STUDIES ON CHROMIUM EXCRETION IN THE DOG

Thesis for the Degree of Ph. D.

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Robert James Collins

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This is to certify that the

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W. D. Collings
Major professor

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STUDIES ON CHROMIUM EXCRETION IN THE DOG

By

ROBERT JAMES COLLINS

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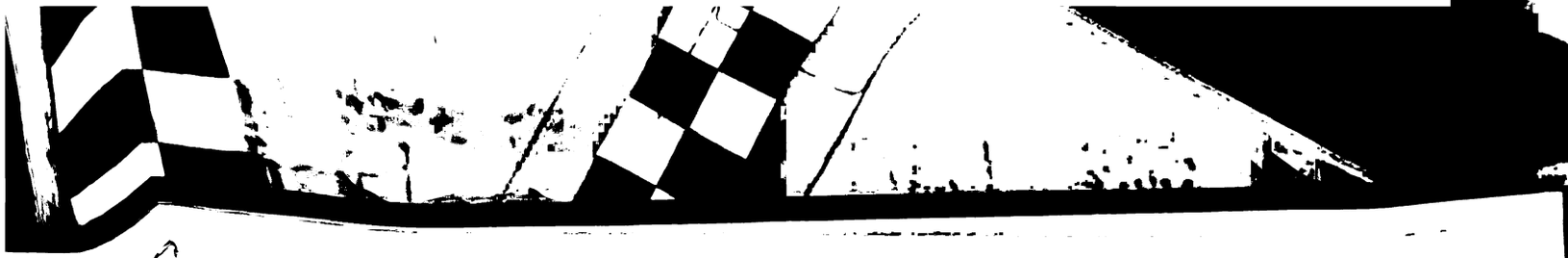

ABSTRACT

No demonstrable biologic function has been reported for chromium although it is omnipresent in trace quantities in man, other animals, food products, soils, and water supplies. In addition, extensive use of chromium in industry frequently results in exposure of animal tissues. Toxicity and tissue distribution of chromium compounds is well documented but little information exists on its excretion. Objectives of this study were to investigate the routes and mechanisms involved in chromium excretion as well as to compare some of the physical and chemical properties of chromium in biological fluids to its characteristics before injection.

Routes of excretion were studied in acute anesthetized dogs over a four-hour period and in bile fistula dogs for four days after intravenous injection of Cr^{51} as chromic chloride or sodium chromate. These studies showed that urine was the major route of excretion of chromium of either valence after intravenous injection; excretion in bile and feces was negligible.

Mechanisms employed in renal excretion of chromium were studied by the renal clearance technique. Renal clearance of chromium decreases exponentially with time after a single intravenous

injection of $Cr^{51}Cl_3$ from 2.5 or 3 ml. per min. at one hour after injection to less than 1 ml. per min. eight hours postinjection. Measurement of plasma-dialyzable chromium by equilibrium dialysis showed that the percentage not bound to plasma proteins in vivo also decreased exponentially with time after injection. These observations led to determination of a dialyzable chromium clearance ($C_{d\text{-}chr.}$). The mean and standard deviation of $C_{d\text{-}chr.}$ on eight dogs measured over periods up to ten hours after injection was 36.3 ± 16.6 ml. per min. This clearance indicates that glomerular filtration and tubular reabsorption are two mechanisms of renal function involved in the handling of unbound chromium. Tubular excretion of dialyzable chromium may occur; however, no reduction in $C_{d\text{-}chr.}$ at high plasma levels favors the conclusion that tubular excretion is of minor importance. Simultaneous creatinine and PAH clearances were normal, demonstrating that renal function was not impaired by large doses of chromium given intravenously.

Measurement of trivalent and hexavalent chromium in urine after the intravenous injection of sodium chromate showed that reduction readily occurs in vivo. Chromium excreted in urine and bile or present in a plasma dialyzate is anionic as shown by ion exchange absorption after intravenous administration of cationic chromic chloride.

Paper chromatography and precipitation procedures on urine and plasma dialyzate indicate, but do not prove that Cr^{51} is excreted in part in organic combination.

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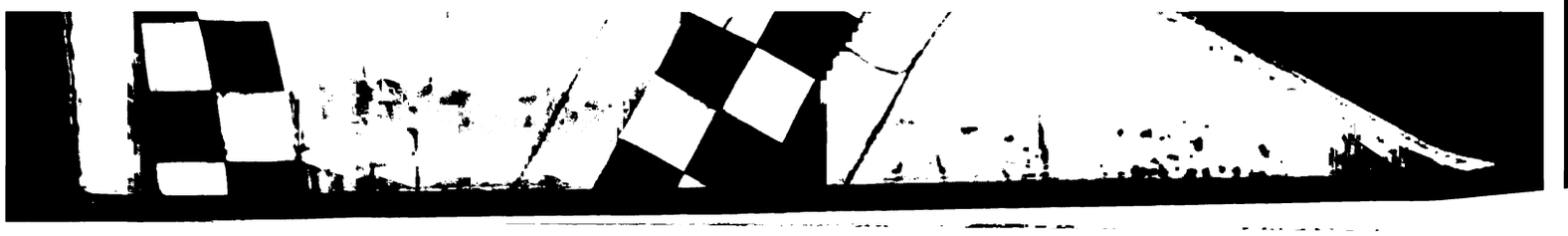

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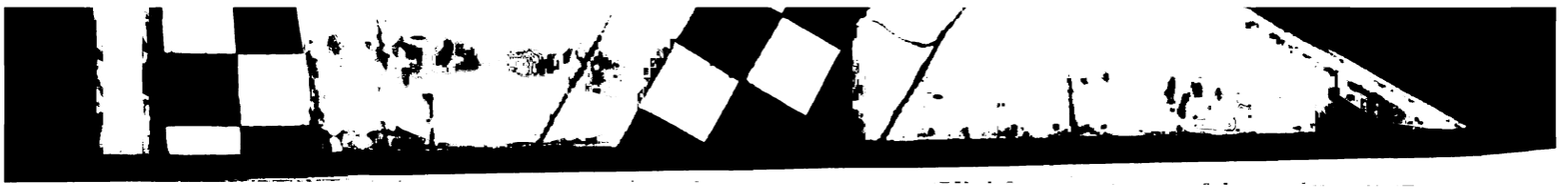

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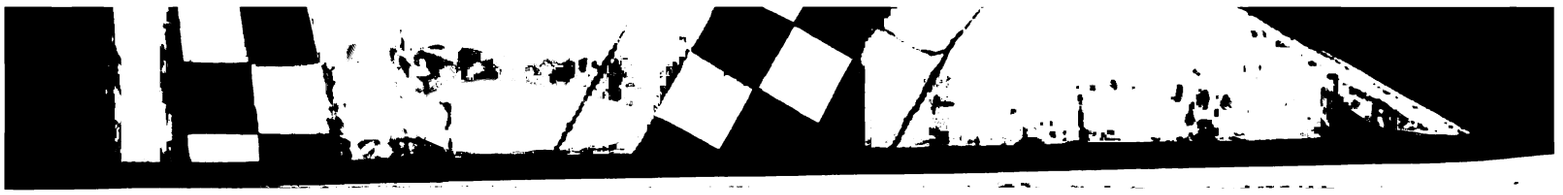
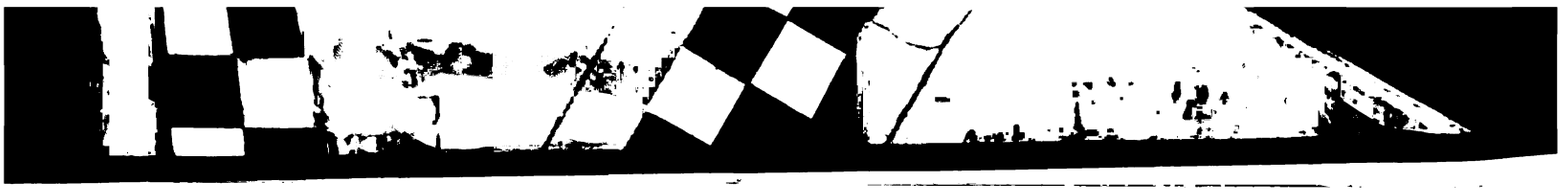




