Project Report

[^{14}C]Chromium Picolinate Monohydrate: Disposition and Metabolism in Rats and Mice

SUBMITTED TO:

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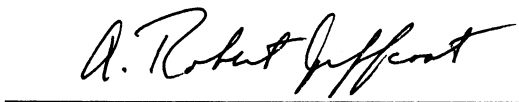

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Summary

Chromium picolinate (chromium trispicolinate, chromium tripicolinate, Cr3P) is a synthetic compound that has widespread use as a nutritional supplement. Chromium (Cr) is an essential element to life, being required for proper glucose metabolism and potentiation of the action of insulin. Cr3P is claimed to have enhanced bioavailability of chromium compared with chromium from dietary sources although Gargas et al. reported that the extent of absorption of Cr3P by adult volunteers was still low ($2.8 \pm 1.1\%$). Although Cr3P toxicity has not been found in in vivo studies, in vitro studies have shown that Cr3P produces chromosomal aberrations in Chinese hamster ovary cells and that Cr3P is reduced by biological reductants in vitro, ultimately leading to the generation of hydroxyl radicals and the cleavage of DNA. The goal of our studies was to determine the disposition of both the chromium and the organic portions of Cr3P in rats and mice to aid in interpretation of prior studies and as an aid in planning any future toxicity studies.

Incubations of Cr3P with rat stomach and small intestine contents demonstrated that Cr3P was stable to both during the time intervals that Cr3P could be expected to be present in the stomach and small intestine following oral dosing. Urinary excretion of radiolabel (between 42 to 56% and 26 to 42% of the administered dose for rats and mice, respectively) following oral doses of [^{14}C]Cr3P to rats and mice was nearly all in the form of a single metabolite that has been identified as N-picolinoylglycine (NPG). Urinary excretion of Cr was much lower than urinary excretion of radiolabel with only 1.3 and 1.5% of the administered dose in rats and less than 5% of the administered dose in mice being excreted in urine for orally dosed animals. For rats, 98% of the Cr was excreted in feces. The stability of Cr3P to gut contents coupled with the absorption of the organic portion of Cr3P, but not the chromium, led to the conclusion that most of the Cr3P is broken down in or near the intestinal wall. The organic portion is then carried into the systemic circulation where it is conjugated with glycine and excreted in urine. Almost all of the chromium is returned to (remains in) the intestinal tract and is excreted in feces.

As has been suggested by Gonzalez-Vergara, et al. for chromium nicotinate, small quantities of Cr3P are absorbed into the systemic circulation intact. Pharmacokinetic parameters were estimated for this material by comparing iv and oral doses of Cr3P. The terminal elimination half-life of Cr3P after oral administration (153 min) is approximately six times longer than that observed following iv administration (24 min). The longer half-life after oral administration is likely due to very slow absorption of Cr3P from the gut. The proportion of total radiolabel in blood attributable to Cr3P is much lower following oral administration as compared with iv administration of Cr3P, further evidence that Cr3P is degraded in the gut prior to adsorption of the radiolabel.

Rats dosed orally with [^{14}C]Cr3P water slurry at 17.4 mg/kg had 90, 87, and 78%, respectively, of the ^{14}C-dose in their GI tract at 1, 2, and 4 h postdose. Blood, adipose, liver, and muscle from these animals were analyzed for total radioactivity, Cr content and for three specific compounds: Cr3P, picolinic acid (Pcl-H), and NPG. These three compounds accounted for 70–90% of the radiolabel present in the acetonitrile extracts of the tissues. Of the tissues analyzed, liver had the highest concentration of Cr3P at

all time points. Adipose, blood, and liver taken from these animals had concentrations of chromium that were 2-6 times those of undosed animals. Chromium concentrations in these tissues were higher than that attributable to the concentrations of Cr3P in the same tissue samples. This result showed that small amounts of chromium are delivered to tissues by oral doses of Cr3P that are not eliminated from the tissues as Cr3P. Since almost all of the reported toxicities of Cr3P have been based on results of in vitro experiments, the knowledge that tissues are actually exposed in vivo to small amounts of intact Cr3P and much larger amounts of its metabolites is important in assessing the relevance of these in vitro studies.

Since Cr3P appears to be metabolized almost completely through Pcl-H, the disposition of [^{14}C]Pcl-H was briefly investigated. Rats dosed orally with [^{14}C]Pcl-H in water at 17.2 mg/kg excreted an average of 83% of the ^{14}C-dose in urine and less than 1% in feces in 6 h. A total of 1% and less than 3% of the ^{14}C-dose administered were found in the nondigestive and digestive tissues, respectively, at 6 h postdosing. A second group of rats also dosed orally with [^{14}C]Pcl-H in water at 17.2 mg/kg excreted an average of 93% of the ^{14}C-dose in urine, 1.7% in feces, and 0.3% as CO_2 in breath in ca. 20 h. The major radioactive peak found in the urine of rats receiving [^{14}C]Pcl-H orally coeluted with NPG, the major urinary metabolite of [^{14}C]Cr3P.

Project Report

on

[^{14}C]Chromium Picolinate Monohydrate:
Disposition and Metabolism in Rats and Mice

RTI Protocol No. RTI-6855-15

Research Triangle Institute
P.O. Box 12194
Research Triangle Park, NC 27709

Study Initiated (protocol signed): September 24, 1999
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to

National Institute of Environmental Health Sciences
Post Office Box 12233
Research Triangle Park, NC 27709

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[a] B = Biologist, C = Chemist; S = Secretary; SD = Study Director, V = Veterinarian
[b] Ex = Conduct of Experiment; R = Preparation of Report

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1.0 INTRODUCTION

Chromium picolinate monohydrate (chromium picolinate, chromium trispicolinate, chromium tripicolinate, Cr3P) is a synthetic compound that has widespread use as a nutritional supplement. Chromium picolinate monohydrate is often referred to as chromium trispicolinate. Two other chromium picolinate complexes have been reported (Evans and Pouchnik, 1993), chromium monopicolinate (Cr1P) and chromium bispicolinate (Cr2P). For clarity, the term chromium trispicolinate will be used when it is known that there are three picolinic acid ligands per atom of chromium.

Chromium (Cr) is an essential element to life, being required for proper glucose metabolism and potentiation of the action of insulin (for reviews see Schroeder, 1968; Mertz, 1969; National Research Council, 1989; USEPA, 1984; Kahn et al., 1990; Gargas, et al, 1994). Only 2–3% of dietary chromium is absorbed systemically (Anderson et al., 1993; Gargas et al., 1994). Because of this low systemic bioavailability of chromium from dietary sources, forms of chromium having higher bioavailability were sought. Cr3P is claimed to be such a compound although Gargas et al. (1994) reported that the extent of absorption of Cr3P by adult volunteers was $2.8 \pm 1.1\%$.

The preparation and use of picolinate complexes of essential metals are covered by USDA patents #4,315,927 (Evans, 1982) and reissue #33,988 (Evans, 1992). Gary W. Evans is listed on the patent and its reissue as the sole inventor. The patent rights have been leased to Nutrition 21, a subsidiary of AMBI. No longer at USDA, Dr. Evans continues as a major proponent of the use of Cr3P, touting its purported beneficial effects for fat loss, for hypoglycemia, for lower cholesterol, for muscle mass, for insulin control and for longer life (Evans, 1996). Evans (1996) also states that “If the product (chromium picolinate) you buy doesn’t have one of these [patent] numbers (as listed above), it may be completely ineffective.” A recent report (McLeod et al., 1999) suggests that nicotinate and picolinate complexes of Cr may have antidepressant activity in humans.

Anderson et al. (1997) administered Cr3P to rats in their diet over 20 weeks and at concentrations of 5, 25, 50, and 100 μg Cr/g diet. No toxicity was observed. At the end of the treatment period, concentrations of Cr in the livers were proportional to the concentrations of Cr in the diet; rats fed diets containing 5 and 100 μg Cr/g diet had concentrations of Cr in livers of 26 and 542 ng Cr/g dry weight, respectively. Cr concentrations in kidneys were 4–5 times those measured in livers (Anderson et al., 1993), possibly because absorbed Cr is excreted almost entirely in urine.

In another study Anderson et al. (1996) incorporated Cr3P, chromium (tris?)nicotinate (Cr3N), or complexes of Cr^{+3} and mixtures of several amino acids into rat chow at concentrations of 5 μg Cr/g diet. Groups of weanling rats were then fed these diets for 3 weeks. Concentrations of Cr in liver following exposure to diets containing no added Cr, Cr3P, or Cr3N were 4, 50, and 13 ng/g dry weight whereas concentrations of Cr in kidneys from the rats were 23, 368, and 166 ng/g dry weight, respectively. In heart and leg muscle, Cr concentrations above those of controls were found in animals treated with Cr3P,

but not with Cr3N. It thus appears that exposure to Cr3P and Cr3N leads to somewhat different patterns of Cr concentrations in tissues.

In an in vitro assay with Chinese hamster ovary cells, Cr3P, but not Cr3N, produced chromosomal aberrations that were 3 to 18 times that of controls (Stearns et al., 1995). Speetjens, et al., (1999) have shown that Cr3P is reduced by biological reductants in vitro, ultimately leading to the generation of hydroxyl radicals and the cleavage of DNA.

The purposes of the present study are to determine the stability of Cr3P to gastrointestinal conditions, the absorption of Cr3P into the systemic circulation, and the fate of both the Cr and the picolinate parts of Cr3P.

2.0 STUDIES PERFORMED

- Analysis of test chemicals
- Determination of solubilities of Cr3P in water and selected organic solvents
- Analysis of Cr3P dietary supplements
- Incubation of [^{14}C]Cr3P with rat stomach and small intestine contents
- In vivo pilot studies with [^{14}C]Cr3P administered to male rats
- 15.3 mg/kg definitive oral excretion study with male rats (Study A).
- 0.22 mg/kg definitive oral excretion study with male rats (Study D).
- 0.18 mg/kg iv blood and tissue distribution study with male rats (Study Ha).
- 17.4 mg/kg oral excretion study with male rats (Study Ba).
- 17.2 mg/kg [^{14}C]picolinic acid (Pcl-H) oral excretion study with male rats (Study PAa).
- 17.8 mg/kg oral blood study with male rats (Study Hb).
- 17.4 mg/kg oral tissue study with male rats (Study F).
- Isolation and identification of urinary metabolite.
- 17 mg/kg [^{14}C]Cr3P or 9 mg/kg [^{14}C]Pcl-H oral study with male rats; binding to blood proteins, (Study T).
- 20.5 mg/kg oral excretion study with male mice (Study J).
- 19.0 mg/kg oral excretion study with male mice (Study Jb).

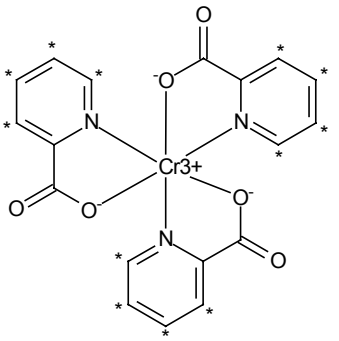
3.0 MATERIALS AND METHODS

3.1 Test Chemical

3.1.1 Chemical Structure of Cr3P

Molecular Formula: $C_{18}H_{12}CrN_3O_6 \cdot H_2O$

Formula Weight: 436.32



3.1.2 Nonradiolabeled Cr3P

Nonradiolabeled Cr3P was obtained from ICN Biomedicals Inc. (hereafter referred to as ICN), lot #0061974 (this material was shown not to be Cr3P), and from Spectrum Quality Products, Inc. (hereafter referred to as Spectrum), lot #NG0569. Aliquots of each lot were examined by HPLC using HPLC System 1 described in Section 3.8, mass spectrometry, and NMR.

In addition, the Cr^{+6} and total chromium contents were determined for each lot. (No Cr^{+6} should be present. Cr^{+3} content is calculated as the difference between total chromium and Cr^{+6} content.) Samples of the Cr3P standards, ICN lot #0061974 and the Spectrum lot #NG0569, were dissolved in distilled water to produce solutions with Cr3P concentrations of 0.0909 mg/mL and 0.0709 mg/mL, respectively. For Cr^{+6} determinations, samples were diluted with deionized water and analyzed by ion chromatography with post column colorimetric detection (IC-PCR). In this mode, the eluting Cr^{+6} ion is reacted with diphenylcarbazide and the absorbance of the resulting complex measured at 530 nm. For measurement of total Cr, the samples were diluted with dilute nitric acid and assayed by graphite furnace atomic absorption (GFAA) directly.

3.1.3 [^{14}C]Cr3P

Radiolabeled Cr3P, labeled with ^{14}C at multiple sites in the aromatic rings, was synthesized by Wizard Laboratories at a specific activity of 52.0 mCi/mmole of Cr3P (17.3 mCi/mmole of each of the three picolinate ligands). The product data sheet is presented in Figure 1. The [^{14}C]Cr3P was supplied as a neat solid.

The radiochemical purity of the [^{14}C]Cr3P was determined by HPLC. Following injection of [^{14}C]Cr3P, the column effluent was collected in fractions. The radioactivity eluting in each fraction was measured by liquid scintillation spectrometry (LSS). The identity of the [^{14}C]Cr3P was tentatively confirmed by comparison of its retention time in HPLC System 1 with that of the Cr3P standard purchased from Spectrum. Wizard Laboratories also used Cr3P purchased from Spectrum as their standard for establishing the identity of the [^{14}C]Cr3P.

3.1.4 Picolinic Acid (Pcl-H)

Name: Picolinic acid (2-pyridinecarboxylic acid, Pcl-H)

CAS Registry Number: 98-98-6

Molecular Formula: $C_6H_5NO_2$

Formula Weight: 123.11

3.1.5 [^{14}C]PcI-H

Source: Wizard Laboratories

Specific Activity: 17.3 mCi/mmole

Lot No.: 990419

3.2 Cr3P Solubilities in Water and in Selected Organic Solvents

The solubilities of Cr3P from Spectrum were determined in the following solvents: water acidified to pH 3 with hydrochloric acid, water at pH 7, 30:70 (v:v) methanol:water, 50:50 (v:v) methanol:water, methanol, propylene glycol, acetone, ethyl acetate, 2-propanol, tetrahydrofuran and methyl t-butyl ether. Saturated solutions of Cr3P were prepared in each solvent. Following filtration through a 0.45 μM filter, each solution was diluted and analyzed by HPLC using HPLC System 2 described in Section 3.8. Standard solutions of Cr3P in 30:70 (v:v) methanol:water were also prepared and analyzed along with the solvent samples to generate a calibration curve.

3.3 Synthesis of Cr1P, Cr2P, and Cr3P

Synthesis of chromium picolinate complexes was attempted according to the methods of Evans and Pouchnik (1993),Anderson, et al. (1996), and Stearns and Armstrong (1992). Reaction mixtures were analyzed by HPLC using HPLC System 4 described in Section 3.8.

3.4 Synthesis of $[Cr(P)_2OH]_2$

The synthesis of $[Cr(P)_2OH]_2$ from the reaction of chromium chloride and picolinic acid in basic solution according to the method of Stearns and Armstrong (1992) was repeated. A solution of $[Cr(P)_2OH]_2$ in methanol was analyzed by HPLC using HPLC System 11 described in Section 3.8 and by mass spectrometry as described in Section 3.9.

3.5 Analysis of Cr3P for Water

Weighed aliquots of Cr3P from two different lots from Spectrum Quality Products, Inc. were analyzed in triplicate for water content by the Karl Fischer method. These samples were not dried prior to analysis. A second weighed aliquot from each lot was dried under vacuum at room temperature over night and then analyzed in triplicate for water content by the same method.

3.6 Samples of Cr3P Dietary Supplements

Samples of Cr3P dietary supplements were purchased from local grocery stores, a local drug store, and a local discount retailer to ascertain the types of formulations currently available as consumer products and the quantity of Cr3P in these products and to use in the development of analytical methods to assay Cr3P in these products. The following products were purchased:

Chromium Picolinate Supplement (200 μg elemental Cr), Nature Made Nutritional Products

Chromium Picolinate (200 μg Cr), Nature's Bounty, Inc.

Chromium Picolinate (200 μg Cr), Fields of Nature

Chromium Picolinate Dietary Supplement (200 μg Cr), Eckerd Drug Company

Chromium Picolinate (400 μg Cr), Spring Valley, manufactured by Rexall Sundown, Inc. (WalMart brand)

Fortified Chromium Picolinate (200 μg Cr), Sundown Vitamins

Chromium Picolinate (200 μg Cr), Kroger Company

3.7 Analysis of Cr3P Dietary Supplements

Individual tablets (25% of the tablets in each bottle) from each source of Cr3P dietary supplement were weighed and the mean weight determined for tablets from each source.

