes. One wonders whether the compound decomposed in the serum before being added to the assay. This paper also contains a note that ICN was the source of the $[Cr(pic)_3]$ and that the integrity of the $[Cr(pic)_3]$ was not checked by the authors [21]; $[Cr(pic)_3]$ sold by ICN had previously been shown in 2002 to not actually be $[Cr(pic)_3]$ [40]. Shrivastava *et al.* determined the compound was more cytotoxic to human dermal cells than $CrCl_3$ [22].

Two additional studies have failed to detect deleterious signs from $[Cr(pic)_3]$ treatment of cultured mammalian cells. Witmer *et al.* observed no reactive oxygen species being generated in A549 cells [23]. However, they dissolved the $[Cr(pic)_3]$ in methanol, another potential radical trap, readily explaining this discrepancy. Hininger *et al.* found pre-treatment of human HaCaT keratinocytes with $[Cr(pic)_3]$ followed by exposure to H_2O_2 resulted in protective effects against oxidative DNA damage as measured in Comet assays [16]. No cytotoxic effects were observed when cells were exposed to 120 μM $[Cr(pic)_3]$ and production of 8-OHdG was decreased [23]. This study is clearly at odds with the other reports.

Thus, when the use of DMSO and methanol as solvents is accounted for, mammalian cell culture studies consistently (with a single exception) point toward DNA damage and mutagenicity from chromium picolinate.

9.1.3 *Drosophila* Studies

However, these studies suggesting the ability of chromium picolinate to damage DNA are only significant if the body is unable to repair this damage; these concerns have been examined by Hepburn and coworkers using *Drosophila* as a model organism [24, 25]. The ability of chromium picolinate, chromium nicotinate, $CrCl_3$, chromium(III) tris(2,2′-bipyridine), $[Cr(bpy)_3]^{3+}$, (a known mutagen) [41], and Cr3

and their ligands to generate developmental delays and decreases in success rates of hatching and eclosion have been examined. 2,2′-Bipyridine and $[Cr(bpy)_3]^{3+}$ were acutely toxic. Of the other compounds at 260 μg Cr/kg food, or the equivalent if the Cr complexes dissociated releasing the ligands, only chromium picolinate and to a lesser extent picolinic acid resulted in appreciable delays in development of larvae and adults and lower success rates in pupation and eclosion; this Cr dosage is approximately equivalent to a human consuming one 200 μg Cr-containing supplement a day [24]. This was observed independent of whether the compound was prepared in the laboratory or by Nutrition 21. Additionally, of the non-toxic compounds, only chromium picolinate and to a lesser extent picolinic acid had detrimental effects on the longevity of male and female *Drosophila* at the nutritionally relevant dosage. Thus, $[Cr(pic)_3]$ and picolinic acid have detrimental effects on essentially all stages of the life cycle of *Drosophila*. Because of these effects, the ability of chromium picolinate to generate heritable mutations and dominant female sterility were probed [24]. At the same dosage, $[Cr(pic)_3]$ was found to generate approaching one mutation per chromosome per individual and 12% sterility.

The ability of $[Cr(pic)_3]$ to generate chromosomal aberrations in polytene chromosomes of the salivary glands of *Drosophila* larvae was also examined. In the chromium picolinate-treated group, 53% of the identified chromosomal arms were positively identified as containing one or more aberrations, while no aberrations were observed for the identified chromosomal arms of the control group [25].

9.1.4 Mammalian Studies (Intravenous or Intraperitoneal)

Hepburn and Vincent and co-workers intravenously injected rats with 5 μg of chromium as chromium picolinate daily for 60 days [26]. For a 65 kg human and assuming 2% absorption of orally consumed $[Cr(pic)_3]$, this would be equivalent to a human taking over 30 mg of Cr per day. Supplementation resulted in significant increases in urinary 8-hydroxydeoxyguanosine (8-OHdG), a product of oxidative damage DNA damage, in urine commencing after 32 days of treatment. At the end of the 60 day study, 8-OHdG levels were significantly greater in the liver and kidney tissue. Additionally, levels of lipid peroxidation in the tissues were significantly increased, which can in turn lead to DNA and chromosome damage [42]. However, this study only indicates

potential toxic effects from an upper limit of chromium picolinate intake assuming 100% absorption as the intact compound.