INTRODUCTION

No demonstrable biologic function has been reported for chromium; however, trace quantities have been found in tissues of man, other mammals, and food products (Grushko, 1948; Van der Walt and Van der Merwe, 1938; Koch et al., 1956; Udy, 1956). Access of chromium compounds to animals and plants is provided by its wide distribution in soils and water supplies (Braidich and Emery, 1935; Davidson and Mitchell, 1940; David and Lieber, 1951; Udy, 1956).

Walsh (1953) has reviewed hazards to workers in industry where chromates are used. In chrome plating and metal anodizing by electrolysis of a chromic acid bath, hydrogen bubbles produce a mist of chromic acid which causes ulcers and respiratory inflammation. Chromate solutions used to control corrosion in various water recirculating systems are responsible for dermatitis and chrome ulcers. Chromium ore dust as well as chromate have been implicated in liver damage and lung cancer. Chromate workers have a death rate due to lung cancer twenty-nine times greater than non-chromate workers (Brinton et al., 1952). Chromate hazards may also result from its use in batteries, cement, and primer paints.

The despicable practice of coloring food with yellow lead chromate has fortunately been discontinued (Monier-Williams, 1949).

Exposure to sufficient quantities of chromium can also cause dermatitis, ulceration (Sollman, 1957) and possibly cancer among workers in the leather-tanning industry. The finished leather contains 3.9 to 6.9 per cent chromium that occasionally causes irritation and sensitization.

With the advent of radioisotopes, chromium compounds containing Cr^{51} have been used in research, diagnosis, and therapy. These uses of radio-chromium result in exposure of animal tissues even though large quantities are not used. Colloidal chromic phosphate has been used in therapy of blood diseases (Fields and Seed, 1957) and as a reticulo-endothelial function test (Gabrieli, 1951). Hexavalent Cr^{51} has found application in the diagnosis of hemolytic anemias (Korst et al., 1955), measurement of red cell volume (Sterling and Gray, 1950) and half life (Read et al., 1954), location and quantitation of intestinal bleeding (Owen et al., 1954) and as a tag for leucocytes (McCall et al., 1955). Trivalent chromic chloride is used for measuring plasma volume (Frank and Gray, 1953).

Although toxicity and tissue distribution of chromium compounds is reasonably well documented in mammals, there is little information available on routes and physiological mechanisms

involved in the excretion of chromium once it gains entrance to animal tissues. For this reason a study of the following aspects of chromium excretion was made: (1) the quantitation of excretion of trivalent and hexavalent chromium in urine, bile, and feces after intravenous injection; (2) plasma binding of chromium in vivo; (3) physiological mechanisms of chromium excretion by the kidney; (4) reduction of hexavalent chromium in vivo; and (5) investigation of the excretion of "bound" chromium.

Mongrel dogs were used in all experiments. Investigation of the routes and relative amounts of chromium excreted by each route were done in two ways: (1) acute experiments on anesthetized dogs in which the ureters and bile duct were cannulated, and (2) experiments on unanesthetized dogs with bile fistulas. Determination of plasma binding and renal mechanisms involved in chromium excretion were done by dialysis and the renal clearance technique. Analysis for hexavalent chromium, paper chromatography, ion exchange, and dialysis procedures were used to elucidate chromium reduction and the possible excretion of "bound" chromium.

LITERATURE REVIEW

Inorganic Chemistry of Chromium

For didactic reasons a paragraph on the valence states of chromium is presented here.

Chromium is a member of Group VI of the periodic table and is characterized by its ability to serve both as a metal and as a nonmetal. Generally it behaves as a metal at lower valences while the characteristics of an acid grow stronger as the valence is increased. Chromium forms compounds in which it is divalent, trivalent, or hexavalent. In both the trivalent and hexavalent state it forms two groups of compounds which differ in degree of hydration. Consequently the following five classes of chromium compounds exist: (1) chromous (basic), (2) chromic (weakly basic and amphoteric), (3) chromite (feebly acidic), (4) chromate (acidic), and (5) dichromate (acidic) (Hopkins, 1942). In addition, univalent and pentavalent chromium are encountered in certain reactions, but only the trivalent and hexavalent states exhibit sufficient stability to exist in biological systems. Of these two, trivalent is more stable than hexavalent at pH's below 7.

Hexavalent chromium, when dissolved in water, exists in true solution regardless of the hydrogen ion concentration or the presence of other ions. However, trivalent chromium, when added to water, exists in solution as a complex or as a colloidal or flocculent precipitate depending on the pH and dissolved substances. The chemistry associated with solvation of trivalent chromium is complex and remains in part theoretical. A brief resumé of the interactions of chromium with aqueous solvents is pertinent (Gustavson, 1956; Udy, 1956). When chromic chloride is dissolved in water, hydration or coordination occurs so that the complex $[Cr(H_2O)_6]^{+3} + 3Cl^-$ is formed. This simple hydration does not satisfy the binding forces of the chromium atom; consequently, one or more oxygen atoms are drawn within the coordination sphere of the chromium atom. The oxygen atom of the water molecule becomes positively charged and so it releases a proton (H^+). The net result is that the chromium cation formed has an ionic charge of +2 instead of +3.

$$[Cr(H_2O)_6]^{+3}\ Cl_3 \rightleftharpoons [Cr^{OH}(H_2O)_5]^{+2}\ Cl_2 + HCl$$

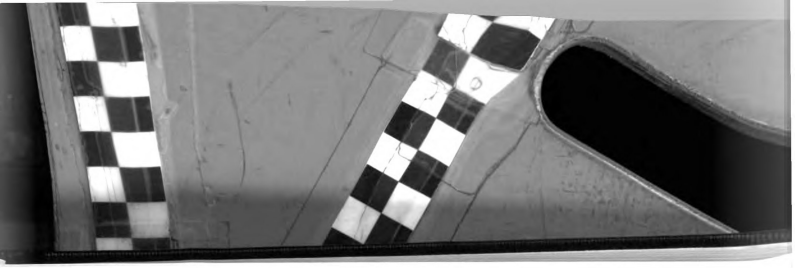
Protolysis or hydrolysis, as this process is called, is favored by the removal of hydrogen ions from the system. The tendency of the chromium atom is to attain the electronic structure of krypton. This can be accomplished by further hydrolysis so that chromium

complexes with charges of +1, 0, -1, -2, and -3 are produced by the successive hydrolysis if protons produced by the reaction are removed. In addition to water, trivalent chromium readily forms coordinate complexes with a large number of neutral or charged groups, some of which are NH_3, $(NO_3)^{-1}$, C_2H_5OH, NO_2, $(OH)^{-1}$, and $(SO_4)^{-2}$ (Udy, 1956). These complexes can also undergo successive hydrolysis.