Four tablets from each source were extracted with 30:70 methanol:water and the extract analyzed for Cr3P by HPLC using HPLC System 3 described in Section 3.8. Weighed aliquots of Cr3P were dissolved in 30:70 methanol:water to prepare standards that were analyzed along with the tablet extracts for construction of the calibration curve used for quantitation of Cr3P in the extracts. The amount of chromium in each tablet analyzed was also calculated using the following formula:

$$Cr\ (\mu g) = [mg\ Cr3P \times MW\ of\ Cr \times 1000]/MW\ of\ Cr3P$$

3.8 HPLC

HPLC analysis was performed on a system consisting of two Waters 6000A pumps, a Waters 715 ULTRA WISP, an ABI 785A Programmable Absorbance Detector set at 264 nm, and an IN/US Systems β-Ram Radioactivity Detector. Mobile phase solvents were combined by volume and all mobile phase gradients were linear. A Waters μ Bondapak™ column (3.9 x 300 mm) was used for both HPLC Systems 1 and 2. For HPLC System 1, the mobile phase was isocratic for 8 min at 20:80 methanol:water and then changed over 2 min to 80:20 methanol:water and held for 2 min. The flow rate was 2 mL/min. For HPLC System 2, the mobile phase consisted of 30:70 methanol:water at a flow rate of 2 mL/min.

A Phenomenex Luna™ C18 (2) column (4.6 x 150 mm) was used for HPLC Systems 3, 4, 5, 8, 9, 10, and 11. For HPLC System 3, the mobile phase was isocratic at 10:90 methanol:water with a flow rate of 2 mL/min. For HPLC System 4, the mobile phase was isocratic for 8 min at 10:90 methanol:water and then changed over 2 min to 80:20 methanol:water and held for 2 min. The flow rate was 2 mL/min. For HPLC System 5, the mobile phase was isocratic at 10:90 0.01% trifluoroacetic acid in methanol:0.01% aqueous trifluoroacetic acid for 2 min and then changed over 8 min to a 30:70 ratio of these solvents where it was held for 2 min. The flow rate was 2 mL/min. For HPLC System 8, the mobile phase was isocratic at 10:90 0.01% trifluoroacetic acid in methanol:0.01% aqueous trifluoroacetic acid for 2 min and then changed over 12 min to 30:70 ratio of these solvents. The flow rate was 1 mL/min. For HPLC System 9, the mobile phase was isocratic at 100% aqueous 0.01% trifluoroacetic acid for 2 min and then changed over 10 min to 30:70 ratio of 0.01% trifluoroacetic acid in methanol:0.01% aqueous trifluoroacetic acid. The flow rate was 1 mL/min. For HPLC System 10, the mobile phase was isocratic at

10:90 0.01% trifluoroacetic acid in methanol:0.01% aqueous trifluoroacetic acid for 2 min and then changed over 12 min to 50:50 ratio of these solvents. The flow rate was 1 mL/min. For HPLC System 11, the mobile phase was isocratic at 10:90 0.01% trifluoroacetic acid in methanol:0.01% aqueous trifluoroacetic acid for 2 min and then changed over 15 min to 40:60 ratio of these solvents. The flow rate was 1 mL/min.

A Phenomenex Luna™ phenyl hexyl column (4.6 x 150 mm) was used for HPLC System 6 and HPLC System 7. For HPLC System 6, the mobile phase was isocratic at 10:90 0.01% trifluoroacetic acid in methanol:0.01% aqueous trifluoroacetic acid for 2 min and then changed over 8 min to a 1:1 ratio of these solvents and held there for 2 min. The flow rate was 2 mL/min. For System 7, the mobile phase was isocratic at 25:75 0.01% trifluoroacetic acid in methanol:0.01% aqueous trifluoroacetic acid. The flow rate was 0.5 mL/min.

3.9 Mass Spectrometry

Electrospray mass spectra (including MS/MS and MS^3 spectra) of nonlabeled Cr3P, urinary metabolite Ur1, the glycine conjugate of picolinic acid (N-picolinoyl glycine, NPG), and $[Cr(P)_2OH]_2$ were obtained on a Finnegan LCQ mass spectrometer and are shown in Figures 2, 3, 4, and 5, respectively. Solutions containing the analytes of interest were introduced by direct infusion at a rate of 3.0 μL/min or by HPLC.

3.10 Animals

Source: Adult male Fischer 344 (F-344) rats and $B6C3F_1$ mice were purchased from Charles River Laboratories, Inc. (Raleigh, NC). The animals were quarantined at least one week before they were used in a study. Animal ages and weights at the time they were used in studies are shown in Table 1.

Diet: Animals were fed Certified Purina Rodent Chow #5002 and were furnished tap water *ad libitum*.

Identification: Individual ear tags were used to identify animals.

Housing: Animals were stored in standard polycarbonate cages until they were used in an experiment. Following dosing, the animals were housed in individual glass metabolism chambers that provided for separate collection of urine, feces and breath (volatile organics and CO_2).

3.11 Incubation of Cr3P with Rat Stomach and Small Intestine Contents

Four adult male F-344 rats were sacrificed by carbon dioxide asphyxiation. Stomach and small intestines were excised inside a glove bag purged with nitrogen gas. The contents of the small intestines were collected inside the nitrogen-purged glove bag. While still under a nitrogen atmosphere in the glove bag, aliquots of the small intestine contents were transferred to screw-cap culture tubes and treated with $[^{14}C]$Cr3P in water. The capped culture tubes containing the $[^{14}C]$Cr3P spiked small intestine contents were then transferred to a 37 °C heater/vortex mixer (Labconco) and incubated in duplicate under nitrogen for 1, 2, and 4 h. The contents of the stomachs were collected outside the glove bag. Aliquots of the stomach contents were then transferred to screw-cap culture tubes and treated with $[^{14}C]$Cr3P in

water. The capped culture tubes containing the [^{14}C]Cr3P spiked stomach contents were then transferred to a 37 °C heater/vortex mixer (Labconco) and incubated in duplicate for 1 and 2 h.

At the end of each prescribed incubation time, 2 mL of methanol was added to each incubation vial. The contents and methanol were mixed on a vortex mixer and then centrifuged. Each supernatant from the stomach and small intestine contents extractions was collected and assayed for radiochemical content by LSS. Repeat methanol (1 mL each) extractions were performed on each incubation mixture and assayed for radiochemical content until it was determined that an additional extraction would not yield appreciable recovery of radiolabel.

The methanol extracts of the duplicate incubations of small intestine contents with [^{14}C]Cr3P for 4 h and duplicate incubations of stomach contents with [^{14}C]Cr3P for 2 h were analyzed by HPLC using HPLC System 4 described in Section 3.8. Eluate fractions were collected and analyzed by LSS.

A second incubation of stomach and small intestine contents with [^{14}C]Cr3P using two adult male F-344 rats that were sacrificed by carbon dioxide asphyxiation. Stomach and small intestines were excised and prepared as described above for the first incubations. One tube of stomach contents was treated with [^{14}C]Cr3P in water and the other with [^{14}C]Cr3P in 1,2-propanediol. The amount of test material mixed with the stomach contents was calculated to approximate the amount of dose delivered to an animal in an oral dosing experiment (5 mL dose volume per kg body weight). The capped culture tubes containing the [^{14}C]Cr3P spiked stomach contents were then transferred to a 37 °C heater/vortex mixer (Labconco) and incubated for 1 and 2 h.

At the end of each prescribed incubation time for stomach contents, weighed aliquots of the [^{14}C]Cr3P spiked stomach contents were transferred to screw-cap culture tubes. A 4 mL aliquot of methanol was then added to each tube. At the end of each prescribed incubation time for small intestine contents, 4 mL of methanol was added to each incubation vial containing small intestine contents. The contents and methanol were mixed on a vortex mixer and then centrifuged. Each supernatant from the stomach and small intestine contents extractions was collected and assayed for radiochemical content by LSS. Repeat methanol (4 mL each) extractions were performed on each incubation mixture and assayed for radiochemical content until it was determined that an additional extraction would not yield appreciable recovery of radiolabel.

The first methanol extracts of the duplicate incubations of small intestine contents with [^{14}C]Cr3P for 4 h and incubations of stomach contents with [^{14}C]Cr3P for 2 h were analyzed by HPLC with flow through radioactivity detection using HPLC System 4 described in Section 3.8.

3.12 In Vivo Pilot Studies with Male Rats

Two dose formulations of [^{14}C]Cr3P were prepared. In one preparation, [^{14}C]Cr3P was dissolved in 1,2-propanediol at a concentration of 0.38 mg (47.3 μCi) Cr3P per g of dose preparation. A second dose was dissolved in water (pH 7) at a concentration of 0.23 mg (28.7 μCi) Cr3P per g of dose preparation. The dose preparations were analyzed for radiochemical purity using HPLC System 4 described in Section 3.8. A total of 8 male rats were dosed, two orally and two ip with each dose

preparation. For the animals dosed with [^{14}C]Cr3P in 1,2-propanediol, urine, feces, and breath (volatile organics and CO_2) were collected for 72 h. For the animals dosed with [^{14}C]Cr3P in water, urine and feces were collected for 72 h.

At the end of the 72 h period, the two animals that received ip doses of [^{14}C]Cr3P in 1,2-propanediol were redosed ip with [^{14}C]Cr3P in 1,2-propanediol, and the two animals that received [^{14}C]Cr3P in water (pH 7) orally were redosed orally with [^{14}C]Cr3P in 1,2-propanediol. At selected time points after receiving the second dose, these animals were sacrificed and blood collected from each animal. Plasma from one of the animals receiving both doses ip was analyzed by HPLC/LSS using HPLC System 15.

The remaining animals were sacrificed 72 h postdosing. The following tissues were collected (and assayed for radioactivity) from the two animals that received [^{14}C]Cr3P in 1,2-propanediol orally: adipose (two sites), muscle (two sites), liver, kidney, lung, spleen, bladder, blood, and GI tract and contents.

Aliquots of 0–24 h urine collections from animals receiving [^{14}C]Cr3P in 1,2-propanediol were analyzed by HPLC with flow through radioactivity detection using HPLC Systems 5 and 6 described in Section 3.8.

3.13 Study A: Single 15.3 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

A dose formulation of [^{14}C]Cr3P dissolved in 1,2-propanediol at a concentration of 2.84 mg (33.6 μCi) Cr3P per g of dose preparation was prepared. The dose preparation was analyzed for radiochemical purity using HPLC System 4 described in Section 3.8. Single oral doses were given by gavage to 4 male rats.

Urine and feces were collected until excretion was essentially complete (52 h) and analyzed for total radiolabel by LSS and Cr by the method described in Section 3.25. Exhaled organic volatiles and CO_2 were collected for 24 and 48 h, respectively. The animals were sacrificed at 52 h postdosing. The following tissues were collected: adipose (two sites), muscle (two sites), bladder, blood, brain, heart, kidney, liver, lung, skin (ear), spleen, testes, stomach (and contents), small intestine (and contents), large intestine (and contents), and cecum (and contents). Initially, all of the collected tissues for one animal were analyzed for total radiolabel by tissue solubilization followed by LSS. Selected tissues (kidney, liver, lung, and spleen) from the three other animals were analyzed for Cr as described in Section 3.25. The acid digests from the Cr determinations for these tissues were also analyzed for total radiolabel by LSS. The remaining collected tissues were stored at ca. -20 °C until analyzed for total radiolabel by tissue solubilization followed by LSS.

Aliquots of the 8 and 24 h urine collections were analyzed for Cr3P and metabolites by HPLC/LSS using HPLC System 5 described in Section 3.8.

3.14 Study Ba: Single 17.4 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

A solution of [^{14}C]Cr3P was prepared in water at a concentration of 9.93 μCi per mg Cr3P. Weighed aliquots of the solution were transferred to silylated glass vials to prepare individual doses for each animal in the study. The contents of the vials were frozen and lyophilized to dryness. Following the addition of a second weighed aliquot of the solution to each vial, the freezing and drying steps were repeated to complete the transfer of [^{14}C]Cr3P to the individual dose vials. Immediately prior to dosing, 1.5 mL of water was added to each dose vial. Following mixing on a vortex mixer to form a Cr3P water slurry, doses were given by gavage to 8 male rats. The remaining undosed slurry from one animal was dissolved in water and analyzed for radiochemical purity using HPLC System 8 described in Section 3.8.

For 4 of the animals, urine was collected for 2 h and analyzed for total radiolabel by LSS. These animals were then sacrificed at 2 h postdosing. The following tissues were collected: adipose (two sites), muscle (two sites), bladder, blood, brain, heart, kidney, liver, lung, skin (ear), spleen, testes, stomach (and contents), small intestine (and contents), large intestine (and contents), and cecum (and contents). Initially, all of the collected tissues for one animal were analyzed for total radiolabel by tissue solubilization followed by LSS. The remaining collected tissues were stored at ca. -20 °C until analyzed for total radiolabel by tissue solubilization followed by LSS.

For the remaining 4 animals, urine and feces were collected at 2 (urine only), 4, 8, 12, 24, and 48 h and analyzed for total radiolabel by LSS and Cr by the method described in Section 3.25. Exhaled CO_2 was collected from these animals at 8, 24, 30, and 48 h. These animals were sacrificed at 48 h postdosing. The following tissues were collected: adipose (two sites), muscle (two sites), bladder, blood, brain, heart, kidney, liver, lung, skin (ear), spleen, testes, and gastrointestinal (GI) tract (and contents). Initially, all of the collected tissues for one animal were analyzed for total radiolabel by tissue solubilization followed by LSS. The remaining collected tissues were stored at ca. -20 °C until analyzed for total radiolabel by tissue solubilization followed by LSS.

3.15 Study D: Single 0.22 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

A dose formulation of [^{14}C]Cr3P dissolved in 1,2-propanediol at a concentration of 0.0446 mg (5.53 μCi) Cr3P per g of dose preparation was prepared. The dose preparation was analyzed for radiochemical purity using HPLC System 4 described in Section 3.8. Single oral doses were given by gavage to 4 male rats.

Urine and feces were collected until excretion was essentially complete (52 h) and analyzed for total radiolabel by LSS. Exhaled organic volatiles and CO_2 were collected for 24 and 48 h, respectively. The animals were sacrificed at 52 h postdosing. The following tissues were collected: adipose (two sites), muscle (two sites), bladder, blood, brain, heart, kidney, liver, lung, skin (ear), spleen, testes, stomach (and contents), small intestine (and contents), large intestine (and contents), and cecum (and contents). All of the collected tissues for one animal were analyzed for total radiolabel by tissue solubilization followed by LSS. To provide a profile of radiolabel in tissue, the tissue samples collected from the other three rats were stored at ca. -20 °C until the results of the analysis for Cr in the tissues

collected from animals in Study A could be evaluated. The tissue from these three rats was later analyzed for total radiolabel by tissue solubilization followed by LSS.

Composites (based on volume excreted) of the 8 h and the 24 h urine collections were analyzed by HPLC with flow through radioactivity detection using HPLC System 5 described in Section 3.8.

3.16 Study F: Single 17.4 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

A solution of [^{14}C]Cr3P was prepared in water at a concentration of 27.7 μCi per mg Cr3P. Weighed aliquots of the solution were transferred to silylated glass vials to prepare individual doses for each animal in the study. The contents of the vials were frozen and lyophilized to dryness. Following the addition of a second weighed aliquot of the solution to each vial, the freezing and drying steps were repeated to complete the transfer of [^{14}C]Cr3P to the individual dose vials. Immediately prior to dosing, 1.5 mL of water was added to each dose vial. Following mixing on a vortex mixer to form a Cr3P water slurry, single oral doses of Cr3P water slurry were given by gavage to 12 male rats. The remaining undosed slurry from one animal was dissolved in water and analyzed for radiochemical purity using HPLC System 8 described in Section 3.8.

The animals were sacrificed in groups of 4 at 1, 2, and 4 h postdosing. The following tissues were collected: adipose, muscle, bladder, blood, brain, heart, kidney, liver, lung, skin (ear), spleen, and testes. The collected tissues were stored at ca. –20 °C for analysis of Cr3P. Stomach (and contents), small intestine (and contents), large intestine (and contents), and cecum (and contents) were also collected and were analyzed for total radiolabel by tissue solubilization followed by LSS. Aliquots of blood and homogenized liver were also analyzed for total radiolabel directly by tissue solubilization followed by LSS.

Aliquots (ca. 1 mL) of blood collections were extracted with 2 portions of acetonitrile (ca. 9 mL followed by 6 m). The two portions were combined, analyzed for extracted radioactivity, and then concentrated. The collected livers were homogenized, and aliquots were analyzed for total radiolabel by tissue solubilization followed by LSS. An additional aliquot of each homogenized liver (ca. 1 g) was extracted with ca. 10 mL acetonitrile: 2 mL water followed by 6 mL acetonitrile. The two portions were combined, analyzed for extracted radioactivity, and then concentrated. Aliquots of muscle and adipose were finely minced while still frozen and extracted as described for liver aliquots above. The muscle and adipose residues following extraction were analyzed for radiolabel by tissue solubilization followed by LSS. The total radioactivity in muscle and adipose aliquots was determined by adding the amounts of radioactivity extracted with acetonitrile and that remaining in each aliquot following extraction.