Andersson *et al.* [21] have shown that a single intraperitoneal injection with up to 3 mg/kg body mass of $[Cr(pic)_3]$ to mice does not result in a change in frequency of micronucleated polychromatic erythrocytes in peripheral blood or detectable DNA damage by Comet assay in lymphocytes or hepatocytes. This work is part of the study above in which authenticity of the $[Cr(pic)_3]$ used might be questionable.

9.1.5 Mammalian Studies (Oral)

In contrast to the studies described above, *in vivo* studies with orally administered chromium picolinate almost universally result in no detectable deleterious effects from the supplement. The focus here is rodent and human studies where certain toxic effects were specifically examined (Table 9.1). In addition to these studies, numerous nutritional and pharmacological studies have been performed in which no deleterious results from chromium supplementation were noted.

Studies done by Anderson *et al.* [29] demonstrated that $[Cr(pic)_3]$ is not an acute toxin. Four-week old rats were fed diets containing up to 1000 mg Cr as $[Cr(pic)_3]$ per kg diet for 24 weeks. Histological evaluation of liver and kidney tissues revealed no effects from the supplement. However, Cr concentrations in the liver and kidney increased linearly with the amount of Cr in the food [29]. The dosage used by Anderson was equivalent to human consumption of 750 mg of Cr per day, ~25 000-fold more than the AI. The results of Rhodes *et al.* are entirely consistent with those of Anderson and co-workers. Rats and mice were fed diets containing 80, 240, 2000, 10 000, or 50 000 ppm chromium picolinate monohydrate for 13 weeks. The supplement had no effects on body mass or composition at these enormous dosages (consistent with results described above), and no changes were observed in haematology and clinical chemistry parameters monitored [29]. The most definitive study has been performed by Stout *et al.* [34]. Male and female rats and mice were provided diets containing up to 5% chromium picolinate for 2 years. $[Cr(pic)_3]$ had no effect on body mass, food intake, survival, or non-plastic lesions. A statistically significant increase in the incidence of preputial gland adenoma in male rats at a diet concentration of 1% was considered as an equivocal finding. Similar results (in terms of body mass, food intake and survival) were obtained in a similarly designed study that ran for 3 months [34].

Studies have been performed which specifically looked for ROS dependent DNA damage generated by [$Cr(pic)_3$] supplementation. In a 56 day study, ten obese women were given 400 μg of [$Cr(pic)_3$] daily. Supplementation had no effect on 5-hydroxymethyl uracil, a product of oxidative DNA damage [32]. [$Cr(pic)_3$] treatment of Zucker obese rats has been found to reduce elevated urinary 8-OHdG levels and improve markers of inflammation [33]. Rats received either 0.19 or 0.41 mg Cr/kg body mass for 20 weeks. Preuss *et al.* [27] have also examined the effect of $Cr(pic)_3$ on lipid peroxidation in rats. Spontaneously hypertensive rats were fed a diet containing 5 mg/kg 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. 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. Given the short lifetime of [$Cr(pic)_3$] in the cells and the instability of peroxidized lipids, termination of the supplementation could have allowed for the repair/replacement of any damaged cellular components making interpretation difficult.