A further complication in chromium chemistry is that divalent chromium cations produced by a single hydrolysis may interact to form a diol with two chromium atoms per molecule and an ionic charge of +4.

$$2[Cr^{/OH}(H_2O)_5]^{+2}\ Cl_2^- \rightleftharpoons [(H_2O)_4\ Cr^{/OH\backslash}_{\backslash OH/}Cr\ (H_2O)_4]^{+4}\ Cl_4^- + 2H_2O$$

The addition of one mole of sodium hydroxide per mole of chromium hydrate results in a quantitative formation of the diol. More sodium hydroxide results in large polynuclear complexes containing great numbers of chromium atoms in each molecule. Finally, if protons are removed as they are formed, the chromium complexes become so large that they can no longer be accommodated by the solvent and precipitation occurs. Thus the solvation of chromium can result in a conglomeration of chromium molecules varying greatly in size and ionic charge.



Nature of Chromium-Protein Bond

The tanning of leather with chromium salts has led to extensive investigation of the interaction of chromium complexes with collagen and gelatin. Bowes and Kenten (1949) demonstrated the importance of carboxyl groups in the fixation of chromium by proteins. Esterification of these groups led to decreased fixation of chromium by proteins. Strakhov (1951) reported that collagen binds more chromium than silk fibroin presumably because it has more free carboxyl and amino groups. An e-capralactam polymer, devoid of amino and carboxyl groups, was unable to fix chromium at all. By treating collagen with formaldehyde which blocks free amino groups, the initial reaction of chromium in cationic form was shown to be with carboxyl groups, while anionic chromium was found to react first with amino groups.

Early theories of the possible mechanism involved in binding chromium to proteins are reviewed by Shuttleworth (1950) who found 154 articles worthy of mention. While no single theory explains all the data or is accepted by all leather chemists, Gustavson's (1949, 1956) concept of the formation of chelate compounds is attractive. According to this concept, the initial reaction is an ionic attraction of cationic chromium complexes such as $[Cr_2(OH)_2SO_4]^{+2}$ with the

charged carboxyl groups of collagen represented by $COO^{-}\cdot P\cdot NH_3^{+}$, the sulfate ions being compensated by the NH_3 ions. Carboxyl groups, having a great tendency for complex formation and for a direct attachment to chromium, penetrate into the coordinate sphere forming a covalent-coordinate bond. Since several chromium atoms are present in large chromium complexes, there is the possibility of a multipoint interaction of a 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 chromium bridge.

That this view of the nature of the chromium-protein bond may be incomplete is shown by Green's (1953) observation that hydroxyl and amino groups as well as carboxyl groups of proteins are involved in chromium fixation. He found that increased acetylation of free amino or hydroxyl groups of collagen decreased fixation of either cationic or anionic chromium complexes.

The Binding of Chromium by Tissue Proteins

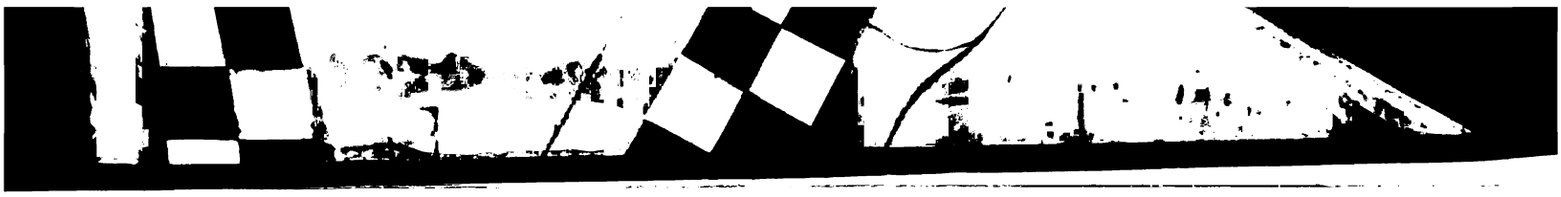
Brard (1935) first pointed out the selective partition of chromate between erythrocytes and plasma proteins. However, Gray and Sterling (1950) investigated the fundamental aspects of the binding of chromium to proteins and erythrocytes. They demonstrated

that erythrocytes have a marked affinity for anionic chromate. When small quantities of sodium chromate were incubated with a saline suspension of erythrocytes, over 97 per cent of the chromium was taken up by the red cells and a semilogarithmic plot of the uptake indicated a first order reaction. Under their experimental conditions the uptake half-time of chromium was 38 minutes. The constant for this reaction was approximately 0.02, indicating that about 2 per cent of the chromium remaining in the saline was bound each minute. The large uptake and the exponential nature of the reaction suggested that chromium was bound or modified after gaining entry into the cell. This aspect was investigated by lysing red cells tagged with Cr^{51} labeled chromate and comparing the counts of each fraction with that of the intact cell. Hemoglobin was found to contain 97 per cent of the activity of the red cells, while only 2 per cent of the activity was in the washed stroma. This indicates that chromium after gaining entry into the red cell is bound to hemoglobin rather than changed to an indiffusable molecule. Fractionation of the hemoglobin into globin-HCl and hemin showed that chromium is bound to the globin and not to the heme. The firmness of the chromium-globin bond was indicated by noting that the addition of nonradioactive chromate to a Cr^{51} tagged red cell suspension did not result in elution of the tag from the red cells. This observation also shows

that chromium binding is essentially irreversible or at least that the equilibrium is largely in favor of the bond. As further evidence of the firmness of the chromium bond it was shown that continuous shaking of a tagged red cell suspension for periods up to 43 hours resulted in little loss of the tag to the saline diluent.

Trivalent chromium binding was also studied by Gray and Sterling. Chromic chloride when injected intravenously in dogs did not enter the erythrocytes but remained in the plasma for a considerable time suggesting protein binding. This was verified by studying the binding of trivalent chromium by albumin. Radioactive chromic chloride was incubated with crystalline bovine albumin and the percentage bound determined by dialysis. At low molecular ratios of chromium to albumin (1:1) 70 per cent of the chromium was bound, while at higher ratios (100:1) only 40 per cent binding occurred. The firmness of the chromium-albumin bond was indicated by prolonged dialysis which resulted in little or no further loss of chromium from the albumin, and by the addition of nonradioactive chromium to tagged albumin, with the result that only a small loss occurred on dialysis. As with the tagging of erythrocytes with chromate, the binding of trivalent chromium to albumin is essentially irreversible or the equilibrium is greatly in favor of the bond.

Gray and Sterling suggested that hexavalent chromium binds to hemoglobin within the red cell only after becoming reduced to the trivalent state. Several facts can best be explained by this hypothesis. They showed that under identical circumstances trivalent chromium would bind to albumin to a considerably greater extent than would hexavalent chromium. Also trivalent chromium was much more effective in tagging cell-free hemoglobin. A reasonable explanation of these observations was that hexavalent chromium diffused into the erythrocytes was reduced to the trivalent state and bound to hemoglobin. The reason greater binding occurred within the cell than with cell-free hemoglobin may have been that the chromium, once reduced, could not leave the red cell and so remained in close proximity with the hemoglobin. The small amount of hexavalent chromium bound to albumin may be hexavalent chromium that had undergone reduction. The work of Grogan and Oppenheimer (1955) lends support to this concept. They were unable to demonstrate the binding of hexavalent chromium to egg albumin or human plasma proteins by paper electrophoresis at pH 7.35. In dialysis experiments at pH 4.14, where hexavalent chromium is less stable, considerable binding of hexavalent chromium could be demonstrated. However, at hydrogen ion concentrations above pH 7 all hexavalent chromium could be dialyzed away from the protein by



changing the dialyzate once. Grogan and Oppenheimer suggest that the binding of anionic chromium at higher pH's is electrostatic in nature.