A solution of nonradiolabeled Cr3P was added to each concentrate prior to analysis by HPLC/LSS using HPLC System 11 described in Section 3.8. The concentration of Cr3P in each tissue was determined from the amount of radiolabel that coeluted with the added nonradiolabeled Cr3P. The concentrations of picolinic acid and the previously identified Cr3P urinary metabolite, NPG, were determined by the amount of radiolabel that eluted at the appropriate retention times for those compounds.

3.17 Study Ha: Single 0.18 mg/kg Intravenous Dose of [^{14}C]Cr3P to Rats

A dose formulation of [^{14}C]Cr3P dissolved in 0.9% sodium chloride at a concentration of 0.187 mg (23.2 μCi) Cr3P per g of dose preparation was prepared. The dose preparation was analyzed for radiochemical purity using HPLC System 8 described in Section 3.8. Single oral doses were given intravenously to 6 male rats that had been implanted with indwelling jugular cannulae on the previous day. Three additional male rats, also with indwelling jugular cannulae, received intravenous doses of 0.9% sodium chloride only.

After dosing, blood (approximately 450 μL) was sampled from the cannula of each of the 9 animals at 10, 30, and 60 min postdosing prior to reinfusion of an equivalent volume of plasma. Blood was also collected through the cannula at sacrifice at 120 min postdosing. Following sacrifice, the following tissues were collected: adipose (two sites), muscle (two sites), bladder, blood, brain, heart, kidney, liver, lung, skin (ear), spleen, and testes. For all collected tissues except heart, bladder and liver, the tissues were divided into duplicate weighed aliquots. One aliquot of each tissue was stored at ca. -20 °C for possible later use in determination of Cr3P in tissue. The second aliquot of each tissue (along with bladder and heart collected from three of the rats) was dried by lyophilization in preparation for analysis for Cr. Liver collected from each rat was homogenized and aliquoted for analysis for radiolabel and Cr. All of the collected aliquots were later analyzed for total radiolabel by tissue solubilization followed by LSS. Small intestine (and contents), large intestine (and contents), and cecum (and contents) were also collected as a single collection and digested in 2 N ethanolic sodium hydroxide prior to analysis for radiolabel.

Collected blood was transferred to a tared collection vial. Two weighed aliquots (ca. 60 μL each) were dispensed. One aliquot was dispensed into a scintillation vial containing ca. 2 mL Soluene-350® prior to determination of radiochemical content by LSS. The second aliquot was transferred to an empty vial and stored at ca. -20 °C for later Cr content determination. The vial containing the collected sample was then reweighed and placed in a 2-proponal/dry ice bath to flash freeze the blood prior to storage at ca. -20 °C.

The flash frozen blood collections were extracted with 2 portions of acetonitrile (ca. 3.5 mL each). Following analysis for extracted radioactivity, the two portions were combined and concentrated. A solution of nonradiolabeled Cr3P was added to each concentrate prior to analysis by HPLC/LSS using HPLC System 8 described in Section 3.8. The concentration of Cr3P in blood was determined from the amount of radiolabel that coeluted with the added nonradiolabeled Cr3P.

A pilot study with two additional rats that had been implanted with indwelling jugular cannulae on the previous day was conducted using the same dose formulation and procedure described above. For these animals, a single blood collection was made at 5 h postdosing. A weighed aliquot of each collection was dispensed into a scintillation vial containing ca. 2 mL Soluene-350® prior to determination of radiochemical content.

3.18 Study Hb: Single 17.8 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

A solution of [^{14}C]Cr3P was dissolved in water at a concentration of 26.5 μCi per mg Cr3P. Individual doses were prepared as described in Section 3.16. Single oral doses were given by gavage to 4 male rats that had been implanted with indwelling jugular cannulae on the previous day. The remaining undosed slurry from one animal was dissolved in water and analyzed for radiochemical purity using HPLC System 8 described in Section 3.8.

After dosing, blood (approximately 400 μL) was sampled from the cannula of each of the 4 animals at 0.5, 1, 2, 4, 6, 9, 12, and 15 h postdosing prior to reinfusion of an equivalent volume of plasma. Urine and feces were collected at 4, 8, 12, and 24 h postdosing. Blood was also collected through the cannula at sacrifice at 24 h postdosing. Following sacrifice, the following tissues were collected: adipose, muscle, and liver. Aliquots of the collected tissues were analyzed for total radiolabel by tissue solubilization followed by LSS.

Collected blood was transferred to a tared collection vial. Two weighed aliquots (ca. 60 μL each) were dispensed. One aliquot was dispensed into a scintillation vial containing ca. 2 mL Soluene-350® prior to determination of radiochemical content by LSS. The second aliquot was transferred to an empty vial and stored at ca. –20 °C for later Cr content determination. The vial containing the collected sample was then reweighed and placed in a 2-proponal/dry ice bath to flash freeze the blood prior to storage at ca. –20 °C.

The flash frozen blood collections were extracted with 2 portions of acetonitrile (ca. 3.5 mL each). Following analysis for extracted radioactivity, the two portions were combined and concentrated. A solution of nonradiolabeled Cr3P was added to each concentrate prior to analysis by HPLC/LSS using HPLC System 8 described in Section 3.8. The concentration of Cr3P in blood was determined from the amount of radiolabel that coeluted with the added nonradiolabeled Cr3P.

3.19 Study J: Single 20.5 mg/kg Oral Dose of [^{14}C]Cr3P to Mice

A solution of [^{14}C]Cr3P was dissolved in water at a concentration of 30.4 μCi per mg Cr3P. Weighed aliquots of the solution were transferred to silylated glass vials to prepare individual doses for each animal in the study. The contents of the vials were frozen and lyophilized to dryness. Immediately prior to dosing, 0.5 mL of water was added to each dose vial. Following mixing on a vortex mixer to form a Cr3P water slurry, single oral doses of Cr3P water slurry were given by gavage to 4 male mice. The remaining undosed slurries from two animal were dissolved in water and analyzed for radiochemical purity using HPLC System 11 described in Section 3.8.

Urine and feces were collected at 6, 12, 24, and 48 h and analyzed for total radiolabel by LSS. Exhaled CO_2 was collected at 12, 24, 32, and 48 h. The remaining urine and feces were stored at ca. -20 °C for later analysis for Cr3P, metabolites and Cr. The animals were sacrificed at 48 h postdosing. The following tissues were collected: muscle, adipose, liver and blood and stored at ca. -20 °C for later

analysis. The remaining carcass from each animal was digested in ca. 70 mL of 2 N ethanolic sodium hydroxide.

Aliquots of the 6, 12, and 24 h urine collections were analyzed for Cr3P and metabolites by HPLC/LSS using HPLC System 11 described in Section 3.8.

3.20 Study Jb: Single 19.0 mg/kg Oral Dose of [^{14}C]Cr3P to Mice

A dose formulation of [^{14}C]Cr3P dissolved in 1,2-propanediol at a concentration of 1.58 mg (39.1 μCi) Cr3P per g of dose preparation was prepared. The dose preparation was analyzed for radiochemical purity using HPLC System 11 described in Section 3.8. Single oral doses were given by gavage to 4 male mice.

Urine and feces were collected at 6, 12, 24, and 48 h and analyzed for total radiolabel by LSS. The remaining urine and feces were stored at ca. -20 °C for later analysis for Cr3P, metabolites, and Cr. The animals were sacrificed at 48 h postdosing. The following tissues were collected: muscle, adipose, liver, and blood and analyzed for total radiolabel by tissue solubilization followed by LSS. The remaining carcass from each animal was digested in ca. 70 mL of 2 N ethanolic sodium hydroxide and analyzed for radiolabel by LSS.

Aliquots of the 6, 12, 24, and 48 h urine collections were analyzed for Cr3P and metabolites by HPLC/LSS using HPLC System 11 described in Section 3.8.

3.21 Study PAa: Single 17.2 mg/kg Oral Dose of [^{14}C]Pcl-H to Rats

A dose formulation of [^{14}C]Pcl-H dissolved in water at a concentration of 2.90 mg (6.03 μCi) Cr3P per g of dose preparation was prepared. The dose preparation was analyzed for radiochemical purity using HPLC System 9 described in Section 3.8. Single oral doses were given by gavage to 8 male rats.

For 4 of the animals, urine and feces were collected at 2 (urine only), 4, and 6 h and analyzed for total radiolabel by LSS. These animals were then sacrificed at 6 h postdosing. The following tissues were collected: adipose (two sites), muscle (two sites), bladder, blood, brain, heart, kidney, liver, lung, skin (ear), spleen, testes, stomach (and contents), small intestine (and contents), large intestine (and contents), and cecum (and contents). Aliquots of the collected tissues were analyzed for total radiolabel by tissue solubilization followed by LSS.

For the remaining 4 animals, urine and feces were collected at 2 (urine only), 4, 8, 12, and ca. 20 h and analyzed for total radiolabel by LSS. Exhaled CO_2 was collected from these animal at 8 and ca. 20 h. Urine collected at 2, 4, 8, and 12 h postdosing from these animals was analyzed by HPLC with flow through radioactivity detection using HPLC System 10 described in Section 3.8.

3.22 Study T: Determination of Binding to Blood Protein and DNA/RNA

A dose formulation of [^{14}C]Cr3P as an aqueous slurry was prepared as described in Section 3.16 for Study F. A dose formulation of [^{14}C]Pcl-H dissolved in water was prepared as described in Section 3.17 for study PAa. Single oral doses of the [^{14}C]Cr3P were given to three animals, and single oral doses of [^{14}C]Pcl-H were given to two additional animals. Two hours after dose administration, the

animals were anesthetized with carbon dioxide; and as much blood as possible was removed by cardiac puncture. The animals were then immediately sacrificed.

Aliquots of the blood were assayed for total radioactivity. The remainder was separated into red blood cells and plasma by centrifugation. The red blood cells were rinsed with equal volumes of normal saline four times. The resultant plasma and red blood cells were frozen.

Protein was precipitated in weighed aliquots of the plasma samples by addition of nine volumes of methanol. After decanting the supernatant, the precipitated pellet was washed four times with methanol. The original supernatant and the methanol washes were assayed for radiolabel. The process was repeated using acetonitrile in place of methanol for additional aliquots of several plasma samples.

3.23 Collection and Storage of Urine, Feces, Cage Rinse, and Expired Breath

Urine, Feces, and Cage Rinse: Urine and feces were collected separately into receivers cooled over dry ice. At the end of each collection time, these receivers were emptied into tared scintillation vials. At the end of the in-life portion of the study, the metabolism chambers were rinsed with water and ethanol and the total rinse collected into a tared bottle. Urine, feces, and cage rinse samples were stored in the dark at ca. -20 °C until analyzed for radiochemical or Cr content.

Expired Volatile Organic Compounds and Carbon Dioxide: Room air (200–600 mL/min) was passed through each metabolism chamber and a series of four traps designed to remove expired volatile organic compounds and carbon dioxide or only through the two traps designed to remove expired carbon dioxide. The first two traps (to remove volatile organic compounds) contained 50–75 mL of absolute ethanol cooled to 0 °C and -60 °C with an ice/water bath and dry ice/isopropanol slurry, respectively. The last two traps (to remove carbon dioxide) were each filled with ca. 500 mL of 1 N sodium hydroxide. Air was passed through stainless steel frits (10-μm pore size) within each trap to facilitate efficient gas to liquid transfer. The weights of solution in both ethanol traps and both sodium hydroxide traps were recorded. Aliquots (ca. 20 mL) of the ethanol and sodium hydroxide trapping solutions were retained. The ethanol and sodium hydroxide samples were stored at room temperature until analyzed for radiochemical content.

3.24 Analysis of Biological Samples for Carbon-14 Content

Samples were assayed for carbon-14 either directly (after dissolution in a scintillation cocktail) or following solubilization in 2 N ethanolic sodium hydroxide or Soluene-350. Samples that were too dark were bleached and neutralized (perchloric acid/H_2O_2) prior to analysis by LSS. Ultima Gold scintillation cocktail was used in all determinations of radiochemical content.

Controls: Samples of urine and feces excreted during the acclimation period were collected immediately prior to dosing for determination of background carbon-14 levels.

Urine and Cage Rinse: Duplicate aliquots of urine and cage rinse were weighed into scintillation vials containing ca. 15 mL of scintillation cocktail for carbon-14 analysis.

Feces: Fecal samples were homogenized after adding an approximately equal mass of water. The total homogenate weight was recorded. Triplicate homogenate aliquots were weighed into scintillation vials containing 2 mL of Soluene 350®. After dissolution, scintillation cocktail was added.

Breath: Duplicate aliquots of the trapping solution collections were weighed into scintillation vials containing scintillation cocktail.

Tissues, Blood and the Residual Carcass: Tissue and blood samples added to Soluene 350® were agitated using a reciprocating shaker until they were fully dissolved. Liver, lung, blood, and kidney samples were then neutralized with 125 μL of perchloric acid and decolorized with 300 μL of hydrogen peroxide. Scintillation cocktail was added to all tissue vials. Stomach (and contents), cecum (and contents), small and large intestines (and contents), and the residual carcass placed in 2 N ethanolic sodium hydroxide were also agitated until dissolved. Aliquots were weighed into scintillation vials containing ca. 15 mL of scintillation cocktail.

3.25 Analysis of Urine, Feces and Tissues for Total Cr

Selected tissues (kidney, liver, lung and spleen) from three of the animals and aliquots of urine and feces from all animals receiving 15.3 mg/kg [^{14}C]Cr3P in 1,2-propanediol (Study A) and aliquots of adipose, blood, liver, and muscle from animals that received single oral doses of [^{14}C]Cr3P (17.4 mg/kg) as an aqueous slurry and sacrificed 1, 2, or 4 h after dosing (Study F) were analyzed for Cr. Aliquots of feces collected from the two pilot study animals that received ip doses of 1.8 mg/kg [^{14}C]Cr3P in 1,2-propanediol were also analyzed for total Cr. For measurement of total Cr, feces homogenates, liver homogenates, and whole organs were lyophilized prior to analysis and dissolved in nitric acid. Urine samples were diluted with dilute nitric acid. The resulting solutions were assayed by graphite furnace atomic absorption (GFAA) directly in a Perkin Elmer 4100 ZL instrument.

Aliquots of urine and feces from four of the animals receiving 17.4 mg/kg [^{14}C]Cr3P in a water slurry (Study Ba) were analyzed for total Cr. The samples were prepared for analysis as described above. The resulting solutions were analyzed for Cr using a Perkin Elmer Optima 4300 DV inductively coupled plasma atomic emission spectrometer (ICP-AES).

Aliquots of urine and feces from the four mice receiving 20.5 mg/kg [^{14}C]Cr3P in a water slurry (Study J) and from the four mice receiving 19.0 mg/kg [^{14}C]Cr3P in 1,2-propanediol (Study Jb) were analyzed for total Cr. The samples were prepared for analysis as described above. The resulting solutions were analyzed for Cr using a Perkin Elmer Optima 4300 DV inductively coupled plasma atomic emission spectrometer (ICP-AES).

3.26 Isolation and Identification of Urinary Metabolite

Urinary metabolite Ur1, the single major radioactive peak found in the urine of rats receiving [^{14}C]Cr3P orally, was isolated from urine by multiple HPLC injections (System 5). The fractions containing the metabolite were collected, combined, and concentrated. The isolated metabolite was analyzed by HPLC/MS/MS using the conditions of HPLC System 7 on a Finnegan LCQ mass

spectrometer. The isolated metabolite was also diluted with dilute nitric acid and assayed directly for total Cr by graphite furnace atomic absorption (GFAA).

The glycine conjugate of picolinic acid (N-picolinoyl glycine, NPG) was synthesized as described by Reddy et al. (1990) from picolinic acid and glycine ethyl ester using DCC as the coupling agent followed by hydrolysis of the resulting ethyl ester. The synthetic product and isolated Ur1 were cochromatographed using HPLC Systems 5 and 6. The synthetic NPG was also analyzed by HPLC/MS/MS using the same conditions as those for the isolated urinary metabolite.

4.0 RESULTS

4.1 Analysis of Nonradiolabeled Test Chemicals

The chromatograms from the HPLC analyses of the ICN (lot #0061974) Cr3P standard (top trace) and the Spectrum (lot #NG0569) standard (bottom trace) are shown in Figure 6 (HPLC System 1). The chromatogram of Spectrum Cr3P contains a single major peak with a retention time of 5.6 min. The chromatogram of the ICN Cr3P was devoid of significant peaks with absorptions at 264 nm, the reported UV absorption maximum for Cr3P (Evans and Pouchnik, 1993). A peak appeared in the chromatogram of the ICN Cr3P at 11.5 min during the wash-off phase of the chromatographic run.

The electrospray mass spectrum of the Spectrum Cr3P shown in Figure 2 contained a base peak at m/z 441, corresponding to $(Cr3P + Na)^+$ and a smaller peak at m/z 859 corresponding to $[2(Cr3P) + Na]^+$. MS/MS experiments showed that the m/z 859 peak fragmented to produce the peak at m/z 441 and that the m/z 441 peak produced daughter ions at m/z 397 (loss of CO_2) and m/z 319 (loss of 1 picolinic acid).