Komorowski *et al.* of Nutrition 21 have examined the effects of a single dose of chromium picolinate (33, 250, or 2000 mg [$Cr(pic)_3$]/kg body mass) on bone marrow cells of Sprague–Dawley rats [31]. No DNA damage was observed. Providing 200, 800, 1600, or 3200 mg Cr/kg diet as [$Cr(pic)_3$] to growing finishing pigs resulted in increased serum superoxide dismutase and kidney catalase at the highest concentrations but no effects on malondialdehyde in tissues and blood serum, urinary 8-OHdG and DNA strand breaks in liver and kidney [43]. Mahmoud *et al.* have found that rats receiving 15 mg [$Cr(pic)_3$]/kg body mass for 8 weeks had lower cornea glutathione levels and higher malondialdehyde levels and morphological and histological changes in the cornea; 8 mg/kg doses had no effects [37]. Reproducing this study could be interesting.

Finally, developmental studies have also been performed using orally dosed mammals. Rasco and co-workers have recently reported cervical arch defects in the offspring of pregnant mice fed a diet containing 200 mg Cr/kg diet as chromium picolinate but not as $CrCl_3$ [30]. A similar trend was observed in a subsequent study, but the effect was not statistically significant [35]. These researchers have also found no

statistically significant effects of chromium picolinate or picolinic acid supplementation of mated mice on neurological effects of offspring [36]. Feeding a diet containing [$Cr(pic)_3$] to breeding male mice also has been recently shown not to lead to any skeletal defects in offspring [44].

Isolated incidents of deleterious effects of [$Cr(pic)_3$] supplementation of humans have been reported: weight loss, anaemia, thrombocytopenia, liver dysfunction and renal failure [45]; renal failure [46]; rhaddomyolysis [47]; dermatitis [48]; acute, short-lasting cognitive, perceptual and motor changes [49]; exanthematous pustulosis [50]; and hypoglycaemia [51]. The significance of these isolated incidents is difficult to ascertain, especially given the lack of notes of isolated incidences in the clinical trials using chromium picolinate (see Chapters 4 and 5).

9.1.6 Neurological Effects

A potential beneficial effect of chromium picolinate on antidepressant pharmacotherapy for dysthymic disorder has been reported [52, 53]. Chromium picolinate has also been reported to lower cortisol response to 5-hydroxytryptophan precursor [54]. These reports and others discussed in Chapter 5, in combination with the isolated report of [$Cr(pic)_3$] possibly causing perceptual and motor changes, may potentially be concerning. Picolinic acid/picolinate is a natural catabolite of the amino acid tryptophan, generated as an end product in the kynurenine pathway in the body [55]. Molecules generated along this pathway tend to have neurological effects [56]. This raises concerns about [$Cr(pic)_3$] in the body as the [$Cr(pic)_3$] should deliver and release three picolinates (or its degradation products) [57] per Cr. Thus, effects on perceptual and motor function and dysthymic disorder could potentially be related to picolinate released in the body; this is an area that requires more investigation as recommended in Chapter 5.

9.1.7 *In Vitro* Studies

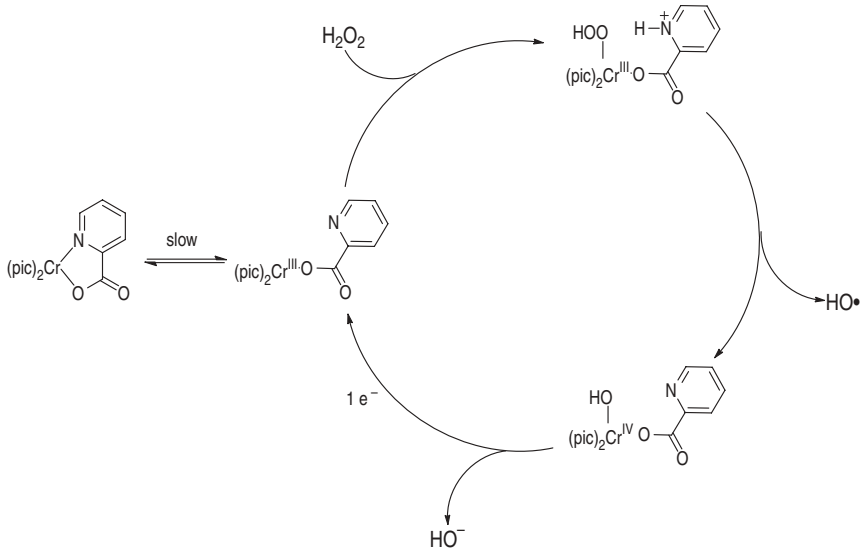
In vitro studies testing whether chromium picolinate can generate high valent chromium species, reactive oxygen species and oxidative DNA damage are uniformly positive (Table 9.1). An *in vitro* study by Speetjens *et al.* [2] used physiologically relevant concentrations of chromium picolinate (as low as 120 nM) and demonstrated the compound's ability to