Nechels et al. (1953) were interested in using radioactive sodium chromate for the study of the survival of red blood cells in vivo and so investigated the stability of the chromium tag. They found that erythrocytes tagged with radioactive chromate lost approximately 1 per cent of their remaining activity each day. In addition, they reported that hexavalent chromium even in large quantities had little detrimental effect on erythrocytes. They found that whole blood underwent 0.4 per cent hemolysis in four days while the addition of 30 μg. chromium per milliliter of blood increased this hemolysis to only 0.6 per cent. Sixty μg. chromium per ml. blood caused only a slight additional increase in hemolysis.

The ease with which hexavalent chromium undergoes reduction to the trivalent state is indeed surprising. MacKenzie (1957) introduced radioactive hexavalent chromium by stomach tube into two groups of rats, one starved and the other fed. After four hours blood was collected, centrifuged, and the plasma and erythrocytes counted separately. In the starved animals each ml. of plasma contained 4.8 times as much activity as an equal quantity of packed red cells. In the nonstarved animals the plasma contained 8.8 times as

much activity as an equal volume of erythrocytes. The experiment was repeated using radioactive trivalent chromic chloride; here essentially all the activity was in the plasma. In order to interpret these results it is necessary to assume that ingested hexavalent chromium will tag erythrocytes while trivalent chromium will bind to plasma proteins. If this is true then the greater part of the hexavalent chromium taken orally is reduced in the alimentary canal or during the absorptive process. This experiment also shows that food in the digestive tract will cause reduction of hexavalent chromium. In another experiment the chromium was injected directly into the duodenum. Much less reduction of hexavalent chromium was found as shown by the increased quantity of chromium tagged to the erythrocytes. This would indicate that considerable reduction of hexavalent chromium occurs in the stomach. Since hexavalent chromium is least stable in an acid medium considerable reduction would perhaps be expected here.

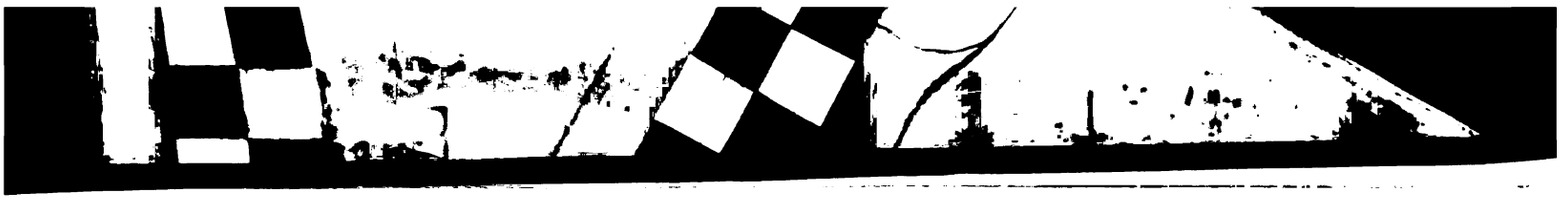
Cunningham et al. (1957) noted that the addition of sodium chromate to an acid citrate dextrose solution before the addition of red cells resulted in comparatively poor erythrocyte labeling. Further investigation revealed that labeling was adversely affected by citrate, dextrose, decreased pH, and by exposing the acid citrate dextrose and sodium chromate mixture to light. Inquiry into the

nature of this change by spectrographic analysis for trivalent chromium established that chromate was undergoing reduction to the chromic state.

Grogan and Oppenheimer (1955), by adding large quantities of chromium to egg albumin, were able to observe by color changes the reduction of hexavalent chromium. Reduction readily occurred below pH 6.4 but not above in these solutions.

Absorption, Distribution, and Elimination of Chromium

Absorption of chromium compounds from the digestive tract depends greatly on the chromium compound present. Brard (1935) found considerable absorption of chromates that were introduced by stomach tube. Conn et al. (1932) found little absorption of chromic phosphate in milk fed to rats as indicated by the trace quantities present in tissues and excreted in the urine. MacKenzie (1957) fed rats small quantities of radiochromate by stomach tube and found a maximum of 5.5 per cent chromium present in the tissues or excreted in the urine in seven days in starved animals. In nonstarved animals less than half this quantity was recovered in the urine and tissues. By comparing the tissue levels of two groups of rats fed 25 p.p.m. sodium chromate or chromic chloride for one year he concluded that five times more hexavalent than trivalent chromium



was absorbed. In another experiment in which rats were fed various quantities of chromate in their drinking water for periods of up to one year he noted that levels of chromate below 5 p.p.m. resulted in little accumulation, whereas between 5 and 10 p.p.m., an appreciable increase in the rate of accumulation occurred.

The absorption of chromium compounds through the respiratory tract has not been studied, but that some absorption may occur was indicated by the tissue and urinary levels of chromium found in chromate workers (USPHS, 1952). Some chromium compounds are probably absorbed through the skin. White (1934) reports of twelve deaths that followed the use of an antiscabetic ointment in which a chrome preparation was substituted in place of sulfur.

The fate of chromium compounds when intravenously injected has been studied by Kraintz and Talmage (1952). They injected rabbits and rats with Cr^{51} labeled chromic chloride in acetate buffer, sacrificed the animals at intervals of up to twenty-four hours, and counted various tissues. At one hour after injection the kidney showed the highest activity per gram of wet tissue followed by the blood, bone, and liver, each with the same activity. The spleen and muscle contained the least chromium. After twenty-four hours the activity in the liver and blood decreased, while that of the spleen

and bone increased. Forty per cent of the injected dose was excreted in the urine but none in the feces in twenty-four hours.

Treatment of the plasma of one animal with trichloro-acetic acid indicated that 80 to 90 per cent of the chromium was associated with the precipitated plasma proteins. Saline extracts of liver and spleen homogenates contained 40 per cent of the radioactivity, 80 to 90 per cent of which was precipitated with trichloro-acetic acid.

Visek et al. (1953) used a method similar to that of Kraintz and Talmage to study the metabolism of chromium compounds. They employed two hundred rats sacrificed at intervals of up to forty-three days after injection. Further, they correlated the electrophoretic behavior of each chromium compound present in serum with its tissue distribution. Of the total sodium chromite ($Na_3Cr^{51}O_2$) injected, which was colloidal in serum, 90 per cent appeared in the liver and tissues of the reticulo-endothelial system. Chromic chloride also behaved as a colloid in serum and its distribution in liver, spleen, and bone marrow reflected reticulo-endothelial accumulation. The reduced uptake of chromic chloride compared to sodium chromate that they observed was probably due to its binding to plasma proteins. Chromic chloride buffered by either acetate or citrate existed in the serum as a complex and was not found in high

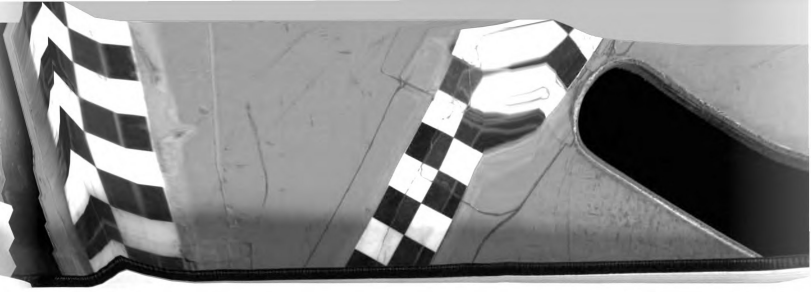
concentration in any organ. Sodium chromate was ionic in serum and its distribution pattern resembled the buffered chromic compounds. The excretion values for each compound in urine and feces respectively were as follows: colloidal sodium chromate 0.6 and 1.6, colloidal chromic chloride 15 and 20, chromic chloride complexed with citrate 75 and 17, and ionic sodium chromate 35 and 17 per cent of the injected dose excreted in four days. The low excretion values for chromate, which was ionic in serum, and therefore should be excreted rapidly, was attributed to its binding by erythrocytes.