The electrospray mass spectrum of the ICN Cr3P had a base peak at m/z 355 and smaller peaks at m/z 775 $[(752 + Na)^+]$, m/z 1528 $[(2 \times 752 + Na)]^+$, m/z 1151, m/z 731, m/z 377, and m/z 273. These peaks could not be correlated to any anticipated peaks arising from Cr3P.

The Spectrum Cr3P was determined to contain 12.68% Cr (calculated for Cr3P, 12.44%). The ICN Cr3P was determined to contain less than 0.01% Cr. Each sample contained less than 20 ppm Cr^{+6}.

From this data it was concluded that the Spectrum Cr3P was approximately 95–98% chemically pure Cr3P, whereas the ICN material contained no Cr3P!

The two samples of Cr3P analyzed without drying prior to analysis were found to contain 5.51 ± 0.15% and 5.70 ± 0.67% water. The dried samples were found to contain 3.01 ± 0.22% and 3.61 ± 0.38% water. The number of equivalents of water per equivalent of Cr3P is 1.35 and 1.40 for the undried samples and 0.72 and 0.87 for the dried samples.

4.2 Analysis of Radiolabeled Test Chemical

Radiolabeled Cr3P was 96.5% radiochemically pure by HPLC (HPLC System 1) and contained the same major component as the Spectrum Cr3P standard (Figure 7).

4.3 Analysis of Cr3P Solubilities

The Cr3P solubilities in the solvents tested are shown in Table 2. Cr3P solubilities in acetone, ethyl acetate, 2-propanol, tetrahydrofuran, and methyl t-butyl ether were all below 0.05 mM, the lowest concentration of Cr3P used to establish the calibration curve.

4.4 Synthesis of Cr1P, Cr2P and Cr3P

When synthesis of Cr1P, Cr2P and Cr3P was attempted according to the methods of Evans and Poulchnik (1993), Cr3P was obtained upon mixing chromium(III) chloride hexahydrate with either 1, 2, or 3 molar equivalents of picolinic acid. No Cr1P or Cr2P was observed.

A small amount of Cr3P was prepared by following the method of Anderson et al. (1996,) but the reaction rate was much slower with a much lower yield than that reported in the paper.

Attempted synthesis of the dimer of Cr2P according to the method of Stearns and Armstrong (1992) yielded a mixture of products. When the reaction mixture was analyzed by HPLC, three major products were detected. One product had the same retention time as Cr3P, a second product was more polar than Cr3P, and a third product that appears to be in the largest concentration was less polar than Cr3P.

4.5 Synthesis of $[Cr(P)_2OH]_2$

The most recent reaction of chromium chloride and picolinic acid in basic solution resulted in the formation of a mixture of red and purple crystals, identified by the authors of the method (Stearns and Armstrong, 1992) as Cr3P and $[Cr(P)_2OH]_2$, respectively. After allowing the crystals to remain in the reaction mixture for ca. 1 month, some of the crystals had grown sufficiently large so that a sample of the purple crystals could be separated from the mixture under magnification.

The electrospray mass spectrum of a methanol solution of the purple crystals (Figure 5) contained a base peak at m/z 649, corresponding to $[[Cr(P)_2OH]_2 + Na]^+$ and a smaller peak at m/z 1275 corresponding to $[2[Cr(P)_2OH]_2 + Na]^+$. MS/MS experiments showed that the m/z 1275 peak fragmented to produce a peak at m/z 1134 (loss of 1 picolinic acid and 1 H_20). MS/MS experiments also showed that the m/z 1134 peak fragmented to produce a peak at m/z 1011 (loss of 1 picolinic acid) and that the m/z 1011 peak produced a daughter ion at m/z 967 (loss of CO_2).

Analysis by HPLC using HPLC System 11 described in Section 3.8 of a methanol solution of $[Cr(P)_2OH]_2$ showed that this material was less polar than Cr3P. (See Figure 8.)

4.6 Analysis of Cr3P Dietary Supplement

The mean weights of the tablets from each source, the determined amount of Cr3P in the dietary supplements and the calculated amount of Cr in the dietary supplements are shown in Table 3. Table 4 contains a listing of the other ingredients in each dietary supplement in addition to Cr3P.

4.7 Incubation of Cr3P with Rat Stomach and Small Intestine Contents

The recovery of radiolabel from rat stomach and small intestine contents incubated with $[^{14}C]$Cr3P in the initial experiments is shown in Table 5. The overall recovery of radiolabel from the

incubations was 100% (SD = 3.7%). The recovery of radiolabel from rat stomach and small intestine contents incubated with [^{14}C]Cr3P in the repeat experiments is shown in Table 6. The results of the repeat experiments are essentially identical to those from the initial experiments. The overall recovery of radiolabel from the contents incubations spiked with [^{14}C]Cr3P in water was 95% (SD = 3.0%). A total of four extractions of the spiked stomach contents was necessary to recover greater than 90% of the radiolabel. The overall recovery of radiolabel from the stomach contents incubations spiked with [^{14}C]Cr3P in 1,2-propanediol was greater than 99% in two extractions. As observed in the earlier experiment, there was no evidence of decomposition of Cr3P in either stomach or small intestine contents.

Results of the HPLC analysis of the methanol extracts from the initial 2 h stomach contents and the 4 h small intestine contents incubations are shown in Table 7. There was no evidence of decomposition of Cr3P in either stomach or small intestine contents.

4.8 In Vivo Pilot Studies with Male Rats

The radiochemical purity of [^{14}C]Cr3P dissolved in 1,2-propanediol was determined by HPLC/LSS to be greater than 95% following dosing of the animals. The radiochemical purity of [^{14}C]Cr3P dissolved in water (pH 7) and stored overnight at ca. 4 °C was determined to be only 68% following dosing.

Excretion of radioactivity in rats following administration of Cr3P is shown in Table 8. The two male rats dosed orally with [^{14}C]Cr3P dissolved in 1,2-propanediol excreted an average of 46% of the ^{14}C-dose in urine, 19% in feces, and 7.1% as CO_2 in breath in 24 h. For the two animals receiving [^{14}C]Cr3P dissolved in 1,2-propanediol ip, an average of 75% of the ^{14}C-dose was excreted in urine, 2.1% in feces, and 2.1% as CO_2 in breath in 24 h. Additional small amounts of radiolabel were excreted during the collections at 48 h and 72 h. An average total of 77% and 87% of the ^{14}C-dose was excreted by the animals receiving [^{14}C]Cr3P in 1,2-propanediol orally and ip, respectively, in 72 h.

The two male rats dosed orally with [^{14}C]Cr3P dissolved in water excreted an average of 47% of the ^{14}C-dose in urine and 27% in feces in 24 h. For the two animals receiving ip doses of [^{14}C]Cr3P dissolved in water, an average of 82% of the ^{14}C-dose was excreted in urine and 2.2% in feces in 24 h. An additional 2–9% of the ^{14}C-dose was excreted during the collections at 48 h and 72 h. An average total of 81% and 90% of the ^{14}C-dose was excreted by the animals receiving [^{14}C]Cr3P in water orally and ip, respectively in 72 h in urine and feces. Exhaled breath was not collected in this pilot experiment.

The results of the assay of radioactivity in tissues collected at 72 h postdosing from the two rats dosed orally with [^{14}C]Cr3P in 1,2-propanediol are shown in Table 9. The concentrations of radiolabel in blood and plasma at 4 h from the animals that were redosed with Cr3P dissolved in 1,2-propanediol at 72 h after the initial dose are shown in Table 10. Analysis by HPLC of plasma obtained from a rat 4 h after the second ip dose of [^{14}C]Cr3P in 1,2-propanediol showed that the plasma contained both Cr3P and more polar metabolites.

Analysis by two different HPLC columns (Systems 5 and 6) of 0–24 h urine from animals dosed with [^{14}C]Cr3P in 1,2-propanediol showed that the radioactivity excreted from animals receiving ip doses consisted of two major peaks, one that coeluted with Cr3P and a second that did not (Figure 9). This second major peak also did not coelute with picolinic acid. None of the radioactivity excreted from animals receiving oral doses of [^{14}C]Cr3P was associated with Cr3P, but rather it eluted in a single major peak. (Figure 10). Cochromatography of urine samples from ip and orally dosed animals showed that the same Cr3P metabolite (Ur1) was present after both oral and ip dosing.

Analysis for Cr in feces collected from the two pilot study animals that received ip doses of 1.8 mg/kg [^{14}C]Cr3P in 1,2-propanediol showed that these animals excreted an average of 4.1% (range = 0.1%) of the administered Cr in 24 h and 4.8% (range = 0.1%) of the administered Cr in 48 h.

4.9 Study A: 15.3 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

The radiochemical purity of [^{14}C]Cr3P dissolved in 1,2-propanediol was determined by HPLC/LSS to be greater than 96% following dosing of the animals.

Excretion of radioactivity in rats following administration of Cr3P is shown in Table 11. The rats dosed orally with [^{14}C]Cr3P dissolved in 1,2-propanediol excreted an average of 53% of the ^{14}C-dose in urine, 39% in feces, and 1.5% as CO_2 in breath in 24 h. No measurable dose was collected as volatiles in breath. An additional 8% of the ^{14}C-dose was excreted during the collections at 48 h and 52 h.

The results of the assay of total Cr excreted in urine and feces collected from animals in Study A are shown in Table 11. An average of 1.25 $\pm$ 0.24% and 97.5 $\pm$ 7.4% of the Cr dose was excreted in urine and feces, respectively, in 48 h.

The results of the HPLC assay of the 8 and 24 h urine collections are shown in Table 12. An average of 49.0 $\pm$ 1.2% the ^{14}C-dose received was excreted in 24 h as N-picolinoylglycine (NPG). An average of 1.9 $\pm$ 0.4% and 1.1 $\pm$ 0.7% of the ^{14}C-dose received was excreted in 24 h as picolinic acid and Cr3P, respectively.

The results of the assay of radioactivity in all tissues collected at 52 h postdosing from the four rats in Study A are shown in Table 13. Less than 1% of the ^{14}C-dose was found in these tissues. The results of total Cr analysis of selected tissues are shown in Table 14.

4.10 Study Ba: 17.4 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

The radiochemical purity of [^{14}C]Cr3P water slurry was determined by HPLC/LSS to be 95% following dosing of the animals.

Excretion of radioactivity in rats sacrificed at 48 h following administration of Cr3P is shown in Table 15. The rats dosed orally with [^{14}C]Cr3P water slurry excreted an average of 41% of the ^{14}C-dose in urine, 47% in feces, and 1.4% as CO_2 in breath in 24 h. An additional 3% of the ^{14}C-dose was excreted during the collections at 48 h. Excretion of radioactivity (including bladder contents) in rats sacrificed at 2 h following administration of Cr3P is shown in Table 16. The rats dosed orally with

[^{14}C]Cr3P water slurry excreted an average of 0.1% of the ^{14}C-dose in urine in 2 h. An additional 4.9% ^{14}C-dose was found in the bladder contents at sacrifice at 2 h.

Excretion of Cr from rats sacrificed at 48 h following administration of Cr3P is shown in Table 15. An average of 1.53 ± 0.51% and 97.6 ± 7.4% of the Cr dose was excreted in urine and feces, respectively, in 48 h.

The results of the assay of radioactivity in all tissues collected at 2 h postdosing from four rats and at 48 h postdosing from four rats in Study Ba are shown in Table 17. A total of 2.9% and less than 1% of the ^{14}C-dose administered were found in the non-GI tract tissues at 2 and 48 h, respectively. The GI tract tissues and contents of the animal sacrificed at 2 h contained an average of 92 ± 4.8% of the^{14}C-dose with 64 ± 12% of the dose contained in the small intestine and contents.

4.11 Study D: 0.22 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

The radiochemical purity of [^{14}C]Cr3P dissolved in 1,2-propanediol was determined by HPLC/LSS to be 95% following dosing of the animals.

Excretion of radioactivity in rats following administration of Cr3P is shown in Table 18. The rats dosed orally with [^{14}C]Cr3P dissolved in 1,2-propanediol excreted an average of 40% of the ^{14}C-dose in urine, 27% in feces, and 11% as CO_2 in breath in 24 h. No measurable dose was collected as volatiles in breath. An additional 5% of the ^{14}C-dose was excreted during the collections at 48 h and 52 h.

The results of the assay of radioactivity in tissues collected at 52 h postdosing from the four rats in Study D are shown in Table 19. An average of 2.8 ± 0.5% of the ^{14}C-dose was found in the tissues of these animals.

4.12 Study F: 17.4 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

The radiochemical purity of [^{14}C]Cr3P water slurry was determined by HPLC/LSS to be 95% following dosing of the animals.

The concentrations of radiolabel in stomach (and contents), small intestine (and contents), large intestine (and contents), and cecum (and contents) are shown in Table 20. At 1, 2, and 4 h postdosing, the average amount of radioactivity present in the GI tract was 90 ± 0.7%, 87 ± 1.1%, and 78 ± 1.8% of the ^{14}C-dose. At 1 h postdosing, the largest amount of ^{14}C-dose in the GI tract was found in the small intestine and its contents. By 4 h postdosing, most of the ^{14}C-dose in the GI tract was found in the cecum and its contents.

The results of the assay of blood, adipose, liver, and muscle for Cr3P are shown in Table 21. The average tissue-blood ratios (TBR) for liver at 1, 2, and 4 h postdosing were 2.8 ± 1.1, 3.9 ± 0.6, and 4.0 ± 1.0, respectively. The TBRs for adipose and muscle were 0.4 or less for all time points. Less than 1% of the ^{14}C-dose at any time point was found in these tissues.

The results of the assay by HPLC/LSS of the radioactivity extracted from blood, adipose, liver, and muscle are shown in Table 22. For all tissues and time points analyzed, the largest single component identified in each tissue was picolinic acid. The percent of radioactivity present as Cr3P was

greatest at 1 h for all tissues analyzed with Cr3P accounting for 11%, 14%, 24%, and 6% in blood, adipose, liver, and muscle, respectively.

The results of the assay of chromium in aliquots of tissues from this study are depicted in Figure 11. Concentrations of Cr in blood averaged 11.5, 10.6, and 9.0 ng/g at 1, 2, and 4 h, respectively, after dosing compared to 2 ng/g in undosed animals. Some of this chromium, 3.8, 2.1, and 0.7 ng/g, respectively, was accounted for by the chromium in Cr3P present in blood. Concentrations of chromium in the liver were approximately three times those in blood at each time point with approximately the same proportion accounted for by the chromium in Cr3P. The chromium concentrations in adipose were higher than those in either blood or liver. The chromium concentrations in muscle were highly variable with the concentration in muscle from undosed animals considerably higher than is reported in the literature by Anderson et al. (1996). For these reasons, we plan to confirm our results by analyzing samples form other animals.

4.13 Study Ha: 0.18 mg/kg Intravenous Dose of [^{14}C]Cr3P to Rats

The radiochemical purity of [^{14}C]Cr3P dissolved in 0.9% sodium chloride was determined by HPLC/LSS to be 95% following dosing of the animals.

The concentrations of radiolabel and Cr3P in blood following intravenous administration of Cr3P are shown in Table 23. The mean concentration of Cr3P in blood at 120 min following intravenous administration of [^{14}C]Cr3P was 6 $\pm$ 3 ng/g. For both rats in the pilot study, the concentration of radiolabel in blood at 5 h following intravenous administration of [^{14}C]Cr3P was 11 ng-eq Cr3P/g.

A plot of pharmacokinetic data from this study is shown in Figure 12. The mean half-life for the disappearance of Cr3P from rat blood was 24 min (n = 5, range was 20 to 35 min). The mean pseudo half-life for the disappearance of radiolabel from rat blood was 35 min (n = 5, range was 30 to 49 min). The concentration of radiolabel in blood at 5 h from the two pilot study animals is also shown in Figure 12.

The results of the assay of radioactivity in tissues collected at 120 min postdosing are shown in Table 24. An average of 8.7 $\pm$ 1.8% and 8.2 $\pm$ 1.0% of the administered radiolabel was present in non-GI tract tissues and a portion of the GI tract (small intestines, cecum, and large intestines plus contents), respectively, at 120 min postdosing.

4.14 Study Hb (PK): Single 17.8 mg/kg Oral Dose of [^{14}C]Cr3P to Rats

The radiochemical purity of [^{14}C]Cr3P water slurry was determined by HPLC/LSS to be 94% following dosing of the animals.

The concentrations of radiolabel and Cr3P in blood following oral administration of Cr3P water slurry are shown in Table 25. The mean concentration of Cr3P in blood 0.5 h after dose administration was 19 ng/g, close to the mean C_{max} value of 21 ng/g observed 2 h after dose administration. By 12 h postdose, the mean concentration of Cr3P in blood had fallen to 0.9 ng/g. Cr3P accounted for 7% of the total radiolabel in blood at 0.5 h postdose, but only 0.2% of the total radiolabel at 12 h postdose.