cause single stranded breaks in pUC19 plasmid DNA in the presence biological reductants, such as ascorbate and thiols, and air. In the presence of 5 mM ascorbate (the approximate concentration in cells) chromium picolinate catalytically generated ROS , such as possibly hydroxyl radicals. Individually, equivalent concentrations of picolinic acid and Cr^{3+} (as $CrCl_3$ or Cr3) did not generate clastogenic damage in the presence of biological reductants [2]. This indicates that the catalytic reaction does not occur with all Cr(III) compounds and appears to be ligand specific. These findings are consistent with earlier results that showed mutagenic forms of Cr^{3+} possessed imine chelating ligands (bipyridyl and phenanthroline) that also cause damage in the presence of dioxygen [41].

DNA cleavage by $[Cr(pic)_3]$ has also been observed by two other groups [4, 5]. These ligands can alter the redox potential of the chromium(III) centre so that the chromium becomes susceptible to redox chemistry in the presence of biological oxidizing and reducing agents. While original proposals were that the Cr(III) was reduced by a reducing agent such as ascorbate to Cr(II), which is susceptible to oxidation by dioxygen, generating reactive oxygen species and high valent Cr (i.e. Cr(IV) and/or Cr(V)) [2, 41], the redox potential of chromium picolinate (with three imine ligands) is not as shifted as the compounds with a larger number of imine ligands, such that it should not be susceptible to reduction by thiols or ascorbate (see Chapter 4). In these studies the interaction of biological reducing agents, particularly ascorbate, with dioxygen probably generates reactive oxygen species, which interact with the chromic centre of chromium picolinate generating more ROS (including hydroxyl radical) and high valent chromium species (Scheme 9.1); the interaction of these species with DNA generates the oxidative damage and cleavage. Thus, in a test-tube in the presence of air and an appropriate reducing agent or in the presence of a sufficient concentration of peroxide, chromium picolinate can give rise to reactive species capable of generating oxidative damage, including DNA cleavage.

9.1.8 Reconciling *In Vitro* and *In Vivo* Studies

Now the results in the various studies with chromium picolinate can be reconciled. When chromium picolinate is intact in the presence of appropriate reductants and oxidants, oxidative damage is expected. Thus, chromium picolinate dissolved in water or buffer in a test-tube is susceptible to generating this type of damage. The compound does not cause



Scheme 9.1 Proposed mechanism of hydroxyl radical generation by $[Cr(pic)_3]$ in the presence of peroxide.

damage to bacterial cells in Ames assays because it probably cannot diffuse into the cells.

Mammalian cells with less robust outer membranes allow the diffusion of chromium picolinate into the cells; thus, the mammalian cell culture studies almost uniformly observe oxidative damage or DNA damage. Similarly, if chromium picolinate is administered intravenously, the intact complex has the ability briefly to diffuse into cells and generate damage. In contrast, when given orally to rodents and humans (as discussed in Chapter 4), chromium picolinate readily breaks down in the gastrointestinal tract so that only ~1% of the small amount of absorbed Cr exists as intact chromium picolinate. Thus, when administered orally, the animal is fortuitously not exposed to the form capable of entering into the redox chemistry leading to oxidative damage and DNA cleavage. In the very different digestive system of fruit flies, the complex would appear to be absorbed intact so that it is a potent clastogen, mutagen and developmental toxin. Curiously, a daily dose of 200 or 400 μg Cr as $[Cr(pic)_3]$ to calves has been shown to result in increased lymphocyte micronucleus frequency, malondialdehyde levels and frequency of apoptotic cells [58]. Does chromium picolinate decompose in the ungulate digestive tract similar to in those of rodents and humans or could it remain intact?

