A STUDY OF THE TOXICITY OF HEXAVALENT AND TRIVALENT CHROMIUM IN THE ALBINO RAT

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Robert D. MacKenzie

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A STUDY OF THE TOXICITY OF HEXAVALENT AND TRIVALENT CHROMIUM IN THE ALBINO RAT

By

Robert D. MacKenzie

A THESIS

Submitted to the College of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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VITA

The author was born August 18, 1928 in Chicago, Illinois. After receiving his elementary school education in Chicago, Detroit, and Cincinnati, attended Woodrow Wilson high school in Washington, D. C. He was then in the United States Army for 18 months serving with the 82nd Airborne Division, after basic training. He entered the University of Cincinnati in September 1948, and was graduated in June of 1952 with a Bachelor of Science Degree. During the summer of 1952 he worked at the William S. Merrell Company as an organic chemist. He enrolled in the Graduate School of Michigan State College in the fall of 1952, obtaining a Teaching Assistantship in December of that year. He remained at this position until receiving his Master of Science Degree in June 1954 presenting as his thesis "The Use of Radioactive Ergosterol in the Study of Lipid Absorption in the Albino Rat." He resumed his studies at Michigan State University in the Fall of 1954 as a Special Graduate Research Assistant under a United States Public Health Service Grant, which he held until the completion of his graduate program. He is married and has a son R. Bruce and two daughters Barbara and Catherine.

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ABSTRACT

The extensive industrial use of chromium and the disposal of chromium wastes has resulted in the contamination of river and well water supplies in certain areas of this country. Little is known about the effects of the ingestion of small amounts of chromium over a long period. It was, therefore, the purpose of this study to determine at what concentration in water harmful effects might appear in albino rats. It was hoped that the results might lead to a more realistic chromium standard for potable water supplies.

The initial experiment with 35 day old albino rats involved concentrations of chromate ion ranging from 1 to 25 parts per million in the water supply used in conjunction with an adequate diet. Blood studies were made at monthly intervals over a period of a year. No differences in the hemoglobin level or in the differential white cell count were observed between the experimental and control groups.

At the end of six months one rat of each sex was sacrificed to determine the chromium content of the liver, kidney and femur and to study tissue sections for pathological changes. At the end of the year the remaining animals were sacrificed and the spleen included in the examination of tissues. There was no evidence of pathology in the tissues studied. The accumulation of chromium in the tissues was fairly slow up to 5 ppm but increased rapidly at higher concentrations and continued throughout the period of observation.

In order to determine the influence of food on the absorption of chromate ion, groups of starved and non-starved rats were given radioactive chromium (Cr^{51}) by stomach tube. The rats were placed in metabolism cages and sacrificed at 6, 24, 72, and 168 hours after injection. The activity in the liver, kidney, blood, stomach, intestine, urine and feces was determined. Approximately 5.5% of the dose was absorbed by the starved rats and only 2.3% by the non-starved.

In another series a comparison was made in the absorption of hexavalent and trivalent chromium (Cr^{51}) by starved and non-starved rats, the dose again being given by stomach tube. Four hours after injection the rats were anesthetized, blood removed from the heart and after centrifuging, activity counts made on plasma and red cells. The results indicated that hexavalent chromium was absorbed to a much greater extent than trivalent and confirmed the greater absorption previously observed in the starved rats. Also confirmed was the observation that hexavalent but not trivalent chromium can be absorbed by the red blood cells.

In a third series radioactive chromium in the form of sodium chromate, chromic chloride, and a mixture of equal parts of each was injected into the small intestine. Four hours later blood was removed from the heart as before, centrifuged and the activity of the plasma and red cells determined. The activity observed with the hexavalent form was higher than that found by stomach injection, indicating that considerable reduction to the trivalent state occurs in the stomach. The blood of the animals again showed higher activity than that of the non-starved.

A final comparison was made of the influence of prolonged ingestion of water containing hexavalent and trivalent chromium equivalent to 25 ppm chromium. At the end of a year the rats were sacrificed and the liver, spleen, kidneys and femur analyzed for chromium and sectioned for microscopic study. The tissues of rats fed hexavalent chromium contained approximately 5 times the amount found in the trivalent group. There was no evidence of pathology in any of the animals.

It is apparent that hexavalent chromium is absorbed to a much greater extent than trivalent. The reduction of chromate wastes before their disposal would obviously greatly reduce the potential hazards of water contamination of this type.

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INTRODUCTION

Chromium is classified as a metal and occupies a position in Group VI of the periodic table. Though it is classified as a metal, it is an amphoteric substance depending on the environment and valence. Chromium can exist in three series of compounds 1) divalent chromium (basic), 2) trivalent chromium (weakly basic and amphoteric), and 3) hexavalent chromium (acid). Univalent and pentavalent chromium are also found in certain reactions. However, only the trivalent and hexavalent compounds are actually stable enough to be found in water supplies or in ores. Trivalent chromium is more stable than hexavalent in solution at a pH below 7.

Chromium is found mainly as chromite ore, also called chrome ironstone or ferrous chromite ($FeCr_2O_4$ or $FeO \cdot Cr_2O_3$). Rarer ores such as chrome ochre (Cr_2O_3) and chromitite ($Fe_2O_3 \cdot 2\ Cr_2O_3$) also occur. Chromite is mined in Rhodesia, the Transvaal, Cuba, Greece, Turkey, India, Russia, Yugoslavia, the Philippines and New Caledonia. In the United States it is found in fairly high concentrations in Arkansas in bauxite deposits and in serpentine soils in Maryland and California.

Chromium is used in iron and steel alloys to increase hardenability, strength at high temperatures, and resistance to abrasion, corrosion and oxidation. Chromium is an essential component of high-speed steel, and of many engineering steels, stainless steel, and a large proportion of other corrosion resistant alloys. Chromium

compounds are used in the tanning of leather, for plating and anodizing metals, for the production of catalysts in gasoline and synthetic rubber manufacture, in the refractory industry, and in the manufacture of certain pigments.

In the refining of chrome ores, and in certain industries air contaminated with chromic acid mist from vats or dust of chromates is the principal type of exposure to chromium. Toxicity is usually due to inhalation or topical contact. Hexavalent chromium is generally considered the most toxic by these routes. Dermatitis and perforation of the nasal septum are frequently observed in workers exposed to chromate. When skin abrasions come in contact with chromate liquors, chrome ulcers or "Chrome holes" develop. Several cases of lung cancer were reported in Germany during the World War II in plants where workers were exposed to zinc chromate dust. An analysis of the mortality data of the chromate-producing industry in the United States, revealed that the death rate for cancer of the respiratory system was 21.8 percent of all deaths (16 times the expected incidence). It has been suggested that the monochromates may be the compounds responsible for lung cancer (2). The maximum allowable concentration of chromium as chromate dust or as chromic acid mist has been suggested as 0.1 milligrams of CrO_3 per cubic meter of air.

In recent years, chromium has assumed increased importance as a contaminant of water supplies. Certain industries have disposed of chromate wastes by direct discharge into streams conveniently located.