Pharmacokinetic data from this study is shown in Table 26 and plotted in Figure 13. The mean terminal half-life for the disappearance of Cr3P from rat blood was 153 min (n=4, range was 126 to

180 min). The mean pseudo half-life for the disappearance of radiolabel from rat blood was 8.5 h (n=4, range was 5.1 to 11.6 h).

Excretion of radioactivity in rats following oral administration of Cr3P in a water slurry is shown in Table 27. The rats excreted an average of 49% of the ^{14}C-dose in urine and 31% in feces in 24 h.

The results of the assay of radioactivity in aliquots of blood, liver, muscle, and adipose collected at 24 h postdosing are shown in Table 28. The average tissue-blood ratios (TBR) for liver, muscle, and adipose were 4.71 ± 0.84, 0.25 ± 0.04, and 0.19 ± 0.06, respectively. The remaining unanalyzed portions of these tissues are available to be analyzed for Cr or Cr3P.

4.15 Study J: Single 20.5 mg/kg Oral Dose of [^{14}C]Cr3P to Mice

The radiochemical purity of [^{14}C]Cr3P water slurry was determined by HPLC/LSS to be 91% following dosing of the animals.

Excretion of radioactivity in mice sacrificed at 48 h following administration of Cr3P is shown in Table 29. The mice excreted an average of 26% of the ^{14}C-dose in urine and 59% in feces in 48 h. Less than 0.1% of the ^{14}C-dose was excreted as CO_2 in breath in 48 h. An average of 0.2% of the ^{14}C-dose was recovered in the digested carcasses.

The results of the assay of total Cr excreted in urine and feces collected from animals in Study J are shown in Table 29. An average of 1.1 ± 0.7% and 105 ± 7.8% of the Cr dose was excreted in urine and feces, respectively, in 48 h.

The results of the HPLC assay of the 6, 12, and 24 h urine collections are shown in Table 30. An average of 22.3 ± 4.2% of the ^{14}C-dose was excreted in 24 h as N-picolinoylglycine (NPG), the urinary metabolite isolated and identified from rat urine. An average of 0.29 ± 0.05% and 0.51 ± 0.20% of the ^{14}C-dose was excreted in 24 h as picolinic acid and Cr3P, respectively. Also present in mouse urine was an additional metabolite, MUr1, that accounted for an average of 0.16 ± 0.12% of the ^{14}C-dose.

4.16 Study Jb: Single 19.0 mg/kg Oral Dose of [^{14}C]Cr3P to Mice

The radiochemical purity of [^{14}C]Cr3P dissolved in 1,2-propanediol was determined by HPLC/LSS to be 94% following dosing of the animals.

Excretion of radioactivity in mice sacrificed at 48 h following administration of Cr3P is shown in Table 31. The mice excreted an average of 42 ± 7.8% of the ^{14}C-dose in urine and 55 ± 5.6% in feces in 48 h.

The results of the assay of total Cr excreted in urine and feces collected from animals in Study Jb are shown in Table 31. An average of 3.9 ± 1.1% and 91.3 ± 12.7% of the Cr dose was excreted in urine and feces, respectively, in 48 h.

The results of the assay of radioactivity in tissues, GI tract and contents, and residual carcass collected at 48 h postdosing from the four mice in Study Jb are shown in Table 32. A total of less than 1% of the ^{14}C-dose was found in the tissues of these animals at 48 h postdosing.

The results of the HPLC assay of the 6, 12, 24 and 48 h urine collections are shown in Table 33. An average of 31.9 ± 7.6% of the ^{14}C-dose was excreted in 24 h as N-picolinoylglycine (NPG), the urinary metabolite isolated and identified from rat urine. An average of 1.3 ± 0.8% and 2.8 ± 1.0% of the ^{14}C-dose was excreted in 24 h as picolinic acid and Cr3P, respectively. Also present in mouse urine was an additional metabolite, MUr1, that accounted for an average of 0.25 ± 0.11% of the ^{14}C-dose.

4.17 Study PAa: 17.2 mg/kg Oral Dose of [^{14}C]Pcl-H to Rats

The radiochemical purity of [^{14}C]Pcl-H in water was determined by HPLC/LSS to be 97% following dosing of the animals.

Excretion of radioactivity in rats sacrificed at 6 h following administration of Cr3P is shown in Table 34. The rats dosed orally with [^{14}C]Pcl-H in water excreted an average of 83% of the ^{14}C-dose in urine and less than 1% in feces in 6 h. The results of the assay of radioactivity in all tissues collected at 6 h postdosing from these rats are shown in Table 35. A total of 1% and less than 3% of the ^{14}C-dose administered were found in the non-GI tract and GI tract tissues, respectively, at 6 h postdosing.

Animals in the other group died at ca. 20 h postdosing due to a malfunction of the breath (CO_2) collection system. Cumulative excretion of radioactivity in these rats is shown in Table 36. The rats dosed orally with [^{14}C]Pcl-H in water excreted an average of 93% of the ^{14}C-dose in urine, 1.7% in feces, and 0.3% as CO_2 in breath in ca. 20 h.

The major radioactive peak found in the urine of rats receiving [^{14}C]Pcl-H orally coeluted (on HPLC System 10) with N-picolinoylglycine (NPG), the major urinary metabolite of rats receiving [^{14}C]Cr3P orally.

4.18 Binding of Cr3P and Pcl-H Derived Radiolabel to Plasma Proteins and DNA/RNA

The binding of Cr3P and Pcl-H derived radiolabel was investigated because in previous experiments (see Table 22) the majority of radiolabel in plasma was not extracted by acetonitrile, a solvent that extracts Cr3P from plasma quantitatively (data not shown). Plasma obtained 2 h after a single dose of either [^{14}C]Cr3P or [^{14}C]Pcl-H was used for the study. Methanol extracted >90% of the radiolabel in all cases . Little to no binding of radiolabel to the precipitated protein fraction (that also contains the plasma DNA and RNA) was found.

4.19 Isolation and Identification of Urinary Metabolite

Only a single radiolabeled metabolite (Ur1) was excreted in rat urine following an oral dose of Cr3P. This metabolite was isolated and purified by HPLC. No apparent decomposition was observed during the purification process. Analysis of the isolated and purified metabolite for total Cr showed that Ur1 does not contain Cr. A sample of Ur1 that would have contained 75 μg-eq/L of Cr based on the Cr to ^{14}C ratio in Cr3P was found to contain less than 10 μg/L Cr (limit of quantitation of method).

The isolated urinary metabolite Ur1 and synthetic N-picolinoylglycine (NPG) coeluted when analyzed on two different HPLC columns. HPLC/MS spectra of NPG and Ur1 shown in Figures 3 and 4 each contained a base peak at m/z 181 $(NPG + H)^+$. HPLC/MS/MS experiments showed the m/z peak

181 in both spectra fragmented to produce major peaks at m/z 163 (loss of OH) and m/z 135 (loss of CHO_2).

5.0 DISCUSSION

Cr3P is a relatively insoluble compound that is not amenable to NMR analysis due to the paramagnetic nature of Cr^{+3} or to simple (direct probe) mass spectral analysis. Perhaps due to the relatively high cost of commercial Cr3P, many previous investigators have prepared their own Cr3P for use in toxicity and absorption studies. Unfortunately, sparse analytical work has been reported for these test chemicals. For instance, Anderson et al. (1996, 1997) reported no analytical data for the Cr3P he prepared and used in either of his feeding studies with rats. For other Cr complexes he compares to Cr3P, reaction mixtures containing unisolated, unanalyzed materials are given chemical names and used to dose the animals. Stearns et al. (1995) prepared Cr3P and isolated it from the reaction mixture after a reaction period of 24 h. She reports chromium content of the isolated Cr3P as the only analytical data. However, in her studies of "soluble" Cr3P, upon which much of her conclusions were drawn, she exposed the hamster ovary cells to the contents of the reaction mixture after a reaction period of an hour or less and without any analysis of its contents. Thus, the undefined or uncertain nature of the test chemicals used in these studies should introduce some caution in the interpretation of the study results.

"Standard" Cr3P was initially obtained from ICN. The material purchased from this supplier was found to contain no chromium and produced an uninterpretable mass spectrum. It was therefore not used. A second sample of "standard" Cr3P, obtained from Spectrum Quality Products, gave anticipated results for chromium content, an interpretable mass spectrum, and a single peak when chromatographed by HPLC using conditions similar to those reported by Evans and Pouchnik (1993). The Cr3P purchased from Spectrum appears identical (color, HPLC) to Cr3P we prepared from chromium chloride and picolinic acid by the procedure described by Evans and Pouchnik (1993).

Analysis of Cr3P from two different lots from Spectrum Quality Products, Inc. by the Karl Fischer method showed that the Cr3P we have used in these studies is primarily in the form of the monohydrate.

Evans and Pouchnik reported the preparation of Cr1P and Cr2P by combining 1 equivalent of chromium(III) trichloride with one or two equivalents of picolinic acid, respectively. These authors characterized their reaction products by HPLC and mass spectrometry, reporting different retention times for Cr1P, Cr2P and Cr3P. Because we needed to know that our HPLC system separated Cr3P from its likely metabolites and from decomposition products and impurities, we attempted to synthesize Cr1P and Cr2P by these authors' method. The only crystalline reaction product we obtained in any of the reactions was indistinguishable from Cr3P. Crystalline Cr3P was also produced when we repeated the synthesis reported by Anderson et. al. (1996), but the rate of crystalline product formation was much slower than reported by these authors.

Stearns and Armstrong (1992) report the preparation of a dimer of Cr2P, $[Cr(P)_2OH]_2$, when at least one equivalent of sodium hydroxide is present. Originally when we repeated this experiment, we obtained two intermixed crystalline products, one dark red (the color of Cr3P) and the other pink. The two

crystalline products, when separated by hand, produced two different HPLC peaks, the red crystals producing one identical to that of Cr3P, and the pink crystals a peak that was well resolved from Cr3P. A second attempt at the synthesis of $[Cr(P)_2OH]_2$ by the same method resulted in the formation of dark red (Cr3P) crystals and dark purple crystals ($[Cr(P)_2OH]_2$) that we were able to separate under magnification. The $[Cr(P)_2OH]_2$ produced from this reaction was characterized by mass spectral analysis. Again HPLC analysis showed that this dimer was well resolved from Cr3P itself.

The objective of the synthesis of other chromium-picolinic acid complexes was to show that our HPLC system was able to separate Cr3P from these complexes. Indeed, Cr3P is resolved from $[Cr(P)_2OH]_2$ by our HPLC methods, and we have shown that there is no detectable presence of $[Cr(P)_2OH]_2$ in the Cr3P used as the test chemical. We have also developed several other HPLC systems using three stationary phase chemistries and both neutral and acidic mobile phases. Using these differing HPLC systems, we have been able to manipulate the retention times of Cr3P as well as the relative retention times (and order of elution) of Cr3P and its urinary metabolites (cf. Figure 9). Picolinic acid is not retained on any of the HPLC systems developed for Cr3P.

Many previous investigators have reported the poor solubility of Cr3P and other complexes of Cr with picolinic and nicotinic acids. Even so, the reported solubility for Cr3P in water at pH 7 (0.6 mM) is more than an order of magnitude higher than the reported solubilities of Cr1P, Cr2P, or chromium bisnicotinate (Evans and Pouchnik, 1993). Because of these low solubilities and the reported difficulties in redissolving Cr3P and other chromium complexes, coupled with our need to develop dose formulations, extraction procedures, and perhaps purification methods, we determined the solubility of Cr3P in water (both at pH 7 and pH 3) and in selected organic solvents. The organic solvents were chosen for their potential utility in HPLC mobile phases, in dose formulations or as extraction solvents for tissues or marketed preparations of Cr3P. Solubilities were measured at room temperature (ca. 22 °C) after at least 30 min of dissolution time in an ultrasonic bath. Our measured solubility of Cr3P was identical to that reported by Evans and Pouchnik (1993). Of the solvents tested, Cr3P was most soluble in 1,2-propanediol (propylene glycol, which we have used as a dose vehicle) and methanol (used to extract tissues and Cr3P tablets and as part of the HPLC mobile phase). Cr3P was slightly more soluble in water at pH 7 than at pH 3 (stomach pH). Cr3P was practically insoluble in 2-propanol and in the more lipophilic solvents.

Poor agreement has been reported between the advertised content of many dietary supplements, particularly herbal preparations, and the actual content as determined by chemical analysis. The receipt of a bottle labeled "chromium picolinate" from ICN that on analysis was shown to be devoid of this chemical further raised the possibility that dietary supplements claiming to contain "chromium picolinate" may not contain this chemical at all or may contain amounts that were different from the stated amount. Thus samples of seven brands of "chromium picolinate" preparations, sold locally and thought to be available over a wide area, were analyzed for Cr3P by HPLC. All tested preparations were within 15% of their stated amounts of Cr (as Cr3P) with tablet to tablet CVs of 5% or better.

Previous reports from investigators who followed the disposition of chromium in animals given oral doses of organo-chromium(III) complexes, including Cr3P, have stated that chromium is very poorly (<5%) absorbed (Gargas, 1994; Gonzalez-Vergara et al., 1981), and that virtually all of the absorbed material is excreted in urine. Gonzalez-Vergara et al. (1981) investigated the absorption and excretion of chromium from several chelates and other complexes. They found that "urinary excretion (of chromium) was highest when chelated chromium (such as Cr3P) was administered," and that "this result suggests that chromium chelates are absorbed intact and are rapidly removed from circulation by the glomerulus as small molecules." Anderson et al. (1996) concluded from their work with [^{51}Cr]Cr3P that the absorbed chromium from Cr3P in blood is not in equilibration with chromium in tissues.

We conducted several pilot studies to guide us in the definitive work. These pilot studies consisted of in vitro stability studies, oral gavage studies, and IP dose studies.

Several sets of in vitro studies were conducted to assess the stability of Cr3P to rat stomach and intestinal contents. Incubation of Cr3P with rat stomach and small intestine contents indicated that Cr3P is stable to both during the time intervals that Cr3P could be expected to be present in the stomach and small intestine following oral dosing. Experiments with small intestine contents were conducted with the exclusion of oxygen. Because of the higher than expected absorption of radiolabel, but not Cr, following the oral doses of Cr3P, these in vitro experiments were repeated to ensure that our original conclusions as to the stability of Cr3P to stomach and intestinal contents were correct. The repeat experiments confirmed the original findings.

Following oral administration of a dose preparation of Cr3P dissolved in 1,2-propanediol, greater than 50% of the radiolabel is absorbed. Although most of the absorbed radiolabel is excreted in urine, ca. 8% of the dose was excreted as CO_2, showing that at least a portion of the radiolabel was no longer associated with chromium. Almost all of the radiolabel in an ip dose of Cr3P dissolved in 1,2-propanediol was excreted in urine. Thus, excretion in urine and breath is a reasonable measure of the extent of absorption of radiolabel from oral doses of Cr3P.

Unexpectedly, a dose formulation of Cr3P was found not to be stable in unbuffered water at pH 7. The radiochemical purity of a dose preparation stored overnight at ca. 4 °C decreased from 97% to 68%. Since animals had already been dosed with the impure aqueous solution of Cr3P before this instability was found, the information gained from these animals is not as precise as for the animals dosed with Cr3P in 1,2-propanediol. Based on urinary excretion of radiolabel, at least 50% of the radiolabel from orally administered aqueous Cr3P dose solution was absorbed. Even with a “worst case” assumption that all of the radiochemical impurities (ca. 30% of the dose) were absorbed, at least 20% of the radiolabel arising from Cr3P was also absorbed. Thus oral absorption of radiolabel from [^{14}C]Cr3P dissolved either in 1,2-propanediol or in water is much higher than that reported previously for ^{51}Cr. (This observed instability has not been observed for subsequent aqueous preparations of Cr3P).

The unexpectedly high oral absorption of radiolabel and subsequent excretion of this radiolabel in urine eliminates the potential issue of contamination of urine with water-soluble radiolabel excreted in

feces. In an experiment in which a fecal pellet from an animal given an oral dose of Cr3P was placed in the metabolism cage of an undosed animal and left there for 24 h, less than 1% of the radiolabel was extracted by urine flowing over the pellet.

To provide further information as to the nature of the Cr3P-derived chemicals that tissues are exposed to in vivo, blood was collected from two rats given an oral dose of Cr3P in 1,2-propanediol. One animal was sacrificed 1 h postdosing, and the second animal was sacrificed 2 h postdosing. The concentration of ^{14}C in blood for each of the samples collected 1 or 2 h after an oral dose or 4 h after an ip dose of Cr3P was approximately half the concentration of ^{14}C in plasma derived from that sample (cf. Table 10). Since the hematocrit of rat blood is approximately 50%, this result is evidence that the radiochemicals present in blood are only in the plasma fraction and have not penetrated into red blood cells. The concentrations of radiolabel in tissues 72 h after an oral dose of Cr3P were unremarkable.