Potentially related to this are the results of a recent study which demonstrated that [$Cr(pic)_3$] associates with the lipid interface in reverse-micelle model membranes [59]. Additionally, when added to rat basophilic leukaemia (RBL-2H3) cells, [$Cr(pic)_3$] decreased plasma lipid membrane order along with increasing association of native insulin receptor and phosphorylated IRS-1 and Akt with detergent-resistant membrane microdomains of characteristically high buoyancy [59]. This is consistent with previous reports of the compound increasing membrane fluidity *in vitro* [60]. These effects are probably attributable to the association of the neutral, somewhat hydrophobic intact molecule associating with membranes. Thus, the relationship of these effects to *in vivo* studies is of questionable relevancy [59].

9.2 CHROMIUM NICOTINATE

As reports of potential deleterious effects from chromium picolinate appeared in the mid-2000s, InterHealth began a series of studies to examine the toxicity of chromium nicotinate [61–64]. Previous studies with cultured macrophages had already shown DNA damage from chromium nicotinate, although the extent of damage was less than that resulting from similar amounts of chromium picolinate [11, 14]. The acute oral LD_{50} was found to be greater than 5000 mg/kg; the compound had no effect in Ames assays or in gene mutation tests in mouse L5178Y lymphocytes [61]. Chromium nicotinate in the diet up to 125 ppm Cr/kg food for 9 months had no effects on hepatic lipid peroxidation or DNA fragmentation or on tissue masses, body mass, food intake, or water intake [61].

Providing chromium nicotinate in food at 25 ppm for 52 weeks to male and female Sprague–Dawley was reported to have no effect on hepatic lipid peroxidation or DNA fragmentation or on a variety of haematological or clinical chemical parameters [62]. Body mass gain was lower in both males and females; this was interpreted positively but could be a sign to the contrary. Two additional studies looked at developmental toxicity in Sprague–Dawley rats [63,64]. Chromium nicotinate (60 ppm diet) when provided to adults prior and during breeding and to females during gestation and lactation across two generations had no effects on sexual maturity, fertility and mating, litter properties and development [63]. In another similar study, no effects were noted on parameters associated with pregnancy or on embryo-foetal defects [64]. Additionally, no effects from chromium nicotinate or nicotinic acid have been

observed on the development of *Drosophila*, in contrast to chromium picolinate [25].

9.3 Cr3/KEMTRACE

Cr3 has an oral LD_{50} greater than 2 g/kg body mass in male and female rats, measured using the OECD procedure [65]. In female Wistar rats receiving 100 mg Cr/kg body mass as Cr3 in the diet for 4 weeks, no effects in body mass, organ mass, or feeding efficiency were observed, while Comet assays of lymphocyte DNA revealed no damage [66]. When given orally in doses up to 10 mg Cr/kg body mass to male Sprague–Dawley rats daily for 10 weeks, no deleterious effects were observed [67]. Developmental toxicology studies in both rats [68] and mice [35] observed no deleterious effects. Similarly, in contrast to chromium picolinate and picolinic acid, no developmental delays or changes in hatching or eclosion success have been observed in *Drosophila* with Cr3 or corresponding concentrations of propionate [25]. Because the amount of propionate delivered by administering the supplement at nutritional or pharmacological doses is small compared with amount of propionate generated by bacterial fermentation of fibre in the gastrointestinal tract, beneficial or deleterious effects from propionate should not be anticipated; an attempt to observe effects in rats from propionate at a dose equivalent to taking 1000 mg Cr/kg body mass as Cr3 daily for 24 weeks observed no effects [69].