These results on the metabolism of chromium compounds are in general agreement with the concept that the physical and ionic states of chromium in plasma largely govern its tissue distribution and rate of excretion. Chromium present in the serum in particulate form is rapidly taken up by reticulo-endothelial tissues and so is available for excretion. Additional support is lent to this concept by the work of Brauer, Holloway, and Long (1957). They reported that 75 per cent of a colloidal chromic phosphate preparation was removed from the blood in a single circulation through the liver. Presumably the colloidal particles of the chromic phosphate were larger than those encountered by Visek et al. and so were removed more rapidly.

Little additional information exists on the excretion of chromium compounds other than that already discussed. Edmunds and Gunn (1928) state that chromic acid and its salts seem to be excreted through the kidneys and probably to a less extent through the intestinal epithelium. These authors also report that the metal occurs in urine in part in organic combination. However, no data or references were presented to substantiate this statement. Mancuso and Hueper (1951) are of the opinion that chromium may be excreted through the skin and intestinal mucosa since chromium was found in the hair and feces of a chromate workman whose last exposure to chromium was three years preceding his death. Elimination of chromium through the urine, bile, stomach, and intestinal mucosa of dogs poisoned with sodium chromate or chromic chloride has been reported by Brard (1935).

Renal Clearances

Ludwig in 1844 suggested, purely on anatomical considerations, that urine formation begins as a passive process of filtration of a protein-free filtrate at the glomerulus. However, eighty years elapsed before a quantitative measure of filtration rate was made. Rehberg (1926) measured urine volume and the concentration of creatinine in plasma and urine after orally ingesting creatinine.



By appropriate calculation, not unlike the renal clearance formula used today, he derived the volume of plasma in milliliters per minute necessary to provide the quantity of creatinine excreted in the urine each minute. Moller, McIntosh, and VanSlyke (1929) justly deserve credit for using the term "clearance" and for presenting the clearance formula and concept clearly. They defined urea clearance as the volume of blood cleared of urea by one minute's excretion of urine (UV/B). No attempt was made to explain urea clearance in terms of any particular process of the kidney since they were interested primarily in quantitatively comparing the capacity of normal and diseased kidneys to excrete urea. Jolliffe and Smith (1931) extended the term clearance to the excretion of creatinine. Since then it has been widely used to describe the excretion of a large variety of substances.

The next step in the quantitation of renal function was to find a substance excreted only by glomerular filtration. No one experiment has ever been devised to establish that any given substance is filtered but not reabsorbed or excreted by the kidney tubules. However, it has been concluded beyond all reasonable doubt that inulin clearances in vertebrates and creatinine clearances in the dog measure glomerular filtration (Smith, 1951).

Concurrently with the search for substances that measured glomerular filtration, compounds which would measure other aspects of renal function were sought. The work of Shannon (1935); Goldring, Clarke and Smith (1936); and Smith, Goldring, and Chasis (1938) suggested that phenol red might be a measure of renal plasma flow and tubular function. Subsequently, diodrast and then p-aminohippuric acid (PAH) as the sodium salt have come into use for the measurement of renal plasma flow and tubular excretion.

Data for glomerular filtration rate and renal plasma flow in an animal or between animals of the same species show considerable variation. Houck's (1948) statistical analysis of seventy-five normal unanesthetized female dogs gives a mean and standard deviation of 84 ± 19.1 ml. per min. per sq. meter surface area for creatinine and 266 ± 66 ml. per min. per sq. meter surface area for PAH clearances. Russo et al. (1952) also using female dogs gives slightly different figures. For creatinine 95 per cent of all observations in their study could be expected to fall within the limits of 94 ± 36 ml. per min. per sq. meter surface area and for PAH, 238 ± 133 ml. per min. per sq. meter surface area. The standard deviation of a single determination on any given dog on any one occasion was 9 ml. per min. for creatinine and 32 ml. per min. for PAH expressed per sq. meter surface area.

Anesthesia alone or used with surgical procedures is frequently employed to facilitate the study of renal function. Corcoran and Page (1943) found that sodium pentobarbital anesthesia (30 mg./kg.) produced no consistent change in inulin and diodrast clearances or tubular excretion of diodrast in dogs. Glauser and Selkurt (1952) reported that pentobarbital or barbital anesthesia suitable for surgery of five to six hours' duration has no effect on glomerular filtration but did reduce renal plasma flow and tubular excretion of PAH. Habif et al. (1951) compared the renal clearances of creatinine, PAH, sodium, potassium, and chloride before and during anesthesia with ether, cyclopropane, or thiopental; and during major surgery. Anesthesia depressed all clearances but the surgical procedures had no additional effect.

METHODS

I. Routes of Chromium Excretion

The objectives of experiments discussed in this section were twofold: to investigate the quantity of chromium excreted in urine, bile, and feces, and to compare the excretion of trivalent and hexavalent chromium by each of these routes. Acute measurements on anesthetized dogs gave information on the relative rates of chromium excretion in urine and bile, while prolonged experiments on dogs with bile fistulas were used in order to evaluate chromium excretion in feces as well as urine and bile.

General procedure

Seven healthy male and female mongrel dogs weighing between 7.5 and 15 kg. were used in these experiments. Each animal was housed in an individual cage with a constant supply of food (Borden's Chunx) and water.

Trivalent chromium solutions for administration to these dogs were prepared from stock $Cr^{51}Cl_3$ that ranged in specific activity from 38 to 47 curies per gram. For dogs receiving small quantities of chromium the stock solution was diluted with five parts

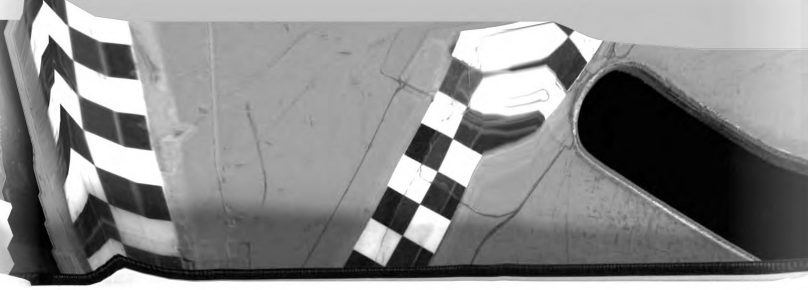
physiological saline and the acidity adjusted to pH 3 to 4 at least one day before use. If a large dose of chromium was to be given $Cr^{51}Cl_3$ was diluted with stock $Cr^{52}Cl_3$ solution and made isotonic by adding the proper quantity of sodium chloride. These solutions were also prepared a day in advance of use and always had a pH of 3 to 4. The reason for aging trivalent dosing solutions before use was to allow time for the $Cr^{51}Cl_3$ to reach ionic stability. Concentration, pH, and other ions present all affect complex formation of trivalent chromium so that $Cr^{51}Cl_3$ added to $Cr^{52}Cl_3$ does not immediately have the same ionic composition as the latter. Isotopic $Na_2Cr^{51}O_4$ was prepared by oxidizing $Cr^{51}Cl_3$ with hydrogen peroxide in alkaline solution (see Appendix B). The method of preparation of hexavalent chromium solutions for injection was the same as for trivalent solutions except that $Na_2Cr^{52}O_4$ was used in place of $Cr^{52}Cl_3$ for large doses and the pH was adjusted to 7. The activity of Cr^{51} in the dosing solutions described above was between 1000 and 1500 μc. per dose.