In other instances such wastes have been dumped into large holes in the ground resulting in contamination of ground water up to 25 ppm of chromium.

Although the effects of acute chromium poisoning are well documented little has been done to study the hazards of prolonged ingestion of water containing small amounts of chromium. There is also a difference of opinion as to whether hexavalent chromium is more or less toxic than trivalent.

At present the allowable chromate concentration in potable water supplies is 0.05 ppm expressed as chromium. This is assumed to be well below the harmful level. However, it is not always practical to maintain so low a concentration. Therefore more work needs to be done to establish the limits of tolerance for chromium so that a more realistic and practical standard may be established. Therefore a study was made of the absorption and retention of chromium at various concentrations in the drinking water and certain tissues examined for pathological changes.

HISTORICAL

HISTORICAL

Occurrence of Chromium

Chromium is found in wide areas all over the world. In the United States it is mined mainly in California and to lesser extent in Wyoming, Maryland and parts of Pennsylvania and Arkansas (1). In other areas it occurs in fairly high concentrations in soil. Davidson and Mitchell (6) reported from 150-350 ppm. of chromium in eight Scottish soils. Two other soils contained as much as 1500-3000 ppm. In the United States concentrations of 150-300 ppm. have been found.

According to Negas (7) inhibitors containing chromium are being sold to prevent corrosion of water pipes. When used as directed a concentration of 0.31 ppm. of sodium chromate or 0.1 ppm. of chromium was found in the water. In one survey (8), the chromium content of twenty-four municipal water supplies in the United States ranged from 0.001-0.04 ppm. In the United States well water has been found to contain up to 25 ppm. of chromium (9).

Chromium Poisoning in Man

Chromium may enter the body in three ways thus causing different effects. Poisoning may result by inhalation of chromic acid mists or dusts, by topical contact with chromic acid solutions, or by ingestion of chromium containing materials. Most of the work done has involved acute toxicity although there has been some evidence of chronic symptoms.

In 1827 Cumming (11) wrote about "chrome holes," ulcers which developed usually on the hands and arms of workers exposed to chromate

solutions. Papules appear first, then change to pustules and finally to deep and penetrating ulcers. Previous abrasion of the skin is essential for this type of damage. The ulcer is characteristically sluggish, the edges markedly indurated and undermined, clear cut, looking as if punched out, hence the name "chrome hole." The center has a scab resting on the slough and the floor underneath is gray. A very common site for this ulcerative process is the septum of the nose. Other sites are the fingernails, the knuckles, and eyelids. Ulcers may also form on the edge of the nostrils, on the toes if the shoes become soaked with chrome water, and, rarely, in the throat. The effect is gradual, and usually not very painful.

Becourt and Chevallier in 1851 (12), apparently without knowledge of Cumming's paper, noted what was to them a new disease of the skin, occurring in some chrome workers in Paris. They wrote to physicians in other countries asking if such things had ever been seen elsewhere and received an answer from Isaac Tyson of Baltimore in 1852, confirming their observations and saying that in Maryland workmen protected themselves by tying a wet sponge over the nose and mouth. Ducatel (13) of the University of Maryland, added details as to the character of chrome ulcers and reported that Baer of Baltimore had seen 20 cases, caused by chrome steam, which healed only when the work was given up. Leymann (14) examined 722 workmen who were exposed to chromates and found ulcers and perforation of the septum in 253 or 35%, respiratory disease in 8.8%, and digestive disorders in 12.3%.

The above description can be said to be that of acute toxicity. The occurrence of possible chronic toxicity in one of the chromate using industries in Germany was reported by Gross (3). He found several cases of lung cancer resulting from exposure to zinc chromate dust during World War II. Machle and Gregorius (4), in a study of the mortality in the chromate producing industry in the United States, reported that 21.8 percent of all deaths were due to cancer of the respiratory system. This is sixteen times the expected ratio.

Poisoning by ingestion has not been reported to any great extent. Only in the case of the accidental ingestion of a large amount of a chromium compound or when water was found to contain chromium have case histories been documented. In a case reported by Krieger (15) biopsy showed parenchymatous degeneration of the liver and on the 2nd day mild kidney damage occurred. The urine had an orange-red color and contained albumin and leucocytes. The symptoms disappeared after three days. Sander and Camp (16) reported a case of poisoning in a 14 month old infant. The infant ingested some chromite ore which was slowly released from the gastro-intestinal tract. One week after ingestion convulsions, stupor, dilation of pupils and fever occurred. There were still some symptoms six months later. David and Lieber (9) reported finding a family using water from a well containing 1.0 ppm of chromium. They used this water for three years with no apparent ill effects. Chromium was detected by spectrographic analysis in the feces of one of the person's checked.

Experimental Poisoning (Animal)

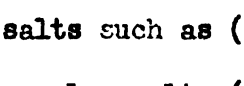
There have been no long term or chronic studies of chromium toxicity reported in the literature. However, there have been many experiments on acute toxicity. Plum (17) reported distinct changes in serum bilirubin in rabbits three weeks after being given a daily intravenous dose of 0.001 grams of chromium per kilogram of body weight. Toxic effects reported were: hemorrhage of the musculture, gastro-enteritis with necrosis in the stomach and small intestine, leukemic changes in the blood, fatty endocarditis, fatty degeneration of the liver and parenchymatous nephritis. In a few cases of post-mortem examination of humans, shrivelled kidneys have also been observed.

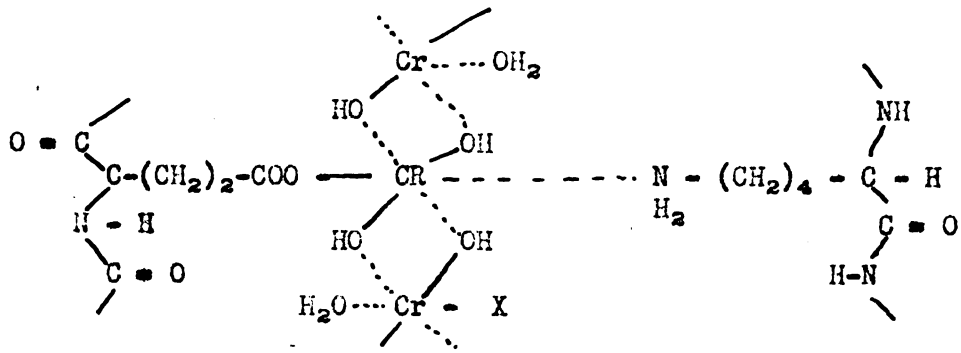
Gross and Heller (18) working with young rats reported that potassium chromate in drinking water caused toxic symptoms in two to three months when the water contained 134 ppm of chromium or more.