The results of the oral pilot studies were confirmed with doses of Cr3P dissolved in 1,2-propanediol. In studies in rats with doses of 0.22 (D) and 15.3 (A) mg/kg, averages of 57 and 60% of the administered radiolabel, respectively, were absorbed and excreted in urine and breath (53–59%) or remained in tissues 52 h postdose (1–2%). In a comparable study in mice (Jb), mice dosed orally with [^{14}C]Cr3P dissolved in 1,2-propanediol at 19.0 mg/kg (743 μCi/kg) absorbed and excreted 42% of the dose in urine. (Exhaled breath was not collected in this study with mice since mice dosed with [^{14}C]Cr3P water slurry excreted less than 0.1% as CO_2 in breath.) Using excretion in urine and breath as a measure of the extent of adsorption of radiolabel from oral doses of Cr3P, radiolabel appears to be more readily absorbed in rats than in mice.

The major radiolabeled urinary metabolite following oral administration of Cr3P in rats and mice has been identified as N-picolinoylglycine (NPG), the glycine conjugate of picolinic acid. NPG is also present in the urine of healthy humans (Liebich and Foerst, 1990; Pinkston et al., 1981).

Urinary excretion of radiolabel following the ip dose of [^{14}C]Cr3P in rats was partially as parent and partially as metabolites. Excretion of radiolabel following oral doses of [^{14}C]Cr3P in rats and mice was primarily in the form of metabolites. Following an oral dose at 15.3 mg/kg (181 μCi/kg) [^{14}C]Cr3P dissolved in 1,2-propanediol (A) in rats, approximately 50% of the ^{14}C-dose received was excreted in urine as NPG. An additional 1.9 ± 0.4 and 1.1 ± 0.7% of the ^{14}C-dose received was excreted in urine as picolinic acid and Cr3P, respectively. Following an oral dose at 20.5 mg/kg (623 μCi/kg) [^{14}C]Cr3P in water slurry (J) in mice, 22.3 ± 4.2% of the ^{14}C-dose was excreted in urine as NPG. The remaining ^{14}C-dose excreted in urine, 0.29 ± 0.05, 0.51 ± 0.20 and 0.16 ± 0.12% dose received, co-eluted with picolinic acid, Cr3P, and an unidentified urinary metabolite (MUr1), respectively. Similar results were found following an oral dose at 19.0 mg/kg (743 μCi/kg) [^{14}C]Cr3P in 1,2-propanediol (Jb) in mice. In this study, 31.9 ± 7.6% of the ^{14}C-dose was excreted in urine as NPG. The remaining ^{14}C-dose excreted in urine, 1.3 ± 0.8, 2.8 ± 1.0 and 0.25 ± 0.11% dose received, co-eluted with picolinic acid, Cr3P, and an unidentified urinary metabolite (MUr1), respectively. MUr1 does not appear to be present in rat urine.

Following intravenous administration of [^{14}C]Cr3P at 0.18 mg/kg (22 μCi/kg) (Ha), the mean half-life of Cr3P in blood was 24 min. The mean concentrations of Cr3P in blood at 60 and 120 min following intravenous administration of [^{14}C]Cr3P at 0.18 mg/kg were 25 and 6 ng/g, respectively. The TBR in liver collected at 120 min is 3. A pilot study was performed using two animals that had been prepared as study extras and not needed for the conduct of the main study. The purpose of the pilot study was to gain information about the concentration of radiolabel in blood following intravenous administration at a time point later than those included in the main study. The concentration of radiolabel in blood at 5 h following intravenous administration of [^{14}C]Cr3P for both rats in the pilot study was determined to be 11 ng-eq Cr3P/g.

An unexpectedly high percentage of the administered radiolabel, 8.2 ± 1.0%, was present in the GI tract at 120 min postdosing.

Three studies have been performed in rats using single oral doses of ca. 17.5 mg/kg of [^{14}C]Cr3P as aqueous slurries. A study has also been performed in mice using a single oral dose of 20.5 mg/kg (623 μCi/kg) of [^{14}C]Cr3P as an aqueous slurry (J). The slurries were prepared by freeze-drying aqueous solutions of [^{14}C]Cr3P followed by taking the residue up in volumes of water appropriate for oral gavage to the study animals. Preparation of slurries by this method in the past has resulted in very small particle sizes, hopefully aiding in the absorption of the chemical from the GI tract.

In the first study (Ba), rats dosed orally with [^{14}C]Cr3P water slurry at 17.4 mg/kg (172 μCi/kg) excreted an average of 40% of the ^{14}C-dose in urine, 46% in feces, and 1.3% as CO_2 in breath in 24 h. An additional 4% of the ^{14}C-dose was excreted during the collections at 48 h. A total of 2.9% and less than 1% of the ^{14}C-dose administered were found in the non-GI tract tissues at 2 and 48 h, respectively. The GI tract tissues and contents of the four animals sacrificed at 2 h contained an average of 92 ± 5% of the^{14}C-dose with an average of 64 ± 12% of the dose contained in the small intestine and contents.

In a comparable excretion study in mice (J), mice dosed orally with [^{14}C]Cr3P water slurry at 20.5 mg/kg (623 μCi/kg) excreted an average of 24% of the ^{14}C-dose in urine, 58% in feces, and less than 0.1% as CO_2 in breath in 24 h. In mice, an additional 3% of the ^{14}C-dose was excreted during the collections at 48 h. Again, as was seen when comparing oral doses of Cr3P dissolved in 1,2-propanediol using excretion in urine and breath as a measure of the extent of adsorption of radiolabel from oral doses of Cr3P, radiolabel appears to be more readily absorbed in rats than in mice.

The second study (Hb) in rats was conducted to determine the oral pharmacokinetics of Cr3P from an aqueous slurry and to determine the most suitable time points for analysis of Cr3P in tissues. Higher amounts of radiolabel (472 μCi of Cr3P per kg body weight) were required for this study than were used in the excretion study in rats described above. Following oral administration of an aqueous slurry of [^{14}C]Cr3P (17.8 mg/kg), blood C_{max} values for Cr3P (mean = 21 ng/mL) were only 30% of the Cr3P concentration found 0.5 h after an iv dose at 0.18 mg/kg. The oral bioavailability of Cr3P in the aqueous slurry, calculated from blood AUC data (oral vs. iv) is 0.7% (Table 26). The terminal elimination half-life of Cr3P after oral administration (153 min) is approximately six times longer than that observed following iv

administration (24 min). The longer half-life after oral administration is likely due to very slow absorption of Cr3P from the gut. The proportion of total radiolabel in blood attributable to Cr3P is much lower following oral administration as compared with iv administration of Cr3P, further evidence that Cr3P is degraded in the gut prior to adsorption of the radiolabel. Common time points examined following the two dosing routes were 0.5, 1, and 2 h.

Rats in the PK study excreted an average of 49% of the ^{14}C-dose in urine in 24 h, slightly more than the 41% dose that was excreted in urine in 24 h by rats in the first aqueous slurry dose. Conversely, less dose was excreted in feces in 24 h by rats in the PK study (31%) compared with those in the excretion study (47%). Radiolabel remaining in the GI tract was not measured in rats in the PK study.

A third study (F) was conducted in rats to measure the tissue concentration of Cr3P at 1, 2, and 4 h following oral administration of [^{14}C]Cr3P water slurry at 17.4 mg/kg (482 μCi/kg). The specific activity of the [^{14}C]Cr3P used in this study was comparable to that used in the oral PK study. The concentrations of Cr3P as well those of picolinic acid and NPG, the previously identified urinary metabolites of Cr3P, were determined in blood, liver, adipose, and muscle at 1, 2, and 4 h. For all tissues and time points, the amount of ^{14}C-dose found in tissue was less than 1%. Liver was determined to be the tissue with the highest concentrations of Cr3P with mean concentrations of 78 ± 10, 66 ± 12, and 23 ± 7.1 ng/g of tissue at 1, 2, and 4 h, respectively. The Cr3P concentrations in blood for the same time points were 31 ± 8.7, 17 ± 1.1, and 6.0 ± 1.7 ng/g of tissue, respectively. The mean concentrations of Cr3P in adipose and muscle were all 6 ng/g of tissue or less.

Additionally, the amount of radioactivity in the GI tract at sacrifice at 1, 2, and 4 h postdosing was determined. At 1, 2, and 4 h, 90, 87, and 78% of the ^{14}C-dose, respectively, was still present in the GI tract. In the first of these three studies involving oral administration of [^{14}C]Cr3P as aqueous slurries, the GI tract tissues and contents of the animals sacrificed at 2 h contained an average of 92% of the^{14}C-dose with an average of 64% contained in the small intestine and contents.

In the studies (A and Ba in rats and J and Jb in mice) where chromium content was determined in urine and feces, chromium was almost entirely excreted in feces (a range of 91 to 105% of the chromium dose administered) with very little chromium was excreted in urine. Following administration of Cr3P dissolved in 1,2-propanediol, an average of 1.3 ± 0.2% and 3.9 ± 1.1% of the administered chromium was absorbed and excreted in urine in rats and mice, respectively. For Cr3P water slurry, an average of 1.5 ± 0.5% and 1.1 ± 0.7% of the administered chromium was absorbed and excreted in urine in rats and mice, respectively.

It thus appears that the majority of the Cr3P complex is broken apart at or very near to the intestinal wall. The resultant picolinic acid is absorbed into the systemic circulation and excreted primarily as NPG, although a measurable amount is converted to CO_2. The chromium is almost all returned to the intestine and excreted in feces. Studies on the EDTA complex with ^{51}Cr, used as a measure of intestinal damage (Bjarnason et al., 1985; Choi et al., 1995), indicate that it, or the chromium in it, crosses the

intestinal wall in a paracellular mode. In healthy individuals, approximately 2% of the administered chromium is excreted in urine. The nature of the absorbed species has not been reported.

Although it is well accepted that chromium is generally excreted from the systemic circulation in urine, no data had been presented to identify the route of excretion of Cr from Cr3P. We measured Cr in the feces from animals (pilot study) given ip doses of Cr3P. Less than 5% of the administered Cr was excreted in the feces of these animals. Little to no Cr from Cr3P absorbed into the systemic circulation would be expected to be excreted in feces.

Adipose, blood, and liver taken 1 h to 4 h postdose from animals that had been given a single oral dose of [^{14}C]Cr3P had concentrations of chromium that were 2-6 times those of undosed animals. (Concentrations of chromium in muscle were quite variable and are not deemed sufficiently reliable for conclusions to be formed.) Chromium concentrations in these tissues were higher than that attributable to the concentrations of Cr3P in the same tissue samples. This result shows that small amounts of chromium are delivered to tissues by oral doses of Cr3P that is not eliminated from the tissues as Cr3P. As seen in Table 14, concentrations of Cr in tissues taken 52 h postdose from animals given a single 15.3 mg/kg dose of [^{14}C]Cr3P were close to background and/or close to or below the limit of quantitation of the method. Background concentrations of Cr are approximately equivalent to 1% of the 15.3 mg/kg dose of Cr3P distributed evenly throughout the rat's body. It is unlikely that measurable Cr concentrations will be found in tissues taken at the same postdose time from animals given lower doses of Cr3P.

The radiochemical purity of the [^{14}C]Cr3P in the water slurry (J) used to dose mice had decreased to 91%, an unacceptably low purity. Additional aqueous slurry dose formulation prepared with purified [^{14}C]Cr3P degraded rapidly. For this reason, the oral excretion study in mice was repeated using a solution of [^{14}C]Cr3P in 1,2-propanediol (Jb). The radiochemical purity of the [^{14}C]Cr3P dissolved in 1,2-propanediol was determined to be 94%. Averages of 25.5 $\pm$ 3.0% and 42.0 $\pm$ 7.8% of the administered radiolabel were absorbed and excreted in urine in 48 h following administration of Cr3P water slurry and Cr3P dissolved in 1,2-propanediol, respectively. Even with a "worst case" assumption that the radiochemical impurities were absorbed at a rate very different from Cr3P itself, radiolabel appears to be more readily absorbed in mice when Cr3P is administered as a solution in 1,2-propanediol than as water slurry. These results are similar to those obtained in rats that received comparable oral doses of Cr3P in water slurry (Ba) or Cr3P dissolved in 1,2-propanediol (A), where the radiochemical purity of the dose in both studies was greater than 95%. For these studies, averages of 43.4 $\pm$ 3.5% and 56.3 $\pm$ 0.9% of the administered radiolabel were absorbed and excreted in urine in 48 h following administration of Cr3P water slurry and Cr3P dissolved in 1,2-propanediol, respectively.

The binding of Cr3P and Pcl-H derived radiolabel to macromolecules in plasma was investigated because of the reports that in vitro Cr3P produces chromosomal aberrations (Stearns et al., 1995; Speetjeans, et al., 1999) and because there was evidence in our studies that the majority of radiolabel in plasma was not extracted by acetonitrile, a solvent that extracts Cr3P from plasma. We determined that

our observations were not due to appreciable binding of Cr3P, Pcl-H, or their metabolites to plasma macromolecules but with the solvent used in the extraction process. Methanol, a solvent used routinely in the isolation of plasma protein and DNA/RNA, extracts the Cr3P and Pcl-H derived radioactivity away from the protein–DNA/RNA precipitate. Unfortunately, Cr3P is not stable in methanol in the presence of extracted plasma components.

In study PAa, animals were dosed orally with [^{14}C]Pcl-H at a dose level comparable to the high dose levels of [^{14}C]Cr3P to provide more information about the absorption of the picolinic acid portion of the Cr3P molecule. A group of 4 rats dosed orally with [^{14}C]Pcl-H in water at 17.2 mg/kg (36 μCi/kg) excreted an average of 83% of the ^{14}C-dose in urine and less than 1% in feces in 6 h. A total of 1% and less than 3% of the ^{14}C-dose administered were found in the non-GI tract and GI tract tissues, respectively, at 6 h postdosing. A second group of 4 rats also dosed orally with [^{14}C]Pcl-H in water at 17.2 mg/kg (36 μCi/kg) excreted an average of 93% of the ^{14}C-dose in urine, 1.7% in feces, and 0.3% as CO_2 in breath in ca. 20 h.

Absorption and subsequent excretion of ^{14}C in urine following [^{14}C]Cr3P administration appears to be related to the availability of the picolinic acid in the dose. Rats dosed orally with [^{14}C]Cr3P water slurry at 17.4 mg/kg, [^{14}C]Cr3P dissolved in 1,2-propanediol at 15.3 mg/kg, or [^{14}C]Pcl-H in water at 17.2 mg/kg excreted an average of 40%, 53%, and 93%, respectively, of the ^{14}C-dose in urine in ca. 24 h. Mice dosed orally with [^{14}C]Cr3P water slurry at 20.5 mg/kg or [^{14}C]Cr3P dissolved in 1,2-propanediol at 19.0 mg/kg excreted an average of 26% and 42%, respectively, of the ^{14}C-dose in urine in 48 h.

The major radioactive peak found in the urine of rats receiving [^{14}C]Pcl-H orally coeluted with N-picolinoylglycine (NPG), the major urinary metabolite of rats and mice receiving [^{14}C]Cr3P orally.

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Appendix A

Protocol and Protocol Amendments

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PROTOCOL	RESEARCH TRIANGLE INSTITUTE POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI 6855-15 Page 1 of 16~~14~~ Effective July 1999

TITLE: DISPOSITION AND METABOLISM OF [^{14}C]CHROMIUM TRISPICOLINATE IN RATS AND MICE FOLLOWING ORAL AND INTRAVENOUS EXPOSURE

SPONSOR:
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Chemistry and Life Sciences
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PROPOSED STUDY DATES: July – December 1999

RTI STUDY DIRECTOR: A. Robert Jeffcoat, Ph.D.

RTI PROJECT NO: 64U-06855-15

SPONSOR STUDY NO: N01-ES-75407

AMENDMENTS:

No.	Date	Section	Pages
1			
2			
3			
4			
5			

PROTOCOL	RESEARCH TRIANGLE INSTITUTE POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	RTI 6855-15 Page 2 of 16~~14~~ Effective July 1999

1.0 INTRODUCTION

Chromium trispicolinate (chromium tripicolinate, chromium picolinate, Cr3P) is a synthetic compound that has widespread use as a nutritional supplement. Although chromium trispicolinate is often referred to as chromium picolinate, two other chromium picolinate complexes have been reported (Evans and Pouchnik, 1993), chromium monopicolinate (Cr1P) and chromium bispicolinate (Cr2P). For clarity, the term chromium trispicolinate will be used when it is known that there are three picolinic acid ligands per atom of chromium.

Chromium (Cr) is an essential element to life, being required for proper glucose metabolism and potentiation of the action of insulin (for reviews, see Schroeder, 1968; Mertz, 1969; National Research Council, 1989; USEPA, 1984; Kahn et al., 1990; Gargas, et al, 1994). Only 2-3 % of dietary chromium is absorbed systemically (Anderson et al., 1993; Gargas et al., 1994). Because of this low systemic bioavailability of chromium from dietary sources, forms of chromium having higher bioavailability were sought. Cr3P is claimed to be such a compound although Gargas et al. (1994) reported that the extent of absorption of Cr3P by adult volunteers was 2.8 ± 1.1%.