The isotope-counting apparatus consisted of Nuclear Corporation's scintillation crystal detector (model D-55), scintillation counter (model 183), and radiation analyzer (model 1810). The minimum detectable activity of Cr^{51} with this apparatus in terms of microcuries required to give a count equivalent to background (approximately 35

c.p.m.) was 4.4×10^{-3} μc. for a five-volt window width setting counted on the middle shelf. Urine and bile samples for counting were pipetted in 4 or 5 ml. quantities into disposable aluminum culture dishes and counted without additional preparation. Fecal samples were well mixed with water to a thin paste consistency and 4 or 5 gram quantities placed in the culture dishes for counting. No correction for self absorption was used or necessary, since all samples and standards from any given dog were counted at the same volume and geometry. The counting of a weight rather than a volume of feces would result in a small counting error but this was ignored. Standards were prepared in duplicate by making a dilution of the injection solution whose concentration was known and counting an aliquot. From the activity and the chromium concentration of the standards the chromium content of urine, bile, and feces could be calculated.

Acute experiments on anesthetized dogs

Five dogs were used in this section of the experiment. Food was withdrawn from each dog the day prior to use. Each animal was anesthetized with 30 mg. sodium pentobarbital per kg. body weight and the ureters cannulated with the proper size polyethylene tubing through a midline abdominal incision. The common bile duct



was similarly cannulated and a serrefine clamp placed on the cystic bile duct.

Radioactive chromium was injected into one of the brachial veins, the time recorded, and urine and bile quantitatively collected in 10 ml. graduates at one-half-hour intervals for four hours. Small urine and bile samples were diluted to 6 ml. to provide an adequate volume for counting.

Prolonged experiments on bile fistula dogs

During the course of a year eleven dogs were operated on to establish functional bile fistulas. Each animal was anesthetized with 30 mg. per kg. sodium pentobarbital and prepared for aseptic surgery. The duodenum was located and the rostral end packed off through a ventral midline incision. A small cut was made in the duodenum about one inch posterior to the pylorus, the ampula of Vater located, and either polyethylene or rubber tubing inserted approximately two inches into the common bile duct. The duodenal incision was closed with interrupted Lambert stitches and the cannula imbedded in the outside surface of the duodenum caudal to the wound with three of four additional stitches. Where the cannula left the gut a purse-string suture was placed for additional insurance against slipping. The free end of the tubing was brought to the

outside through a stab wound and sutured to the skin. The operation was completed by closing the midline incision and taping the cannula to the abdomen. When not on experiment, the bile fistula was closed so that bile could flow around the tubing and into the duodenum.

Two of the eleven dogs died within twelve hours after the operation but the remainder recovered rapidly. Polyethylene tubing was used as cannula material in six of the surviving dogs with poor results. These cannulae invariably pulled away several days to two weeks after surgery. Number 8 Fr. nasal catheters were used in the three remaining animals with excellent results. Two of these dogs were placed on experiment about two weeks after surgery but the third one had a severe biliary infection when operated on (due to a previous unsuccessful cannulation) and was not used.

Just before intravenous chromium injection the bladder of each dog was catheterized, the catheter taped securely to the animal, and a balloon of 200 ml. capacity tied in place. The bile fistula was opened and a similar balloon tied to it. Bile and urine were collected at two, four, eight, sixteen, and twenty-four hours and for the next three days after injection. Because of its size the balloon on the urine catheter had to be changed several times each day for the daily collection periods. Feces were collected after volitional

evacuation by the dog and each day's excrement added to separate bags containing a solution of phenol in water.

II. Mechanisms Involved in the Renal Excretion of Chromium

The purpose of experiments discussed in this section were to (1) evaluate the effects of chromium on kidney function, (2) investigate which aspect of nephron function was most prominent in excreting chromium, and (3) establish a renal clearance for chromium.

Renal clearance procedure

Eleven mongrel dogs weighing 14.5 to 25 kg. were used in the renal clearance measurements. Food was withdrawn from each dog at least twelve hours prior to the experiment. Water was administered to some of the animals by stomach tube to insure adequate urine flow. Sufficient sodium pentobarbital was given intravenously to produce light anesthesia. At no time was more than 25 mg. per kg. of anesthetic necessary to produce the desired effect. In experiments of long duration additional doses of 3 to 5 mg. per kg. pentobarbital were given as necessary when the dog began to show excessive movement. The corneal, palpebral, and paw-pinch reflexes were present at all times in all dogs under this

anesthesia. Sodium pentobarbital anesthesia is known to cause a lowering of body temperature. Furthermore, a change in body temperature affects renal function (Grant and Medes, 1935; Smith, 1939-40); for this reason two heating pads were placed under each dog on experiment and the body temperature, determined rectally, was maintained at 101° to 102.5° F.

Creatinine (0.5 per cent) and 0.25 per cent PAH (para-aminohippuric acid) were infused through a brachial vein with a Sigmamotor infusion pump. The rate of infusion (1.5 to 3.0 ml./min.) varied with the size of the dog but was maintained constant in any one animal. After the infusion was started a priming dose of creatinine (5.0 per cent) and PAH (1.2 per cent), calculated to establish the appropriate plasma level of each was administered intravenously.

Preparation of the chromium solutions for dogs receiving a single injection was the same as in the previous section. When chromium was administered by infusion it was simply added to the creatinine-PAH infusion solution.

Urine samples were collected by an indwelling catheter. The bladder was rinsed by instilling and then withdrawing and discarding 20 cc. water with the aid of a 50 cc. syringe attached to the catheter. The rinsing process was repeated a total of three times. After the last rinse was withdrawn, 30 cc. of air was introduced

into the bladder and aspirated in order to remove the last wash-out fluid. Urine collection periods usually lasted about thirty minutes but this was variable. Two minutes before the end of a collection period the bladder was emptied and the process of rinsing and flushing the bladder repeated. These rinse fluids were collected and added to the urine sample. Urine from dogs Nos. 8 to 13 was collected in 200 ml. volumetric flasks. The volume of urine plus wash was then determined by measuring the amount of water necessary to fill the flask to the mark. For the last five dogs urine was collected and measured in a graduated cylinder.

Blood samples were drawn from a jugular vein into a heparinized syringe. The total number of blood samples drawn from any one dog was usually equal to one more than the number of urine collection periods. These were drawn at approximately equal time intervals throughout the experimental period. Blood samples were centrifuged and the concentration of total chromium, dialyzable chromium, creatinine, and PAH measured on the plasma.