Reaction with Biological Materials

Chromium compounds are found to react with proteins and peptides to form a metal-organo complex, also known as a chelation complex. I. P. Strakhov (19) reported that the cationic or trivalent chromium will chelate with the carboxyl group and the hydroxyl group of hydroxy-proline or with serine whereas the anionic or chromate ion reacts with amino groups. There is no evidence that hexavalent chromium can be chelated. Trivalent chromium, however, is strongly chelated and is the form in which it is bound to the tissue proteins. Gray and Sterling (20) are of the opinion that when hexavalent is absorbed into the red blood

cell it is reduced before combining with the globin moity of hemoglobin.

In the tanning of leather Gustavson (22) has shown that even such small amounts of combined chromium as 0.5-1.5 grams per 100 grams collagen imparts a high degree of stability. Thus chromium influences the properties of the collagen and possibly other proteins to a great extent. He reported that the reaction occurs best with basic chromic salts such as  and forms a modified type of internal complex salts (chelate compounds, involving groups from different protein chains). According to this concept, the initial reaction is an ionic interaction of cationic chromium complexes, such as $(Cr_2OSO_4)n^{2n+}$ with the charged carboxyl groups of collagen, the sulfate ions being compensated by the $-NH_3^+$ ions. The carboxyl groups, having a great tendency for complex formation and for direct attachment to chromium, penetrate into the coordination sphere, forming coordinate-covalent bonds. Since several chromium atoms are present in the large chromium complex, and in view of the secondary aggregation of the fixed chrome complexes by further hydrolysis, possibilities exist for a multipoint interaction of one chainlike chromium complex with several carboxyl ions of the collagen lattice, resulting in the linking of adjacent protein chains by strong bonds, by means of the chrome bridge. Schuttleworth's (21) Conductivity data for gelatin solutions containing chromic salts points to the inactivation of carboxyl ions as the main reaction.



Analytical Chemistry of Chromium

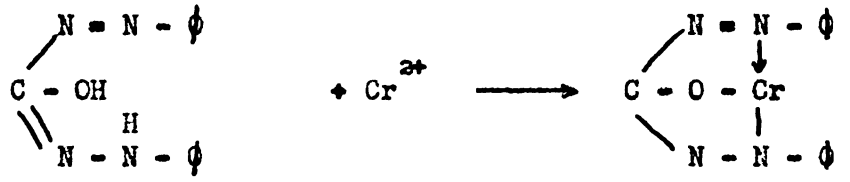
Both colorimetric and spectrographic methods have been developed for the determination of chromium in tissues. The spectrographic method is quite complex and is generally better suited for qualitative analysis. For the colorimetric determination, chromium is oxidized to chromate and treated with s-diphenylcarbazide. By this method as little as one part in twenty million may be determined.

Urone and Anders (23) have reported that wet or dry ashing of tissue followed by oxidation with bromine in alkaline solution gives very good results.

Saltzman (24) reported an improved method for the oxidation step. It consists of wet ashing of the tissue with sulfuric and nitric acid until all organic matter is oxidized, removing the sulfuric acid in a muffle furnace at 550°C. and then washing the sides of the Phillips beakers with aqua regia. After evaporating the solution to dryness on a steam bath, the residue is taken up in 0.5 N sulfuric acid and refluxed for 20 minutes with a slight excess of 0.1 N $KMnO_4$ so that a pink color persists. The excess permangante is decolorized with a 5% solution of sodium azide, avoiding an excess of the latter. Since iron interferes with the color development, it can be removed by adding NaOH

which precipitates the iron as $Fe_2O_3 \cdot H_2O$. After the diphenylcarbazide is added and one minute allowed for development of color, a 4 M solution of NaH_2PO_4 is added to stabilize the color. The optical density is then determined in the Beckman model DU spectrometer.

According to M. Bose (25) the color is due to a complex formed between divalent chromium and diphenylcarbazone. These are formed from an oxidation-reduction reaction in which hexavalent chromium oxidizes the carbazide to carbazone and is itself reduced to the divalent state. The divalent ion forms a stable chelate with the carbazone.



This method does not differentiate between trivalent and hexavalent chromium, because the oxidation conditions would convert trivalent chromium to the hexavalent state. A means of distinguishing between trivalent and hexavalent chromium was suggested by Gray et al. (20) who found that hexavalent but not trivalent chromium was absorbed by red blood cells. The ready absorption of hexavalent chromium and its retention has been used to determine the life span of the red blood cell as well as blood volume. This difference in permeability was used as basis for studying the relative absorption of hexavalent and trivalent chromium from the digestive tract.

Chromium in Tissues

Not too much has been done on the determination of chromium in plant and animal tissues. Stockinger (27) in a study of 107 samples of human blood found values between 0.0 to 0.022 (median 0.012) mg. Cr per 100 gm. of blood. Sixty-one urine samples varied between 0.0 to 0.15 mg. per liter (median 0.041).

In a comprehensive study by Grushko (28) the following amounts of chromium were found in human tissues expressed in mg. percent of wet tissue: hair 0.2, nails 0.12, bile 0.08, salivary gland 0.04, kidney 0.028 (0.027), diaphragm 0.016, large intestine 0.012 (0.063), heart 0.01, liver 0.001 (0.013), brain 0.002, thyroid gland 0.005 (0.004), gastric wall 0.007, cartilage 0.001, hypophysis 0.0006, spleen 0.0005 (0.01), muscle 0.0002, suprarenal gland 0.0005, pancreas 0.0002, small intestine 0.0006, lung 0.0007, blood 0.0035 (0.012), bone (0.085). Grushko also reported values for the rabbit and sheep. For rabbits he reported the following values: stomach contents 0.108, large intestine 0.16, gastric wall 0.07, wall of small intestine 0.013, claws 0.10, bone 0.09, cartilage 0.04, heart 0.043, lung 0.011, liver 0.002, kidney 1.02, spleen 0.01, muscle 0.03. He also analyzed a number of foods and found the following values expressed in mg. percent: flour 0.185, bread 0.087, semolina 0.065, buckwheat 0.028, cabbage 0.031, beets 0.025, carrots 0.011, egg (powdered) 0.003, vernicilli 0.005, fish 0.002, A sample of soil contained 0.0016, water (underground) 0.0009-0.002 mg. per liter, river water 0.0011 (Angara) and 0.0019 (Iskat).

All analyses were made spectrographically. Whether these values give an accurate picture of the normal distribution of chromium is not certain, since it represents the work of only one investigator.