The preparation and use of picolinate complexes of essential metals are covered by USDA patents # 4,315,927 (Evans, 1982) and reissue # 33,988 (Evans, 1992). Gary W. Evans is listed on the patent and its reissue as the sole inventor. The patent rights have been leased to Nutrition 21, a subsidiary of AMBI. No longer at USDA, Dr. Evans continues as a major proponent of the use of Cr3P, touting its purported beneficial effects for fat loss, for hypoglycemia, for lower cholesterol, for muscle mass, for insulin control and for longer life (Evans, 1996). Evans (1996) also states that "If the product (chromium picolinate) you buy doesn't have one of these [patent] numbers (as listed above), it may be completely ineffective." A recent report (McLeod et al., 1999) suggests that nicotinate and picolinate complexes of Cr may have antidepressant activity in humans.

Anderson et al. (1997) administered Cr3P to rats in their diet over 20 weeks and at concentrations of 5, 25, 50, and 100 µg Cr/g diet. No toxicity was observed. At the end of the treatment period, concentrations of Cr in the livers were proportional to the concentrations of Cr in the diet; rats fed

diets containing 5 and 100 μg Cr/g diet had concentrations of Cr in livers of 26 and 542 ng Cr/g dry weight, respectively. Cr concentrations in kidneys were 4-5 times those measured in livers (Anderson et al., 1993), possibly because absorbed Cr is excreted almost entirely in urine.

In another study Anderson et al. (1996) incorporated Cr3P, chromium (tris?)nicotinate (Cr3N), or complexes of Cr^{+3} and mixtures of several amino acids into rat chow at concentrations of 5 μg Cr/g diet. Groups of weanling rats were then fed these diets for 3 weeks. Concentrations of Cr in liver following exposure to diets containing no added Cr, Cr3P or Cr3N were 4, 50, and 13 ng/g dry weight whereas concentrations of Cr in kidneys from the rats were 23, 368, and 166 ng/g dry weight, respectively. In heart and leg muscle, Cr concentrations above those of controls were found in animals treated with Cr3P, but not with Cr3N. It thus appears that exposure to Cr3P and Cr3N leads to somewhat different patterns of Cr concentrations in tissues.

In an in vitro assay with Chinese hamster ovary cells, Cr3P, but not Cr3N, produced chromosomal aberrations that were 3 to 18 times that of controls (Stearns et al., 1995).

The purposes of the present study are to determine the stability of Cr3P to gastrointestinal conditions, the absorption of Cr3P into the systemic circulation, and the fate of both the Cr and the picolinate parts of Cr3P.

The following sections describe studies of Cr3P to be carried out in rats. Studies in mice and other studies in rats will be decided based on the results of the studies described herein and will be added by amendment.

2.0 PERSONNEL

Dr. A. Robert Jeffcoat - Study Director

Ms. Judy Hill - Chemist

Other personnel will be used as required. A full list of study participants will be included in the study report.

3.0 TEST MATERIAL

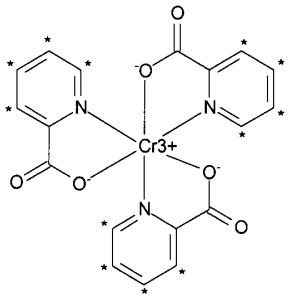
Name: Chromium trispicolinate (chromium tripicolinate, chromium picolinate, Cr3P)

CAS Registry Number: 14639-25-9

Molecular Formula: $C_{18}H_{12}CrN_3O_6$

Formula Weight: 418.3

Structure:



*Position of radiolabel

3.1 Nonradiolabeled Cr3P

Source: To be listed in the final report.

Lot No.: To be listed in the final report.

Identity: The identity of the nonradiolabeled Cr3P will be confirmed by mass spectrometry.

3.2 [^{14}C]Cr3P

Source: Wizard Laboratories

Specific Activity: 52.0 mCi/mmol of Cr3P (17.3 mCi/mmole of each of the three picolinate ligands)

Lot No.: 990415

Identity: The identity of [^{14}C]Cr3P will be confirmed by chromatographic comparison with nonradiolabeled Cr3P.

Purity: The radiochemical purity of [^{14}C]Cr3P will be determined chromatographically.

4.0 ANIMALS

Species and Strain: The test animals will be Fischer 344 rats and B6C3F$_1$ mice.

Sex and Weight: Male rats, 200-280 g; other animals to be added by amendment

Number: Rats, 4 per dose group

Source: Charles River Laboratories, Inc., Raleigh, NC

Justification for Use of Fischer Rats and B6C3F$_1$ Mice: These strains/species will be used in toxicity studies of this test material sponsored by the NTP, and are extensively used as mammalian models. No *in vitro* techniques are available that allow modeling of the metabolism and excretion of this test material by mammals.

4.1 Husbandry

Research Triangle Institute is accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC International). Animal procedures detailed in this protocol are in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996).

4.1.1 Identification

Animals will be identified by individual ear tags. Metabolism chambers will be individually coded by number and color.

4.1.2 Housing

Prior to initiation of the experiments detailed in Section 5.0, rodents will be housed (maximum of 4 per cage) in polycarbonate cages with stainless steel bar lids accommodating a water bottle. Cage sizes are 19" x 10 1/2" x 8" high (143 sq. in. floor space) for rats and 11.5" x 7 1/2" x 5" high (70 sq. in. floor space) for mice. Contact bedding will be Sani-Chips hardwood chips, P. J. Murphy Forest Products Co., Montville, NJ.

During acclimation and following dosing, animals will be housed individually in all-glass Roth-type metabolism chambers that provide for separate collection of urine, feces, CO_2, and expired volatile organic compounds.

4.1.3 Food and Water

Certified Purina Rat Chow (5002) and tap water will be provided *ad libitum*.

4.1.4 Environmental

Air circulation will be 100% fresh filtered air with 12-15 air changes per hour. Fresh outside air is first drawn through an Am-Air 300 prefilter (American Air Filter; Louisville, Kentucky) then through a #6-52C20 High-Flow Filter (Cambridge, Inc.; Syracuse, NY). Room temperature will be maintained at 72 ± 4°F and relative humidity at 35-70% and monitored at least once a day. Light/darkness will be cycled at 12 h intervals.

4.1.5 Acclimation and Quarantine

Rats and mice will be quarantined for a minimum of one week before use on a study.

4.2 Anesthesia and Sacrifice

Anesthesia will be used to avoid undue pain or distress. Rats will be anesthetized with an intramuscular injection of ketamine/xylazine (7:1, 60 mg/kg or to effect) prior to terminal blood collection. Similarly, mice will be anesthetized with an intraperitoneal injection of sodium pentobarbital (180 mg/kg). Rats and mice will be sacrificed by carbon dioxide asphyxiation.

5.0 STUDY DESIGN AND PROCEDURES

In a pilot study, the reaction of Cr3P with stomach contents and small intestine contents from rats will be determined by mixing a formulation of Cr3P with contents at 37°C, then sampling and analyzing for Cr3P following incubation for 1 h (both matrices), 2 h (both matrices), and 4 h (small intestine contents only). Collection of and incubations with intestine contents will be performed under anaerobic conditions.

In a second pilot study, oral and ip doses of Cr3P will be formulated in water or 1,2-propanediol at a dose volume of 5 mL per kg body weight and administered to male rats at dose levels of ca. 2 mg/kg. Urine, feces and radiolabeled components in breath will be collected from these animals to determine (1) if ^{14}C in breath is a significant route of excretion following administration of Cr3P and (2) the major routes of excretion of Cr3P from the systemic circulation, and (3) if urine collected from animals receiving Cr3P will be contaminated by contact with feces that may contain much higher (100 times) levels of ^{14}C.

Other in vitro studies will be conducted as needed. For example, appropriate experiments will be performed to determine the stability of Cr3P in matrices such as water and plasma.

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In vivo studies to be performed in rats are listed below and detailed in Table 1. Studies to be performed in mice will be determined later and incorporated by amendment.

Studies ~~will be conducted~~ to determine ~~and compare~~ the disposition of Cr3P in rats ~~and mice and~~ will include:

High single oral dose

~~A.~~A. ~~Determination of the a~~Absorption ~~and~~, excretion, and metabolism of a single oral dose ~~of Cr3P in male rats following oral administration~~ of 17.5 mg/kg Cr3P dissolved in propanediol ~~[17.5 mg/kg Cr3P (2.2 mg/kg Cr), 5mL/kg]~~.

~~B.~~B. ~~Determination of the a~~Absorption ~~and~~, excretion, and metabolism of a single oral dose ~~of Cr3P in male rats following oral administration~~ of 17.5 mg/kg Cr3P as a slurry in water ~~[17.5 mg/kg Cr3P (2.2 mg/kg Cr), 5mL/kg]~~.

~~C.~~F. ~~Determination~~Distribution of Cr3P and total Cr in selected tissues over time following oral administration of 17.5 mg/kg Cr3P in either 1,2-propanediol or water (depending on the results of A and B).

Mid single oral dose

C. ~~Determination of the a~~Absorption ~~and~~, excretion, and metabolism of a single oral dose following oral administration of 2 mg/kg Cr3P dissolved in 1,2-propanediol.

~~D.~~**Low single oral dose**

D. ~~Determination of the a~~Absorption ~~and~~, excretion, and metabolism of a single oral dose following oral administration of 0.2 mg/kg Cr3P dissolved in 1,2-propanediol.

E. ~~Determination of the a~~Absorption ~~and~~, excretion, and metabolism of a single oral dose following oral administration of 0.2 mg/kg Cr3P dissolved in water.

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Single intravenous dose~~Determination of Cr3P and total Cr in selected tissues following oral administration of Cr3P to male rats at 17.5 mg/kg Cr3P in either propanediol or water depending on the results from the comparison of the two dose vehicles.~~

~~F.~~G. ~~Determination of the~~Metabolism and excretion of an iv dose of Cr3P ~~in male rats~~ at a dose that is comparable to one of the lower oral doses above.

~~G.~~H. ~~Determination of b~~Blood and tissue concentrations of total ^{14}C, C r, Cr3P and radiolabeled metabolites over time after an iv dose of Cr3P to male rats.

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Table 1. Studies to be Performed in Male F-344 Rats

Study ID	Route (length)	Target Dose[1]	Vehicle[2]	Items to be Measured[3]					
				Urine	Feces	Breath[4]	Blood/ Plasma (sac)	Blood/ Plasma (timed)	Tissues[5]
A	oral (72 h)	17.5 mg/kg Cr3P (2.2 mg/kg Cr),	PG	^{14}C, Cr, Cr3P, profile	^{14}C, Cr, Cr3P,	^{14}C, $^{14}CO_2$,	^{14}C, Cr, Cr3P, profile		^{14}C, Cr, Cr3P, profile
B	oral (72 h)	17.5 mg/kg Cr3P (2.2 mg/kg Cr)	slurry in water	^{14}C, Cr, Cr3P, profile	^{14}C, Cr, Cr3P,	$^{14}CO_2$,	^{14}C, Cr, Cr3P, profile		^{14}C, Cr, Cr3P, profile
C[6]	oral (72 h)	2 mg/kg Cr3P	PG	^{14}C, Cr, Cr3P, profile	^{14}C,	$^{14}CO_2$,	^{14}C, Cr, Cr3P, profile		^{14}C, Cr,
D	oral (72 h)	0.2 mg/kg Cr3P	PG	^{14}C, Cr, Cr3P, profile	^{14}C, Cr, Cr3P,	^{14}C, $^{14}CO_2$,	^{14}C, Cr, Cr3P, profile		^{14}C, Cr, Cr3P, profile
E	oral (72 h)	0.2 mg/kg Cr3P	water	^{14}C, Cr, Cr3P, profile	^{14}C, Cr, Cr3P,	$^{14}CO_2$,	^{14}C, Cr, Cr3P, profile		^{14}C, Cr, Cr3P, profile
F	oral (TBD[7]; may be multiple)	17.5 mg/kg Cr3P	PG or water	^{14}C,			^{14}C, Cr, Cr3P, profile		^{14}C, Cr, Cr3P, profile
G[8]	iv (72 h)	TBD[9]	saline	^{14}C, Cr, Cr3P, profile		$^{14}CO_2$,	^{14}C, Cr, Cr3P, profile		^{14}C, Cr, Cr3P, profile (?)
H[8]	iv (TBD[7]; may be multiple)	TBD[9]	saline	^{14}C, Cr				^{14}C, Cr, Cr3P, profile	^{14}C, Cr, Cr3P, profile

Notes

1. oral doses will be given in a volume of 5 mL/kg
2. PG = 1,2-propanediol
3. ^{14}C = total radioactivity, Cr = total chromium, Cr3P = parent Cr3P, profile = profile of Cr3P metabolites containing ^{14}C
4. if >0.5% dose is exhaled as ^{14}C-organics in studies A or D , they will also be measured in studies B, C, E and G
5. analysis of tissues for Cr, Cr3P and metabolite profile will be carried out on selected tissues chosen for each study in collaboration with the project officer.
6. study C may be eliminated if no differences are seen between the results of studies A and D.
7. sampling times to be selected in collaboration with project officer based on results of other studies listed above
8. study may be modified if Cr3P is very unstable in blood or is decomposed after oral dosing before it enters the systemic circulation

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9. dose to be selected in collaboration with the project officer and based on results of other studies listed above
~~H.Comparison of the disposition of Cr3P in male mice to male rats (dose(s) to be chosen after consideration of the data from the experiments above).~~

5.1 Formulation and Administration of Cr3P

In studies A-H, o~~O~~ral ~~and ip (rat pilot study only)~~ doses of Cr3P will be formulated in water (pH ca. 3) or 1,2-propanediol at a dose volume of 5 mL per kg body weight and administered at dose levels of 0.2, 2 and 17.5 mg/kg. Animals will receive between 5 and 250 μCi/kg of [^{14}C]Cr3P depending on the dose level and whether tissues will be collected for HPLC and Cr analysis. Oral doses will be administered from a syringe fitted with a 16 gauge ball-tipped feeding needle~~, and ip doses will be administered from a syringe fitted with a 25 gauge hypodermic needle~~. Intravenous doses will be formulated in saline and administered from a syringe fitted with a 27 gauge hypodermic needle.

5.2 Analysis of Radiolabeled Dose Formulations

The weight and radiochemical content of each radiolabeled oral dose will be measured at RTI on the day of dosing. Radiochemical concentration will be determined by scintillation spectrometric analysis of dose aliquots taken before, during and after the dosing of the animals. Radiochemical purity will be determined by HPLC analysis of an aliquot of the dosing solution.

5.3 Collection and Storage of Biological Samples

Urine and Feces: Urine and feces will be collected separately into receivers cooled over dry ice. ~~For studies A and B, c~~Collections will be made initially at 8 (urine only), 24, 48, and 72 h following the radiolabeled dose unless the results of the pilot study indicate that these times are inappropriate. The excreta collection times for ~~studies C, D, E and F will depend of the results from studies A and B~~the remaining studies may be adjusted with the consent of the project officer. The weight of urine and/or feces collected for each sample interval will be determined. Samples will be stored in the dark at -20 ± 5°C until analyzed.

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Breath: In the ~~in-life pilot study~~initial studies, radiolabeled components in breath will be collected continuously after dosing by passing the air from the metabolism cage (flow = 200-500 mL/min) first through volatile traps, and then through CO_2 traps. The volatile traps will each contain ca. 60 mL of ~~propanediol~~ethanol. The first ethanol trap will be cooled with ice at 4°C, the second trap will be cooled in isopropanol/dry ice at ca. -60°C. The air will next be drawn through two CO_2 traps, each containing ca. 500 mL of 1N NaOH. Traps will be changed at 4~~8~~, 24, 32, 48, 56, and 72 h post dosing. If < 0.2 % of the dose is contained in collections ending at 24 h or later, trapping of exhaled material may be discontinued. The total weight of the solutions in the traps will be measured at the time of changing, and radiochemical content will be determined. If less than 0.5% of the excreted radioactivity from both the oral and ip studies is recovered in the volatile organics traps, ~~breath~~ exhaled volatile organics will not be collected in ~~the definitive~~subsequent studies.

Blood: For studies A, B, C, D, E, ~~and~~ F, and G, at the end of the in-life phase the animals will be anesthetized. Blood will be collected into a heparinized syringe by cardiac puncture prior to sacrifice. For study ~~G~~H, blood will collected from animals at the following time points after dosing: 2, 5, 10 and 30 min and 1, 4, 8, ~~24, 48~~ and ~~72~~ 24 h. These times may be modified with the consent of the project officer. Serial blood samples (ca. 0.15-0.20 mL) will be withdrawn from rats through indwelling jugular cannulas.

Tissues: For studies A and ~~B~~D, the following tissues will be excised~~,~~ and weighed~~, and assayed for radiochemical content~~: samples of adipose (two sites), muscle (two sites), and skin (ears), as well as the following organs in their entirety: brain, lung, heart, spleen, kidneys, testes, liver, small intestine and contents, cecum and contents, large intestine and contents, stomach and contents, and bladder. The results of the analysis of tissues from studies A and ~~B~~ D will be used to determine ~~if~~ the tissues ~~will~~ to be collected for the remaining studies ~~C and D or if solubilization of the whole carcass will be used for mass balance determinations~~. ~~The results of the analysis of tissues from studies A and B will also be used to determine which tissues will be collected in study G.~~ All remaining carcasses and tissues ~~not solubilized or oxidized~~ will be stored frozen until the end of the final report has been submitted or until further directions have been received from the project officer.