Dialysis procedure

An equilibrium dialysis procedure was used to determine the plasma level of dialyzable chromium. This method was applicable because Gray and Sterling (1950) had shown that binding of

chromium to plasma proteins was essentially irreversible; that is, once chromium became bound it could not be dialyzed off the plasma proteins. The dialysis procedure is described below. Visking dialysis tubing (20/32 in. inflated diameter) was wetted and two knots tied in one end. Five ml. of plasma whose radioactive count was known were placed in the sack and the other end tied with two knots. The sack was placed in a test tube and covered with 7 ml. physiological saline. Sacks were prepared for each blood sample withdrawn from the dog. Diffusion equilibrium was allowed for two days at 4° C.; then 5 ml. of the dialyzate was counted. This count was corrected for background, decay, and to 1 ml. volume, then multiplied by 12/5 to give the count equivalent to 1 ml. dialyzable chromium in the original sample. The factor 12/5 represents the correction necessary because 5 ml. of dialyzable chromium in the plasma was diluted to a total volume of 12 ml.

Creatinine and PAH procedures

Creatinine and PAH clearances measured glomerular filtration rates (GFR) and effective renal plasma flows (ERPF) respectively. Plasma creatinine and PAH levels were determined on a trichloroacetic acid filtrate (Greenwalt, as modified by Kennedy et al., 1952). Creatinine determinations on diluted urine and plasma filtrates were

done by the alkaline picrate method (as modified by Brod and Sirota, 1948). PAH was determined by the method of Smith et al. (1945). Creatinine and PAH analysis were done in duplicate but only one protein-free filtrate was prepared for each plasma sample. Methods for these chemical analyses are given in detail in Appendix B.

The plasma concentration in the clearance formula (UV/P) was determined by plotting the plasma level of total chromium, dialyzable chromium, creatinine, or PAH versus time on coordinate graph paper and selecting the plasma value interpolated to coincide with a point three minutes before the midpoint of the urine collection period (Figure 1). The choice of a plasma value three minutes before the midpoint of the urine collection period is arbitrary and compensates for the time required for urine to flow from the nephron to the bladder.

Renal clearances in dogs are almost universally expressed per one square meter surface area. The nomogram found in Smith (1956) was used to estimate surface area of dogs in this experiment. This nomogram is based upon body weight in kg. and length from nose to anus measured over the belly in centimeters.

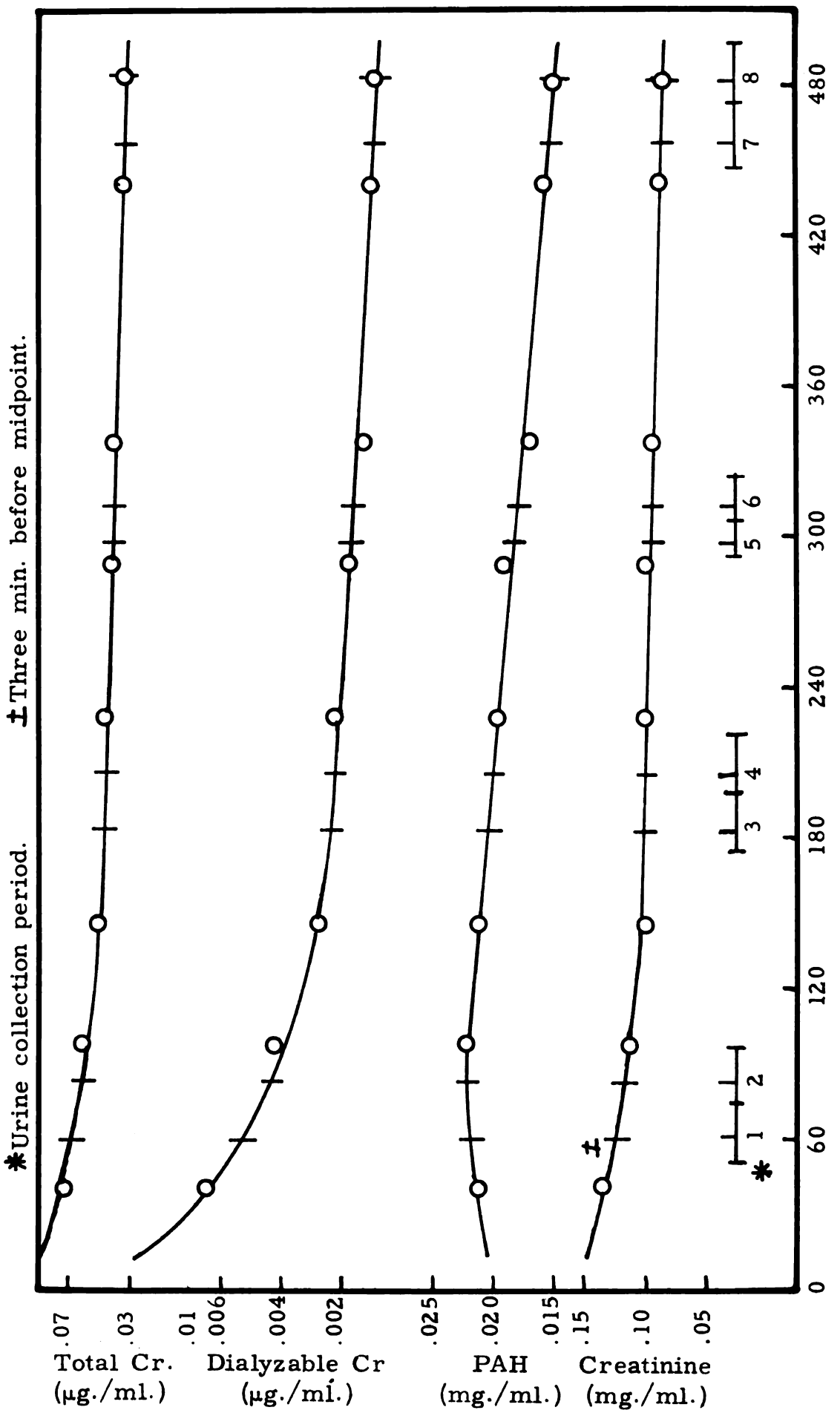


Figure 1. Method used to determine plasma concentration at three minutes before midpoint of urine collection period.

III. Some Physical and Chemical Properties of Chromium in Biological Fluids

Experimental procedures presented here were intended to compare some of the physical and chemical properties of chromium in biological fluids to its characteristics before injection. The possibility that chromium could be excreted in organic combination as Edmunds and Gunn (1928) report was a directing influence in the choice of the following experimental methods.

Dialysis procedures

The equilibrium dialysis procedure, already described for use on plasma, was used without change on urine samples from dogs 14 and 15 to measure percentage of dialyzable chromium.

A rate dialysis procedure was used to compare the diffusion properties of Cr^{51} excreted in urine to that of $Cr^{51}(+6)$ added to normal urine. Twenty ml. of a sample whose rate of diffusion was to be measured was placed in a sack made from dialysis tubing (Cenco, one inch diameter) and dialyzed against 2000 ml. sodium chloride solution made isotonic with the sample as determined by freezing point depression measurements. Two stirrers, one in the sample and one in the dialyzate, assured constant agitation of both liquids. At frequent intervals 2 ml. samples were removed, counted,

and returned to the dialysis chamber. Counts per minute per 2 ml. corrected for background were plotted on the ordinate of semilogarithmic graph paper versus time on the abscissa and a line drawn through the points. The half-time ($t_{1/2}$) was measured from the line and the diffusion rate expressed as the constant k ($k = .693/t_{1/2}$).

Diffusion rates as defined above were made on the following samples: (1) urine from a dog injected intravenously with 8.70 μg. $Cr^{51}(+6)$; (2) Cr^{51} (+6) added to salt solution; (3) urine from a dog injected with 9.81 μg. Cr^{51} (+6); (4) $Cr^{51}(+6)$ added to normal dog urine and dialyzed immediately; and (5) $Cr^{51}(+6)$ added to normal dog urine and dialyzed after five hours. Measurements were made in the order listed and the same dialyzing bag employed so that results could be compared.