Visek et al. (29) did some experiments in rats with trivalent chromium as sodium chromite and chromic chloride and hexavalent chromium as sodium chromate. He injected Cr^{51} (radioactive) intravenously by jugular catheter. Sodium chromite was picked up in extremely large quantities by the tissues of the reticuloendothelial system and the liver accumulated approximately 90% of the dose. The excretion values were correspondingly low. Considering the small amount excreted, and the high liver and spleen values at 21 and 42 days after injection, it is apparent that most of the dose was retained for some weeks after injection. The tibial epiphysis showed a higher concentration of Cr^{51} than did the other skeletal tissues, and the activity was slow in leaving all the tissues except the blood and lungs. The liver and spleen at the end of 42 days contained 33% and 50% respectively of the activity observed at 4 days. In contrast the lung showed only 10% of the activity at this time. No detectable radioactivity was found in the blood after 21 days. Chromic chloride attained its highest concentration in the liver, spleen and bone marrow, although less was retained than in the case of the sodium chromite. Considerable variation between animals, was observed but in all cases the activity was removed very slowly. The value for the liver at 45 days was 35% of the value observed 24 hours after dosing. In contrast, the concentration in all of the other tissues with the exception of the blood and lungs remained practically

constant throughout the 45 day period. The blood contained practically no activity after the seventh day and at 45 days the lungs contained about 15% of the maximum value which was observed at six hours following injection. The spleen appeared to gain activity over the 42 day period. Chromic chloride was the only compound administered orally, intratracheally, and intraperitoneally. Orally administered $Cr^{51}Cl_3$ was almost totally excreted in the feces at the end of four days. The dosage was not given but it was reported that only about 0.5% of the dose was absorbed from the digestive tract. Intratracheal injection indicated that less than 5% was absorbed from the lungs. Intraperitoneal injection of $Cr^{51}Cl_3$ caused extensive necrosis at the site of injection due to the high activity of the solution. Tissue uptake was variable and extremely small. When sodium chromate was injected the major uptake was by the liver, but in much lower amounts than for the trivalent chromium. The excretion was correspondingly higher. Twenty-five percent of the injected dose was deposited in the liver within half an hour after administration and at the end of 42 days less than one percent was present. This shows a marked difference in behavior from both trivalent compounds. All of the other organs lost activity with time except the spleen which gained. The concentration in the blood at the end of 21 days was about one fifth of that found at thirty minutes but declined to practically zero at 42 days. The greater retention by the blood of hexavalent chromium was due to its being absorbed and bound by the erythrocytes.

Effect on Enzymes

The influence of chromium on enzyme systems has not been extensively investigated. Shimizu (31) reported that trivalent chromium stimulated the activity of pancreatic lipase. The only metals that activate it more were calcium, aluminum and ferric ions. According to Curran (32) chromium appreciably increased the hepatic synthesis of cholesterol and fatty acids from acetate (96% to 202%). He reported that hexavalent chromium had a much lower activity than trivalent, and suggested that the hexavalent chromium was reduced to trivalent chromium and that only trivalent chromium was really active.

EXPERIMENTAL AND RESULTS

EXPERIMENTAL AND RESULTS

EXPERIMENT I -- Chronic Toxicity Study

Rat Care and Diets

Sprague-Dawley albino rats 34 days old were used for this experiment. They were assembled into six groups, a control group receiving distilled water (I), and Groups II to VI given water containing 1.0, 5.0, 10.0, 15.0 and 25.0 parts per million (ppm) of chromate ion respectively. The different concentrations were made up by diluting a stock solution (1000 ppm chromate ion) with distilled water. The salt used as a source of chromate ion was potassium chromate (K_2CrO_4). Except for the control group, each contained 8 males and 8 females. The control group contained 10 rats of each sex. The average initial weights are given in Table I.

TABLE I

AVERAGE INITIAL WEIGHTS

Group Number	Body Weight	
	Male	Female
I	85	83
II	81	82
III	86	88
IV	86	83
V	88	84
VI	85	84

The rats were placed in individual raised cages. The room temperature was maintained between 75° and 79°F. throughout the year. All rats received, ad-libitum, the stock diet shown in Table II.

TABLE II

COMPOSITION OF THE STOCK DIET

Constituents	Percent by Weight
Ground yellow corn meal	32.5
Ground whole wheat	25.0
Powdered whole milk	22.5
Linseed oil meal	10.0
Alfalfa	6.0
Brewer's yeast	3.0
Sodium chloride	1.0

Stock diet contained one microgram chromate ion in 50 grams of feed. Weekly records were kept of body weight, food and water consumption.

At the end of six months, one male and one female from each group were sacrificed. Tissues were taken for a study of pathological changes and chromium analysis. After one year the rest of the rats were sacrificed. Rats that died during the experimental period were examined to determine the cause of death and in a few cases tissue sections were prepared.

Blood Studies

Blood analyses were made at monthly intervals on four males and females of each group given chromium and five males and females from

the control group. Blood studies included determinations of total red and white cell counts, differential white cell count and hemoglobin concentration by a modified Sanford method (33).

Pathology[1]

To sacrifice the rats, 0.25 ml. of Halatal (sodium-ethyl, 1-methyl-butyl, barbiturate) was injected into the thoracic cavity. Samples of the following tissues were taken: adrenal glands, liver, kidney, spleen, brain, heart, stomach, duodenum, ileum, colon, and cross-sections of bone marrow from the sternum and femur. Fixatives used were 10% neutral saline-formalin (standard stain procedure for fat), Carnoy's fluid for glycogen, and Zenker's solution. All the tissues were stained with hematoxylin and eosin. Small portions of the liver, kidney, and adrenal gland were stained with Best's Carmine for glycogen and Sudan IV for fat.

Chromium Analysis

The tissues from the rats sacrificed at 6 months were liver, kidney and femur. Those from the rats sacrificed after one year were liver, kidney, spleen and femur. The tissues were frozen in a deep freeze at -15°C. until analyses were performed.

A weighed sample of each tissue was wet ashed on hot plates in 250 ml. Phillips beakers covered with watch glasses using concentrated sulfuric acid to char and then concentrated nitric acid to oxidize the

[1] All pathological studies were performed by the Department of Animal Pathology, U. S. U., under the direction of Dr. Robert F. Langham.

organic material. When all organic material had been oxidized, the beakers were placed in a muffle furnace at 550°C. and the sulfuric acid removed. Subsequently, 5 ml. aqua regia was added (2 HNO_3 : 1 HCl) and heating continued for about 5 minutes. The watch glasses were then removed and the beakers placed on a steam bath until they just became dry. The residue was dissolved in about 10 ml. of 0.5 N H_2SO_4 and 6 N NaOH was added to precipitate most of the iron. This was centrifuged and the supernatant just acidified. A few drops of 0.1 N $KMnO_4$ were then added and the solution refluxed for 15 minutes. If at this stage the pink color disappeared, enough permanganate was added to maintain the color for at least 5 minutes. This solution was then decolorized by adding a few drops of 5% sodium azide solution. The solution and washings were combined, centrifuged and the supernatant transferred to a 25 ml. volumetric flask. Ten milliliters of 0.5 N H_2SO_4 was added, and then 1.0 ml. of diphenylcarbazide reagent (0.625 gm. S-Diphenylcarbazide, 10 gms. phthalic anhydride in 250 ml. of 95% ethanol, See Saltzman (24)). After letting the color develop for one minute 2.5 ml. of 4 M NaH_2PO_4 was added and the solution adjusted to the mark with double distilled water (all reagents were prepared with double distilled water, distilled from glass in the presence of $KMnO_4$). The optical density of each solution was determined about 30 minutes after color development, at 540 m in a Beckman model DU. A standard curve was prepared using known quantities of chromate ion. The standard obtained indicated that Beer's law held between 1.5 ug. and 52 ug. (upper limit of readings) in 25 ml. of solution.