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5.4 Analysis of Biological Samples for Total Radioactivity, Total Chromium (Cr), Parent (Cr3P) and Radiolabeled Metabolites (Metabolite Profile)

See Table 1 for a list of samples to be assayed. Samples will be assayed for total radiolabel by liquid scintillation spectrometry (LSS) either directly (after dissolution in a scintillation cocktail), following solubilization in ethanolic sodium hydroxide or Soluene-350, or after oxidation in a sample oxidizer. Samples that are too dark will be bleached (perchloric acid/H_2O_2) prior to scintillation counting. Ultima Gold scintillation cocktail (Packard Instrument Company, Inc.; Meriden, CT) will be used in all determinations of radiochemical content.

~~The relative suitabilities of solubilization and oxidation of samples will be explored in the pilot studies. In the paragraphs below, solubilization rather than oxidation is listed as the preferred method. Oxidation will be used if the results of the pilot study show it to produce higher recoveries of 14C.~~

The scintillation spectrometers will be calibrated for quench correction using the external standard method.

Total chromium will be determined by graphite furnace atomic absorption (GFAA). For solid samples, e.g., tissues and feces, aliquots of aqueous homogenates will be solubilized and then analyzed. Liquid samples will be analyzed directly. Background chromium levels will be determined using predose samples or samples from untreated animals.

Parent Cr3P and radiolabeled metabolites will be measured using HPLC with radiochemical detection (radio-HPLC). Quantitation will be by LSS. Appropriate HPLC methods will be developed to provide separation of the parent Cr3P and its various metabolites. In some instances , these methods may involve sample clean-up steps prior to analysis by HPLC.

The following procedures will be used:~~The amounts of radiolabeled material may also be calculated from the scintillation counting data for some samples using the internal standard method. The scintillation spectrometers used provide data on the degree of chemiluminescence present.~~

Urine: Duplicate aliquots will be analyzed directly (without solubilization or bleaching) for radiochemical content. Duplicate aliquots will also be analyzed directly for Cr. In designated studies (see

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Table 1), Cr3P, and metabolite profiles will be determined. Samples containing minimal amounts of ^{14}C will be omitted from Cr3P and metabolite profile determinations. With the consent of the project officer, composite samples may also be prepared within a study for Cr3P and metabolite profile determinations.

Feces: Feces in studies A-E will be homogenized with approximately an equal mass of water. The weight of the feces homogenate will be determined, and weighed aliquots (triplicate 0.1 to 0.2 g) will be solubilized in ca. 2 mL of Soluene-350 ~~prior to~~for radiochemical ~~and Cr~~ analysis. Additional aliquots will be assayed for Cr. In studies A, B, D, and E, a composite of the aqueous homogenates will be prepared for each animal, omitting samples containing minimal ^{14}C. Methanol exracts of these homogenates will be analyzed for Cr3P and for metabolites. Unless directed by the project officer, efforts will be applied to separate the metabolites from parent Cr3P, and only limited effort will be made to separate fecal metabolites from each other.

Blood/plasma: Blood will be collected into heparinized syringes. Duplicate aliquots will be weighed, solubilized and analyzed for total radioactivity. Aliquots of plasma prepared from each blood collection will be analyzed for total radioactivity directly. Additional aliquots of blood and plasma will be analyzed for Cr. Methanol extracts will be prepared from all plasma samples that contain sufficient ^{14}C. Cr3P and metabolite profiles for these samples will be measured. Where sample amounts and/or low concentrations of ^{14}C make it advisable, and with the consent of the project officer, composite samples from multiple animals in the same study for a given time point (e.g., the 24 h samples from all animals in study H) may be prepared and analyzed for Cr3P and radiolabeled metabolites.

Tissues: For measurement of total radioactivity, a ~~Tissues~~ liquots of tissues ~~collected for studies A, B, C and D~~ will be solubilized in Soluene-350. ~~Small tissues and organs and small samples of large homogenous tissues (i. e., muscle) will be solubilized in their entirety~~ Large tissues (i.e., liver) will be homogenized, and weighed samples of the homogenate will be solubilized as for small tissues. For tissues designated in collaboration with the project officer, additional aliquots of tissues (or tissue homogenates) will be assayed for total Cr. When selected for analysis for Cr3P and/or metabolite profiling, methanol extracts of the tissue (or tissue homogenate) will be prepared. Hot methanol will be

used where necessary to extract the metabolites. Composite samples will be prepared where necessary/desired by combining samples of a particular tissue type taken from multiple animals in the same study.~~Aliquots of homogenates of tissues collected from animals in study G will be solubilized in Soluene-350 prior to radiochemical analysis.~~

~~**5.5 Analysis of Biological Samples for Cr3P and Cr**~~

~~Urine: For studies A and G, urine will be analyzed for Cr3P by HPLC/LSS and for Cr by graphite furnace atomic absorption (GFAA). Urine from study B will also be analyzed for Cr3P by HPLC/LSS.~~

~~Feces: Methanol extracts of feces from studies A and B will be analyzed for Cr3P by HPLC/LSS.~~

~~Blood: Methanol extracts of all blood collections that contain sufficient total ^{14}C will analyzed for Cr3P by HPLC/LSS. Blood will be analyzed for Cr by GFAA.~~

~~Tissues: Tissues collected during study G will be homogenized, extracted with methanol and analyzed for Cr3P by HPLC/LSS and for Cr by GFAA. Selection of other tissues for analysis for Cr3P and total Cr will be based on the results of the analysis of total ^{14}C in the samples.~~

6.0 SAFETY PRECAUTIONS

a. Precautions for laboratory personnel: All work will be done in well ventilated areas properly designated for use of radiolabeled compounds. Work will be carried out in accordance with RTI regulations for work with radiolabeled compounds. Vials containing volatile radiolabeled compounds will be kept closed at all times.

b. All radioactive wastes will be disposed of in accordance with standard RTI safety policies.

7.0 STORAGE OF RECORDS AND BIOLOGICAL SAMPLES

Records will be maintained in the laboratories of the study personnel while the studies are being conducted. Afterwards, all original study records, protocols, amendments, and final report will be stored in the Research Triangle Institute Archives under the control of the ~~Study Director~~Center for Bioorganic Chemistry. Copies of the final study report are ~~filed with~~maintained in the Chemistry and Life Sciences ~~Center~~Unit, Research Triangle Institute. Chemical and biological samples will be maintained for a

minimum of two years, or for as long as the quality of the preparation affords evaluation, whichever is less.

8.0 REFERENCES

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9.0 APPROVAL SIGNATURES

J B Matthews 9.21.99

Dr. H. B. Matthews Date
Project Officer, NIEHS

Robert Jeffcoat 9/24/1999

Dr. A. R. Jeffcoat Date
RTI, Study Director

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10.0 AMENDMENT 1 TO PROTOCOL RTI 6855-15

The purpose of this amendment (Section 10) is to add studies of [^{14}C]picolinic acid, the organic portion of the Cr3P complex. Studies of picolinic acid (Pcl-H) will be performed as described for Cr3P in the original protocol except for the additions and changes described below.

10.1 Introduction

Studies we have performed with [^{14}C]Cr3P have shown that when [^{14}C]Cr3P is administered orally to rats only ca 1% of the administered chromium is absorbed while ca. 60% of the radiolabeled portion of the molecule is absorbed. This suggests that the chromium and the organic portion of the complex dissociate in the gut. To examine this hypothesis and to determine whether the disposition of material absorbed from Cr3P is essentially that of picolinic acid, we plan to administer [^{14}C]picolinic acid (Pcl-H) orally to rats.

10.2 Personnel

As described in Section 2.0 of the original protocol.

10.3 Test Material

Name: Picolinic acid (2-pyridinecarboxylic acid, Pcl-H)

CAS Registry Number: 98-98-6

Molecular Formula: $C_6H_5NO_2$

Formula Weight: 123.11

10.3.3 Nonradiolabeled Pcl-H

Source: To be listed in the final report.

Lot No.: To be listed in the final report.

Identity: The identity of the nonradiolabeled Pcl-H will be confirmed by NMR or mass spectrometry.

10.3.4 [^{14}C]Pcl-H

Source: Wizard Laboratories

Specific Activity: 17.3 mCi/mmole

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Lot No.: 990419

Identity: The identity of [^{14}C]Pcl-H will be confirmed by chromatographic comparison with nonradiolabeled Pcl-H.

Purity: The radiochemical purity of [^{14}C]Pcl-H will be determined chromatographically.

10.4 Animals

As described in Section 4.0 of the original protocol.

10.5 Study Design and Procedures

(Additions to Section 5.0 of the original protocol)

Studies to determine the disposition of Pcl-H will include:

High single oral dose

PA. Absorption, excretion, metabolism and distribution of Pcl-H in selected tissues at 6 and 48 h following dosing of a single oral dose of 15 mg/kg (0.12 mmol/kg) Pcl-H dissolved in water. This dose is equivalent to the Pcl-H in 17 mg/kg (0.040 mmol/kg) of Cr3P. Additional time points and further analysis of biological materials may be added with concurrence of the project officer.

Additions to Table 1. Studies to be Performed in Male F-344 Rats

Study ID	Route (length)	Target Dose[1]	Vehicle[2]	Items to be Measured[3]					
				Urine	Feces	Breath[4]	Blood/ Plasma (sac)	Blood/ Plasma (timed)	Tissues[5]
PA	oral (6,48 h)	15 mg/kg Pcl-H	water	^{14}C, profile	^{14}C,	$^{14}CO_2$,	^{14}C, profile		^{14}C

10.5.1 Formulation and Administration of Cr3P and Pcl-H

In study PA, oral doses of Pcl-H will be formulated in water at a dose volume of 5 mL per kg body weight. It will be administered at dose levels of 0.12 mmol/kg (15 mg of Pcl-H/kg). Animals will receive between 5 and 50 uCi/kg of [^{14}C]Pcl-H. Oral doses will be administered from a syringe fitted with a 16 gauge ball-tipped feeding needle.

10.5.2 Analysis of Radiolabeled Dose Formulations

As described in Section 5.2 in the original protocol.

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10.5.3 Collections and Storage of Biological Samples

As described in Section 5.3 in the original protocol except for the following.

Urine and Feces: For study PA, urine and feces will be collected separately into receivers cooled over dry ice. For rats sacrificed at 48 h post dosing, collections will be made at 2 (urine only), 4, 8, 12, 24 and 48 h following the radiolabeled dose. These collection times are the same as used with Cr3P in Study B. For rats sacrificed at 6 h post dosing, collections will be made at 2 (urine only), 4 and 6 h following the radiolabeled dose.

10.5.4 Analysis of Biological Samples for Total Radioactivity and Radiolabeled Metabolites (Metabolite Profile)

Samples will be analyzed for total radioactivity as described in Section 5.4 of the original protocol. Parent Pcl-H, the presumed major metabolite (N-picolinylglycine), and other metabolites will be measured by HPLC with radiochemical detection. Metabolite profiles for Pcl-H will be established from urine and feces (unless < 5 % of the dose is excreted in feces).

10.6 Safety Precautions

As described in Section 6.0 in the original protocol.

10.7 Storage of Records and Biological Samples

As described in Section 7.0 in the original protocol.

10.8 References

No changes to Section 8.0 in the original protocol.

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10.9 Approval Signatures

Dr. L. T. Burka Project Officer, NIEHS	5/9/00 Date
Dr. A. R. Jeffcoat RTI, Study Director	5/11/2000 Date

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11.0 AMENDMENT 2 TO PROTOCOL RTI 6855-15

The purpose of this amendment (Section 11) is to provide the details for studies of [^{14}C]Cr3P in mice. Studies of [^{14}C]Cr3P in mice will be performed as described in the original protocol and this amendment. Details for the studies in mice are described below.

11.1 Introduction

We plan to administer [^{14}C]Cr3P orally to mice to compare the disposition of Cr3P in mice with that in rats. We want to compare excretion routes, urinary metabolites and blood contents for the two species. We also plan to perform PK studies that will be planned in consultation with the project officer.

11.2 Personnel

As described in Section 2.0 of the original protocol.

11.3 Test Material

As described in Section 3.0 of the original protocol.

11.4 Animals

(Additions for Section 4.0 in the original protocol.)

Sex and Weight: Male mice, 20-30 g

Number: Mice, 4 per dose group for absorption and excretion studies

Mice, 4 per each blood time point for PK studies

11.4.2 Anesthesia and Sacrifice

Mice will be anesthetized with an intraperitoneal injection of sodium pentobarbital (180 mg/kg) or carbon dioxide inhalation. Mice will be sacrificed by carbon dioxide asphyxiation or by cervical dislocation.

11.5 Study Design and Procedures

(Additions to Section 5.0 of the original protocol.)

Studies to determine the disposition of [^{14}C]Cr3P in mice will include:

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Excretion study: single oral dose in mice

Follow absorption and excretion of [^{14}C]Cr3P over a 48 h following a single oral dose of 15-17 mg/kg Cr3P as an aqueous slurry.

PK study: single oral or iv dose in mice

Determine levels of [14C]Cr3P in blood following a single oral or iv dose of Cr3P. Dose levels and time points will be determined by consultation with the project officer.

Table 2. Studies to be Performed in Male B6C3F1 Mice

Study ID	Route (length)	Target Dose[1]	Vehicle	Items to be Measured[2]					
				Urine	Feces	Breath	Blood/ Plasma (sac)	Blood/ Plasma (timed)	Tissues
J	oral (24 h)	ca. 17.5 mg/kg Cr3P (ca. 2.2 mg/kg Cr)	slurry in water	^{14}C, Cr, Cr3P, profile	^{14}C, Cr	$^{14}CO_2$	^{14}C Cr		^{14}C Cr
K	To be determined in consultation with project officer							^{14}C Cr Cr3P	^{14}C Cr

Notes

1. oral doses will be given in a volume of 10 ml/kg
2. ^{14}C = total radioactivity, Cr = total chromium, Cr3P = parent Cr3P, profile = profile of Cr3P metabolites containing ^{14}C

11.5.1 Formulation and Administration of Cr3P

In study J, oral doses for the excretion study in mice will be formulated in a water slurry at a dose volume of 10 mL per kg body weight. Animals will receive approximately 10 uCi each of [^{14}C]Cr3P at a target dose level of 17.5 mg/kg. Doses will be administered from a syringe fitted with an 18 gauge ball-tipped feeding needle.

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Dose formulations and administration details will be determined for study K in consultation with the project officer. Animals will receive 10-20 uCi each of [^{14}C]Cr3P.

11.5.2 Analysis of Radiolabeled Dose Formulations

As described in Section 5.2 in the original protocol.

11.5.3 Collections and Storage of Biological Samples

As described in Section 5.3 in the original protocol except for the following:

Urine and Feces: For study J, urine and feces will be collected separately into receivers cooled over dry ice. For mice sacrificed at 48 h post dosing, collections will be made at 6, 12, 24 and 48 h following dosing.

Breath: For study J, radiolabeled breath excreted as CO_2 will be collected continuously after dosing by passing the air from the metabolism cage (flow = 200-500 mL/min) through two CO_2 traps, each containing ca. 500 mL of 1N NaOH. Traps will be changed at 12, 24, 32 and 48 h post dosing.

Blood: For study J, at the end of the in-life phase the animals will be anesthetized. Blood will be collected into a heparinized syringe by cardiac puncture prior to sacrifice.

For study K, blood will be collected at time points to be determined in consultation with the project officer. For each time point following dosing, four animals will be anesthetized. Blood will be collected in a heparinized syringe by cardiac puncture prior to sacrifice.

Tissues: For study J, the following tissues will be collected at sacrifice: muscle, liver and adipose. For study K, tissues to be collectioned will be determined by consultation with the project officer.

11.5.4 Analysis of Biological Samples for Total Radioactivity, Total Chromium (Cr), Parent (Cr3) and Radiolabeled Metabolites (Metabolite Profile)

As described in Section 5.4 in the original protocol except for the following:

See Table 2 for list of samples to assayed.

11.6 Safety Precautions

As described in Section 6.0 in the original protocol.

11.7 Storage of Records and Biological Samples

As described in Section 7.0 in the original protocol.

11.8 References

No changes to Section 8.0 in the original protocol.

PROTOCOL	RESEARCH TRIANGLE INSTITUTE POST OFFICE BOX 12194 RESEARCH TRIANGLE PARK, NC 27709	Amendment 2 RTI 6855-15-2 Page 4 of 4 Effective April 2001

11.9 Approval Signatures

Dr. L. T. Burka
Project Officer, NIEHS

Date

Dr. A. R. Jeffcoat
RTI, Study Director

4/18/2001
Date

Appendix B

Individual Animal Data

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