Lay and co-workers have shown that Cr3 can react with hydrogen peroxide (0.1–5 mM) to form high valent chromium species [6,7], which is not surprising as Vincent and co-workers had previously shown that its acetate analogue was susceptible to such oxidation [70]. However, compared with $[Cr(pic)_3]$, Cr3 is poor at cleaving DNA *in vitro* [71]. DNA cleavage by the cation required 120 μM Cr3 in the presence of 215 μM hydrogen peroxide. Cr3 could not cleave DNA in the presence of a physiological concentration of ascorbic acid (5 mM). Thus, at anything approaching physiological conditions, Cr3 did not produce sufficient amounts of either high valent chromium or ROS to cleave detectable amounts of DNA [71].

9.4 CONCLUSIONS

This survey reveals that at nutritional levels and at the pharmacological level used to date that chromium(III) supplementation appears to be

safe. However, as healthy individuals appear to derive no benefit from chromium(III) supplementation, any risk from supplementation would appear to be unjustified. The biochemistry and chemistry of the ligands to the chromic centre (e.g. potential neurological effects from picolinic acid) should be considered as well when considering these supplements. If chromium supplements are convincingly demonstrated in the future to have beneficial effects on animals or humans under particular stresses, then benefits could outweigh the limited potential risks.

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Conclusion

This review of the literature on the biochemistry of chromium(III) lends itself to several conclusions:

(1) At present chromium cannot be considered as an essential element as (i) nutritional data demonstrating chromium deficiency and improvement in symptoms from chromium supplementation are lacking and (ii) no biomolecules have convincingly been demonstrated to bind chromium and have an essential function in the body. The next review of the status of chromium by the Committee on the Scientific Evaluation of Dietary Reference Intakes of the National Academies of Science (USA) must seriously consider revising its status.
(2) The Federal Trade Commission (USA) in 1997 properly stopped claims of body mass loss and changes in body composition from chromium picolinate supplementation as subsequent research has verified that chromium supplementation does not affect body mass or body composition. No beneficial effects have been demonstrated from chromium supplementation by healthy individuals.
(3) The American Diabetes Association position to not recommend chromium(III) supplementation for people with type 2 diabetes or obesity is appropriate given the scientific literature that lacks consistent and reproducible outcomes. Future studies need to be more carefully designed including in terms of number of subjects and amount of chromium being utilized; to use well characterized

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chromium(III) compounds; and to examine whether particular subgroups of type 2 diabetic subjects are likely to benefit from chromium supplementation.

(4) No molecular level mechanism has been clearly established for explaining how chromium could have a beneficial role in carbohydrate and lipid metabolism. Similarly, key details in the transportation, distribution and elimination of chromium(III) from the body at a molecular level are lacking. To address this situation, inorganic chemists, biochemists, nutritionists and medical researchers need to have greater collaboration when designing and performing experiments. For example, an understanding of exactly what form of chromium the cultured cells are exposed to in experiments is crucial to trying to reconcile the conflicting results on the effects of chromium on the insulin signalling cascade. Progress in understanding how chromium could potentially influence carbohydrate and lipid metabolism and using this information to possibly influence the health of humans and animals will not occur until the heterogeneity of results can be reconciled. Recent studies on LMWCr may be a start to finally elucidating the mechanism of chromium action in enhancing insulin sensitivity.

(5) Chromium(III) supplementation appears to be safe at levels currently used in nutritional supplements and in pharmacology studies, in line with assessments by the Food and Drug Administration (USA) and European Food Safety Authority. However, as no benefit has been demonstrated for chromium supplementation of healthy individuals, any potential risk from supplementation would appear to outweigh potential benefits. Risk benefit analysis is more difficult for animals under stress (including type 2 diabetics). Further research is required before proper assessment of the potential risks versus benefits of chromium supplementation can be performed in these cases. Future clinical trials may require the use of larger doses of chromium(III); these trials will require careful monitoring of possible deleterious effects from supplementation.

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