Results

The average weights at various periods given in Table III show that there were no significant differences between the groups. Values for the average food consumption (Table IV) and water consumption (Table V) also show no significant differences between the various groups.

The blood analyses indicate that the experimental groups did not differ significantly from the control group.

In the microscopic study of tissue sections, very little was noticed. The only changes were found at the level of 25 ppm chromate ion and involved the kidneys. The proximal convoluted tubules appeared to be slightly more vacuolated than normal. There was a slight increase in the loss of albumin in the tubules and formation of hyalin casts. However, the changes were so minor that they were considered to be of slight significance.

The tissue analyses for animals sacrificed at 6 months are shown in Table VI. There is an indication that at low concentrations (0-5 ppm chromate ion), relatively little stays in the tissues. However, between 5 and 10 ppm of chromate ion an appreciable increase in the rate of accumulation occurs (Figure I).

The tissue analysis for rats sacrificed at the end of a year are shown in Table VII. The data indicate a similar trend, an increase in accumulation rate above 5 ppm of chromate ion (Figure I).

TABLE III

AVERAGE BODY WEIGHT OF EACH GROUP

Time (in months) / Group Number	$CrO_4^{=}$ ppm	Initial		1		3		6		9		12	
		Male (gms)	Female (gms)	Male (gms)	Female (gms)	Male (gms)	Female (gms)	Male (gms)	Female (gms)	Male (gms)	Female (gms)	Males (gms)	Females (gms)
I	0	85	83	231	190	344	254	386	280	476	285	595	310
II	1	81	82	246	181	385	244	398	261	430	282	450	295
III	5	86	88	249	194	387	253	433	277	508	284	521	308
IV	10	86	83	250	184	400	241	449	262	530	285	564	300
V	15	88	84	232	191	344	256	400	289	505	312	545	338
VI	25	85	84	258	180	402	240	471	267	502	285	526	309

TABLE IV

AVERAGE FOOD CONSUMPTION

Time (in months)		1		3		6		9		12	
Group Number	CrO_4 ppm	Male gm/rat	Female gm/rat	Male gm/rat	Female gm/rat	Male gm/rat	Female gm/rat	Male gm/rat	Female gm/rat	Male gm/rat	Female gm/rat
I	0	602	484	642	540	511	435	384	347	---	383
II	1	589	454	628	505	576	463	464	399	518	393
III	5	580	499	635	523	578	458	554	384	611	391
IV	10	653	528	646	539	581	464	564	419	567	478
V	15	577	479	624	523	690	491	557	382	---	390
VI	25	575	462	612	514	657	511	437	359	493	393

TABLE V

AVERAGE WATER INTAKE OF EACH GROUP

Time (in months)		1 - 3		4 - 6		7 - 9		10 - 12	
Group Number	$CrO_4^=$ ppm	Male ml/rat/day	Female ml/rat/day	Male ml/rat/day	Female ml/rat/day	Male ml/rat/day	Female ml/rat/day	Male ml/rat/day	Female ml/rat/day
I	0	31	30	31	29	33	29	37	35
II	1	32	31	33	32	30	34	30	36
III	5	33	31	31	29	32	34	37	37
IV	10	32	32	31	30	34	33	35	34
V	15	32	30	29	29	32	30	36	30
VI	25	29	29	30	29	28	29	30	28

TABLE VI

CHROMIUM CONCENTRATIONS IN RAT TISSUES AFTER SIX MONTHS EXPOSURE

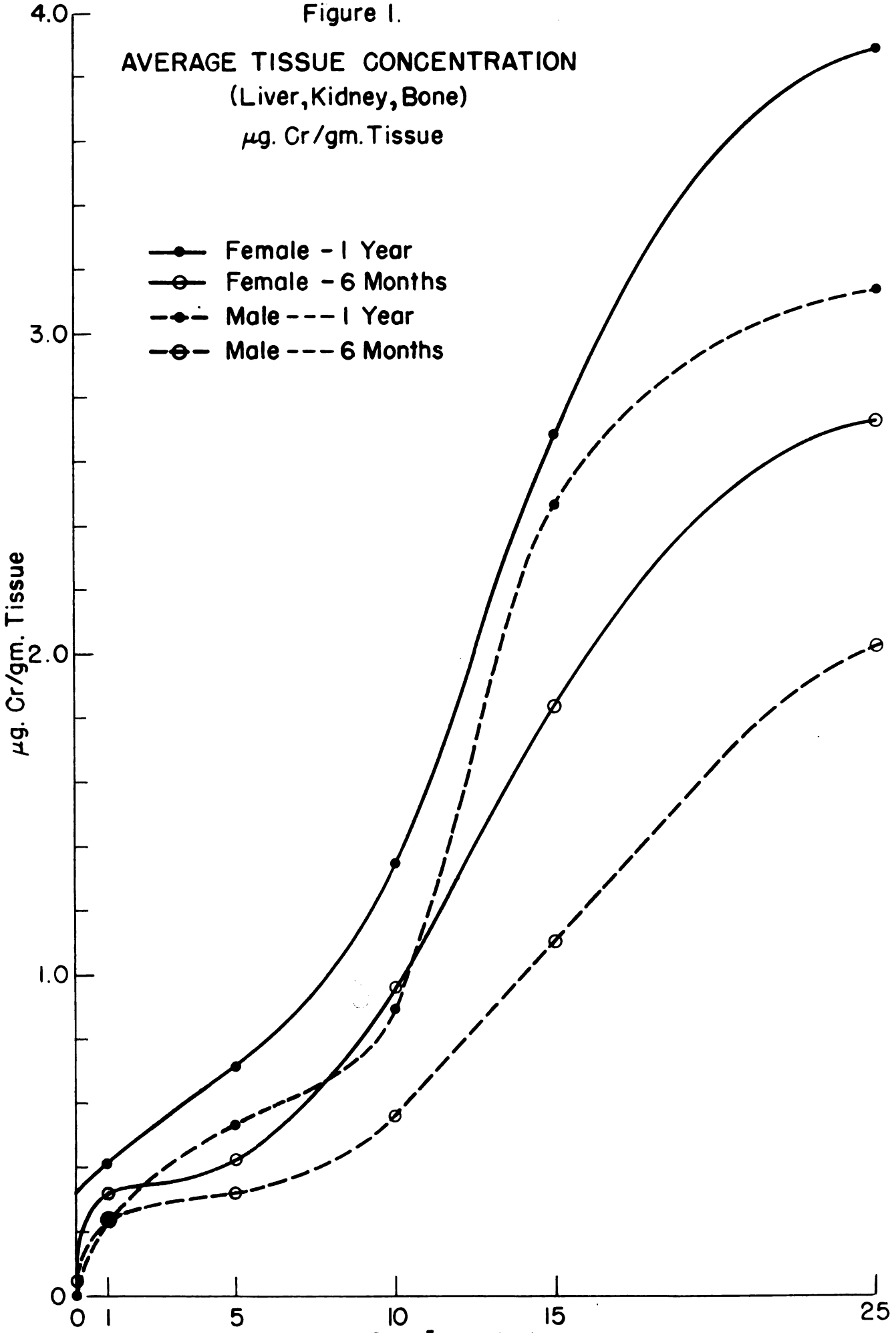
Tissues (Cr/gm tissue) Group Number	$CrO_4^=$ ppm	Liver		Kidney		Bone	
		Male	Female	Male	Female	Male	Female
I	0	0.041	—	—	—	0.525	—
II	1	0.107	0.104	—	—	0.590	0.847
III	5	0.081	0.163	—	—	0.825	1.07
IV	10	0.184	0.16	—	1.0	1.49	1.71
V	15	0.54	0.94	0.45	1.84	2.31	2.7
VI	25	0.8	0.99	2.27	3.0	2.68	4.2

TABLE VII

CHROMIUM CONCENTRATIONS IN RAT TISSUES AFTER ONE YEAR

Tissues (Cr/gm tissue) Group Number	$CrO_4^=$ ppm	Liver		Kidney		Bone		Spleen	
		Male	Female	Male	Female	Male	Female	Male	Female
I	0	—	—	—	0.25	—	0.72	—	—
II	1	0.02	0.08	0.14	0.39	0.58	0.76	0.95	0.91
III	5	0.08	0.17	0.29	0.48	1.27	1.48	0.68	1.14
IV	10	0.15	0.47	0.45	1.09	2.14	2.44	3.41	4.18
V	15	0.70	0.55	3.30	2.39	3.43	5.10	5.24	4.73
VI	25	1.22	1.62	4.40	3.98	3.84	6.06	9.91	11.09

Figure 1.


AVERAGE TISSUE CONCENTRATION
(Liver, Kidney, Bone)
μg. Cr/gm. Tissue
Female - 1 Year
Female - 6 Months
Male --- 1 Year
Male --- 6 Months
μg. Cr/gm. Tissue
4.0
3.0
2.0
1.0
0
0
1
5
10
15
25

EXPERIMENT II -- ABSORPTION AND DISTRIBUTION STUDIES

Experiment IIa -- Distribution and Percent Absorption Study

Procedure

Thirty-two male albino rats of the Sprague-Dawley strain, weighing about 260 gms. (245-271), were divided into two equal groups. One group was starved for 24 hours before injecting the chromium, whereas the other group was fed ad-libitum. The same diet was used as in Experiment I (See Table II). The starved animals did not receive feed until two hours after the chromium was injected.

Each rat was injected by stomach tube with a sodium chromate solution containing radioactive chromium (Cr^{51}). The injecting dose was 57 micrograms of chromium, which contained 22.8 microcuries of Cr^{51} in a volume of 1.5 ml. The sodium chromate was prepared by oxidizing $Cr^{51}Cl_3$ with hydrogen peroxide in 6N NaOH. The excess hydrogen peroxide was removed by long simmering on a hot plate and the pH of the final solution adjusted to 7.5.

After the rats were injected, they were placed in metabolism cages. Feces and urine were collected separately. This was facilitated by spraying the metabolism cages with an acrylic plastic and placing a similarly treated small mesh screen at the bottom to prevent the feces from falling into the beaker used for collecting the urine. The feces were removed from the bottom of the cage daily to minimize contamination of the urine.

The rats were sacrificed at intervals of 6, 24, 72, and 168 hours. Liver, kidney, stomach, intestine, and blood, as well as the urine and

feces were selected for analysis. The samples were prepared for counting by partial oxidation with concentrated nitric acid and some hydrogen peroxide. A 2.0 ml. sample representing all or a known portion of each sample was counted in a well-type scintillation tube containing a thallium activated sodium iodide crystal. The counting was done at 1500 volts and the normal background was 188 counts per minute.

Results

The average activities, expressed in terms of percentage of the injected dose, are found in Table VIII. Since the total amount of chromium injected was only 57 micrograms, this corresponds approximately to what a rat would ingest daily by drinking water containing 2 ppm. The distribution would be comparable to that found at the beginning of a chronic experiment.

It appears that considerably more chromium is absorbed in the starved animals than in the non-starved. This can be seen readily by comparing the activities in the urines of both groups at each interval. It also shows that the greatest part of the injected dose is not absorbed, but is excreted in the feces.

Experiment IIB -- Absorption Study Part I

In experiment IIa, it was found that there was a greater absorption in the starved than in the non-starved animals. The presence of food in the digestive tract obviously decreases the absorption of chromium and it may do so by changing the valence. There is no

TABLE VIII

PERCENT RETENTION IN TISSUES AT VARIOUS INTERVALS

Time (hours)	Percent Injected Dose							
	6		24		72		168	
Condition / Tissue	Starved	Non-Starved	Starved	Non-Starved	Starved	Non-Starved	Starved	Non-Starved
Stomach	7.9	3.46	0.89	1.8	0.14	0.11	0.015	0.010
Intestine	81.1	90.5	21.9	18.2	0.85	1.9	0.012	0.031
Blood	0.162	0.042	0.10	0.032	0.048	0.01	0.003	0.007
Liver	0.13	0.026	0.19	0.029	0.111	0.022	0.0044	0.018
Kidney	0.07	0.012	0.14	0.026	0.104	0.013	0.004	0.018
Urine	0.71	0.26	2.26	1.07	5.3	2.25	5.4	2.4
Feces	1.43	0.0003	62.3	69.7	88.14	88.5	90.3	95.0
Average Recovery	91.4	94.0	87.7	90.9	94.7	92.8	95.7	97.5

satisfactory way to differentiate the valence states of chromium in tissues. Gray and Sterling (20) have shown that erythrocytes will absorb chromate ions but not trivalent chromium. The next two experiments use this difference in absorption by red cells as a means of distinguishing between the two forms of chromium.

Procedure

Sixteen male albino rats weighing about 330 gms (308-375) were equally divided into four groups. Two groups received hexavalent chromium by stomach tube, four rats being starved for 24 hours before injection and the other four fed ad-libitum. The other rats received trivalent chromium, four being starved and four non-starved.

Each rat received by stomach tube 125 micro-curies in 1.0 ml. of solution containing 131 micrograms total chromium. The hexavalent chromium was given as the sodium salt (see Experiment IIA for preparation) at pH 7.5. Chromic chloride at pH 5.0 was the source of trivalent chromium.

Four hours after the rats were injected they were anesthetized with ether and blood removed from the heart before the rats died. The syringes were rinsed with heparin to prevent clotting. The blood was centrifuged and the red cells and plasma partially oxidized with concentrated nitric acid and hydrogen peroxide. As in the previous experiment 2.0 ml. samples were then counted in the scintillation counter at 1500 volts.

Results

The average activities found in the red blood cells and plasma are shown in Table IX. Also shown for comparison are the ratios between counts in the red cells and plasma per unit volume. Although these are probably not absolute values, they do reflect the influences of valence state and alimentation on the amount and distribution of chromium. The results also indicate that even in the starved animals there may be some reduction of the hexavalent chromium before absorption can take place.

TABLE IX

AVERAGE ACTIVITIES IN RED CELLS AND PLASMA

Group	Counts per Minute			
	$NaCr^{51}O_4$		$Cr^{51}Cl_3$	
Conditions	Starved	Non-Starved	Starved	Non-Starved
Avg. Count/ml. Whole Blood	459	261	217	108
Avg. Count/ml. RBC (corrected)*	313	67	0*	0*
Avg. Count/ml. Plasma (corrected)*	1490	592	447	398
Ratio RBC:Plasma	1:4.76	1:8.8	--	--

*The corrections are based on the assumption that red cells absorbed no trivalent chromium. The actual counts found in red cells were 14 and 10 for the starved and non-starved rats respectively and are probably due to incomplete removal of the plasma.

Experiment IIc -- Absorption Study Part II

Is one valence state absorbed at a greater rate than the other or are both valence states absorbed at about the same rate? Why do the starved animals fed hexavalent chromium absorb more chromium than the

non-starved or those fed trivalent chromium? To answer these questions more completely the following experiment was made.

Procedure

Twenty-four male albino rats weighing 345 gms. (330-371) were used in this experiment. The conditions of this experiment were as follows:

TABLE X

CONDITIONS IN EXPERIMENT IIc

Group Number	Condition
1	Na_2CrO_4 injected into starved rats
2	Na_2CrO_4 injected into non-starved rats
3	$CrCl_3$ injected into starved rats
4	$CrCl_3$ injected into non-starved rats
5	Equal mixture of both valence states injected into starved rats
6	Equal mixture of both valence states injected into non-starved rats

Four rats were used in each group.

In this experiment radioactive chromium was injected directly into the intestine. The rats were anesthetized with ether and incisions of about 1-1.5 inches made just below the lower rib in the center of the abdomen. The intestine and stomach were located and the chromium solution injected about 1.5 inches below the stomach.

One milliliter of solution contained 375 micrograms of total chromium and an activity of 125 micro-curies was injected into each rat.

The solutions were prepared as in Experiments IIa and IIb. The equal mixture of both valence states was made by adding 5.0 ml. of the sodium chromate solution to 5.0 ml. of chromic chloride solution. The trivalent chromium formed a slight colloidal suspension. Since the particles were so small, a homogenous solution was easily maintained. The solutions were injected with a 2 ml. syringe equipped with a short 27 gauge needle.

Four hours after the injections, the rats were bled from the heart with a syringe rinsed in heparin. The blood was centrifuged and the plasma carefully removed with a pipette. The red cells were washed three times with isotonic saline solution to remove most of the plasma from the red cells. The red cells and plasma were then partially oxidized with concentrated nitric acid and hydrogen peroxide and 2.0 ml. samples counted in the scintillation counter as previously described.

Results

The average activity in counts per minute for each group is shown in Table XI. The table includes average counts per minute per milliliter of whole blood, average counts per minute per milliliter of plasma and average counts per minute per milliliter of red blood cells. The table also includes the ratios of red cell to plasma counts for each group and condition.

Absorption was greatest in starved rats receiving hexavalent chromium and lowest in non-starved rats receiving trivalent chromium.

TABLE XI

AVERAGE ACTIVITIES IN RED CELLS AND PLASMA

Group Number	Average Counts per Minute: Whole Blood Per ml.	Plasma Per ml.	R.B.C. Per ml.	Ratio RBC:Plasma
1	2,500.	3,135.	1,886.	1:1.66
2	1,098.	1,587.	500.	1:3.17
3	230	653	3	1:217
4	134	433	6	1:72
5	449	737	328	1:2.25
6	285	481	132	1:3.64

It may be noted that the average count per minute per milliliter of whole blood in the starved group given the 50-50 mixture is about the same as that of the starved group receiving an equivalent amount of chromium as hexavalent by stomach tube in experiment IIb. This indicates a definite reduction in the stomach even when devoid of food.

EXPERIMENT III -- A STUDY OF THE RELATIVE TOXICITY OF HEXAVALENT AND TRIVALENT CHROMIUM

Rat Care and Diets

Albino rats of the Sprague-Dawley strain were about 35 days old when the experiment was started. They were separated into three groups each composed of 9 females and 12 males. More males than females were used because of the greater mortality rate of males in Experiment I from respiratory infection. Group I was the control group, Group II was given water containing 25 ppm of chromium as potassium chromate and Group III was given 25 ppm of chromium in the form of chromic chloride solution.

The rats were placed in individual cages. The room temperature was maintained between 75° and 79°F. throughout the year. All rats received the stock diet previously used. Weekly records were kept of body weight, food and water consumption. At the end of one year the rats were sacrificed. Tissues were taken for a study of pathological changes and for chromium analysis as in Experiment I.

Blood Studies

Two weeks before the rats were sacrificed blood samples were taken from two males and two females of each group for the determination of hemoglobin concentration and differential cell count.

Pathology

The rats were sacrificed by the same procedure as in Experiment I. Samples were taken from the following tissues: adrenal glands, liver,

kidneys, spleen, brain, heart, stomach, duodenum, ileum, colon, and cross-section of bone marrow from the sternum and femur. Fixatives used were Zenker's, neutral 10% saline-formalin and Carnoy's fluid. All the tissues were stained with hematoxylin and eosin as in Experiment I. Small portions of the liver, kidney and adrenal gland were stained with Bower's Carmine for glycogen and Sudan IV for fat.

Chromium Analysis

The tissues analyzed for chromium were kidney, femur, liver and spleen. The procedure was that of Saltzman (24) described in Experiment I, but with one modification. The 2.0 ml. of 4 M sodium dihydrogen phosphate (NaH_2PO_4) was added to the sample solution before the diphenylcarbazide reagent. This would bind any iron present and thus prevent its interference in the chromium complex development. A standard curve was made by this modified procedure.

Results

When the weight gains and food consumption were averaged at the end of the year no differences were found between the groups of females. The high incidence of disease and mortality in the males makes any comparison between the male groups meaningless. Nevertheless, it is possible to conclude that the consumption of water containing as much as 25 ppm of chromium in either hexavalent or trivalent form produced no gross changes within a year.

The water intake, however, did show a slight variation (Table XII). Rats given hexavalent chromium drank less than the controls or those

given trivalent chromium. The females consumed only 77% as much water as the controls, while the average consumption of the males was 84% that of the controls. There was no significant difference between the water consumption of the control and trivalent groups.

The blood analysis showed no differences in blood cell morphology or hemoglobin concentration in any of the groups. Microscopic study of tissue sections revealed no evidence of pathology due to the ingestion of either hexavalent or trivalent chromium.

The concentration of chromium in each tissue in the different groups is shown in Table XIII. The data indicate a considerable difference in chromium concentration between Group II and III. Group II, that given chromate, averaged about 5 times the concentration in Group III (trivalent chromium). The values in the tissues of Group III (25 ppm Cr., equivalent to 55.5 ppm chromate ion) correspond to values found in tissues in Experiment I at levels of 5, 10, 15 ppm chromate ion depending upon the tissue compared.

TABLE XII

AVERAGE WATER INTAKE OF EACH GROUP

Time (in months) / Group Number	1		3		6		9		12	
	Male ml/rat/day	Female ml/rat/day	Male ml/rat/day	Female ml/rat/day	Male ml/rat/day	Female ml/rat/day	Male ml/rat/day	Female ml/rat/day	Male ml/rat/day	Female ml/rat/day
I Control	36	34	31	32	35	36	37	36	36	35
II 6+	32	28	30	26	32	28	24	24	29	27
III 3+	36	36	33	31	31	35	34	33	36	36

TABLE XIII

CHROMIUM CONCENTRATION IN RAT TISSUES

Tissues	Kidney		Bone		Liver		Spleen	
	mcg.Cr/gm Tissue		mcg.Cr/gm Tissue		mcg.Cr/gm Tissue		mcg.Cr/gm Tissue	
Group	Male	Female	Male	Female	Male	Female	Male	Female
I Control	0.28	0.35	0.77	0.78	0.05	0.08	0.90	0.91
II Cr^{6+}	10.47	13.79	4.07	8.71	3.2	8.12	23.53	52.85
III Cr^{3+}	1.71	2.07	1.03	1.23	0.35	0.53	3.22	3.61

DISCUSSION

DISCUSSION

The main purpose of this study was to determine the minimum concentration of chromium ingested in water that would cause pathological changes in the rat. No changes were evident after one year at any of the levels used (Experiment I). A study of the concentration of chromium in the tissues indicated that at low concentrations (0-5 ppm of chromate ion) little accumulation occurred. Between 5 and 10 ppm chromate ion there was an appreciable increase in chromium found in the tissues (Figure I). This change in retention was observed both at the end of 6 months and at the end of one year. The results of this study show that there was relatively little retention of chromium when the water contained up to 5 ppm of chromate ion. This indicates that small amounts of ingested chromium are efficiently eliminated. The increased retention at higher intakes means that there is a physiological limit in the elimination of chromium. On the basis of these results it would not be unwarranted to consider 5 ppm of chromate ion as a maximum allowable concentration for a healthy, normal albino rat. Whether this would apply to other animals including man can not be said. Diseases of the kidney might seriously impair the capacity to eliminate chromium and lead to harmful accumulations. Other diseases might make a person much more sensitive to chromium. The establishing of a higher allowable concentration than the present 0.05 ppm of chromium will have to be postponed until further work on the metabolism of chromium has been done.

Experiments I and III indicate the presence of very low concentrations of chromium in the control animals. This can be expected because small amounts of chromium are found in food and water supplies. In the control groups of these experiments the largest concentration of chromium was found in the femur bone. This may suggest a method of controlling the concentration of chromium in the system. The bone may take it up and then slowly eliminate it. This may work efficiently only at very low intakes as chromium is not generally known as a bone seeker. The increase in soft tissues and the bone were similar. Above 5 ppm of chromate ion the soft tissues increased at a greater rate than the bone and at 25 ppm surpassed it in concentration (Cr./gm. tissue). At this level the rate of accumulation in bone decreased.

Experiment II indicated only 5.5% maximum absorption of chromium occurred in the form of hexavalent chromium. Since the hexavalent chromium is apparently reduced to a variable degree in the digestive tract, it is difficult to determine to what extent pure chromate ions would be absorbed if none was reduced. The chromate ion is obviously more readily absorbed than trivalent chromium. However, considerable amount of reduction occurs even in the stomach of the starved animals. Experiment IIc indicates reduction of chromate ion not only in the acid environment of the stomach, but also in the intestine and in the blood stream.

Experiment III shows that about 5 times more hexavalent than trivalent chromium was absorbed. The concentration found in the tissues

of the trivalent chromium group receiving water containing the equivalent of 55.5 ppm chromate ion, correspond to the amounts found in the tissues of the groups given 5, 10, or 15 ppm of chromate ion in Experiment I depending on the tissue. It is evident from these results that trivalent chromium shows a much lower absorption than hexavalent. It is, therefore, suggested that chromate wastes be treated with a reducing agent before they are disposed of.

The conclusion of this study is that the occurrence of hexavalent chromium in water is potentially more hazardous than trivalent chromium, because it is more readily absorbed into the body. No toxic symptoms have been observed at any of the intakes over a period of one year, although quite high concentrations were found in the tissues. Apparently tissues can accumulate considerable quantities of chromium without causing damage.

SUMMARY

SUMMARY

1. Five groups of rats were administered concentrations of chromium, between 1.0 and 25.0 parts per million as chromate ion, in the drinking water. During a one year period there were no differences between these groups and the controls as to water intake, food consumption or weight gain.

2. An analysis of blood at regular intervals for one year showed no significant differences between any of the groups given chromium and the control group. No significant microscopic changes were found in any of the tissues examined.

3. Kidney, liver and femur were analyzed for chromium at the end of six months and the spleen included at the end of one year. The chromium content of the tissues increased from quite low to relatively large amounts. There was an abrupt rise in tissue retention at water concentrations above 5 ppm of chromate ion.

4. Starved and non-starved rats were injected by stomach tube with 57 micrograms of radioactive chromium in the form of chromate having an activity of 22.8 microcuries. They were sacrificed at intervals of 6, 24, 72, and 168 hours. Approximately 5.5% of the injected dose was absorbed from the starved animals, while only 2.5% was absorbed from the non-starved animals. Blood, stomach, intestine, liver, kidney, urine and feces were analysed. The highest activity in

the blood occurred at 6 hours or before, whereas the highest activity in the liver and kidney occurred at about 24 hours, slowly decreasing thereafter. There was more activity in the tissues of the starved animals than in the non-starved. The rest was eliminated in the feces in about a week.

5. Starved and non-starved rats were given radioactive sodium chromate or chromic chloride solution equal to 131 micrograms of chromium with an activity of 125 microcuries by stomach tube and blood samples taken 4 hours later. The activity of the plasma and red cells was determined separately. The starved animals receiving sodium chromate solution absorbed the most chromium while the non-starved group given chromic chloride absorbed the least. There was considerable reduction of the chromate in the digestive tract.

6. Radioactive sodium chromate, chromic chloride or a mixture of equal parts of each were injected into the intestines of three groups of rats. The amount injected in each case was 375 micrograms with an activity of 125 microcuries. Blood samples were taken 4 hours after injection. The plasma and red cells were counted separately. The absorption was greatest in the starved animals given chromate and lowest in the non-starved group given chromic chloride. The fact that much higher blood values were obtained than in the previous experiment indicates that a considerable amount of reduction of chromate occurs in the stomach.

7. Two groups of rats were administered 25 ppm of chromium either as hexavalent or trivalent chromium. During a one year period, no toxic symptoms occurred in either group. However, tissue concentrations were much higher in the group given hexavalent chromium. This indicates a much greater absorption of chromate ion than trivalent chromium.

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