Reduction of hexavalent chromium

The ease with which chromates are reduced in vitro suggests that reduction also may occur in vivo. In order to determine if reduction took place a 10 kg. male dog was injected intravenously with 40 mg. $Na_2Cr^{51}O_4$ and urine samples immediately analyzed for hexavalent and trivalent chromium. The Saltzman (1952) reaction (see Appendix B), but without previous ashing of samples, was used for

qualitative analysis for hexavalent chromium while total chromium was determined by radioassay for Cr^{51}. Schiffman (1957) showed that ashed and nonashed samples of chromate in water gave essentially the same results in this reaction. To test the reducing action of urine in vitro, 100 or 200 μg. quantities of sodium chromate were added to 100 ml. of stored or fresh urine and 5 ml. samples analyzed at intervals for the presence of hexavalent chromium.

Ion exchange procedure

Ion exchange columns were prepared by drawing out one end of 8 mm. I.D. glass tubing, placing a disk of glass wool in the bottom, and washing in sufficient resin to form a cylinder 8 cm. high. The resins used were Amberlite IR-120 (cation exchange) and Dowex 1-X100 (anion exchange). Urine, a plasma dialyzate, and Cr^{51} infusion solution from dog 18 were counted, run through the resin at a rate less than 0.5 ml. per minute and recounted. The fluids mentioned above plus bile from dog 4 were also subject to ion exchange separation.

Chromatographic procedure

Urine, $Cr^{51}(+3)$ infusion solution, and a plasma dialyzate from dog 12 were used in the following procedure. Capillary tubing drawn to a fine point was used to apply each of the chromium

solutions in a fine line to strips of Whatman No. 3 mm. filter paper (3.8 by 46 cm.) then the strips were dried under a heat lamp. It was necessary to repeat this process several times in order to have sufficient Cr^{51} present to count. The solvent used to obtain separation consisted of n-butanol: water: glacial acetic acid: acetoacetic acid ester in the proportions 50:35:10:5 respectively. The ends of the paper strips were dipped in the butanol phase of the solvent, while the aqueous phase was used to saturate the chromatographic chamber. Descending separation was allowed for twenty-four hours, and the strips quickly dried in an air oven at 70° C. The chromatograms were then analyzed to locate the position of Cr^{51}, ninhydrin-positive substances, creatinine, and PAH.

The migration of Cr^{51} on the strips was determined by means of the scintillation detector and radiation analyzer previously used connected to a count rate meter (model 1620) and Esterline Angus recorder (model AW). The recorder was used to drive an actograph (model C-100) which moved the strips under the scintillation detector. To restrict scanning to a narrow band of the paper a lead shield 13 mm. thick with a slot in the middle (3.5 by 3.8 mm.) was placed between the paper and detector. The filter paper strips were moved past the detector at a rate of 0.75 inch per minute, and the peak activity used in calculating R_f values.

Ninhydrin color was developed by spraying the strips with 0.25 per cent ninhydrin in butanol and heating for 15 minutes in an oven at 70° C. This reagent also produced a pink color with PAH. An orange color indicative of creatinine was developed by spraying the filter paper first with 0.75N NaOH, then with 0.4M picric acid.

Chromium precipitation procedure

Normal urine and heparinized blood were collected from a 19 kg. dog; then 13.4 mg. $Cr^{51}Cl_3$ was injected intravenously and urine and blood again collected. The blood samples were centrifuged and the plasma dialyzed as previously described. To the normal urine and dialyzate sufficient $Cr^{51}Cl_3$ injection solution in anionic form was added so that their activity was approximately equal to the samples collected after the dog received the radioisotope. To 7 ml. portions of the two urine and two dialyzate samples an equal quantity of $Cr^{52}Cl_3$ in anionic form was added.

Chromium in $CrCl_3$ solutions is present almost entirely in cationic form. Conversion to the anionic form was done by adjusting the acidity of such solutions to pH 5 or above which favors hydrolysis, allowing them to stand overnight and absorbing any cationic chromium remaining with amberlite IR-120. The reason for adding $Cr^{52}Cl_3$ to the urine and dialyzate samples was to provide sufficient

chromium so that a visible precipitate would form upon the addition of ammonium hydroxide. A brief discussion of precipitation of metals with ammonium hydroxide is given in Willard and Diehl (1950). Two ml. 1 N ammonium hydroxide were added to each preparation, samples were removed for counting, and the remainder centrifuged at 3000 r.p.m. for one-half hour in an International centrifuge (model SBV). The supernatant was then counted and the quantity of precipitated $Cr^{51}Cl_3$ in each sample calculated. The organically bound chromium, if present, would not precipitate with ammonium hydroxide, while free chromium would. Chromium added to urine and plasma dialyzate served as controls to assure that non-bound chromium would precipitate under these conditions.

RESULTS

I. Routes of Chromium Excretion

The data used to compile Figures 2 through 8, expressed as total micrograms chromium excreted for each collection period, are presented in Appendix A. Figures 2 through 6 show the accumulated excretion of Cr^{51} in urine and bile for a period up to four hours after intravenous injection of $Na_2Cr^{51}O_4$ or $Cr^{51}Cl_3$. These dogs were anesthetized and the common bile duct and ureters cannulated to obtain urine and bile samples. Additional experiments (summarized in Figures 7 and 8) extending over a four-day period were essential to evaluate the amount of chromium excreted in feces as well as bile and urine after intravenous injection. Feces were collected after volitional defecation by the animal, while a biliary fistula and indwelling catheter provided quantitative collection of urine and bile. The data for excretion of Cr^{51} after $Cr^{51}Cl_3$ administration were obtained from one dog while two dogs were used with $Na_2Cr^{51}O_4$. Since the bile fistula operations resulted in only two dogs suitable for experiment, one of these (dog 6) was used first with $Cr^{51}Cl_3$, then three weeks later with $Na_2Cr^{51}O_4$. At this time an insignificant

Figure 2. Accumulated excretion of hexavalent chromium in urine.

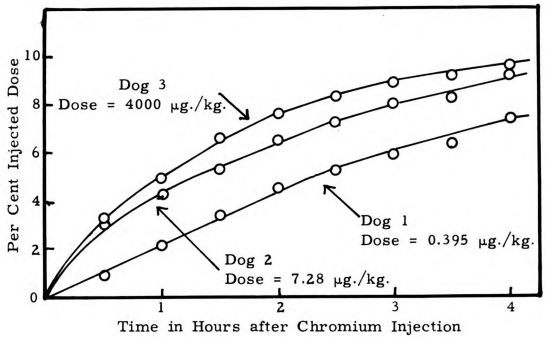


Figure 3. Accumulated excretion of hexavalent chromium in bile.

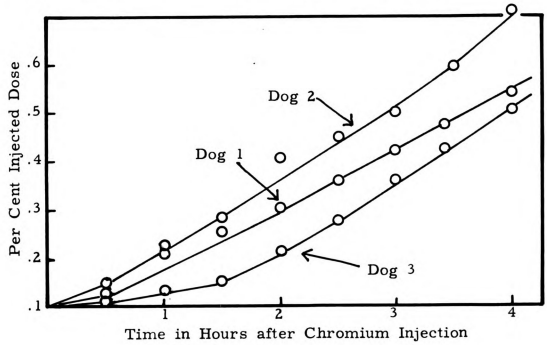


Figure 4. Accumulated excretion of trivalent chromium in urine.

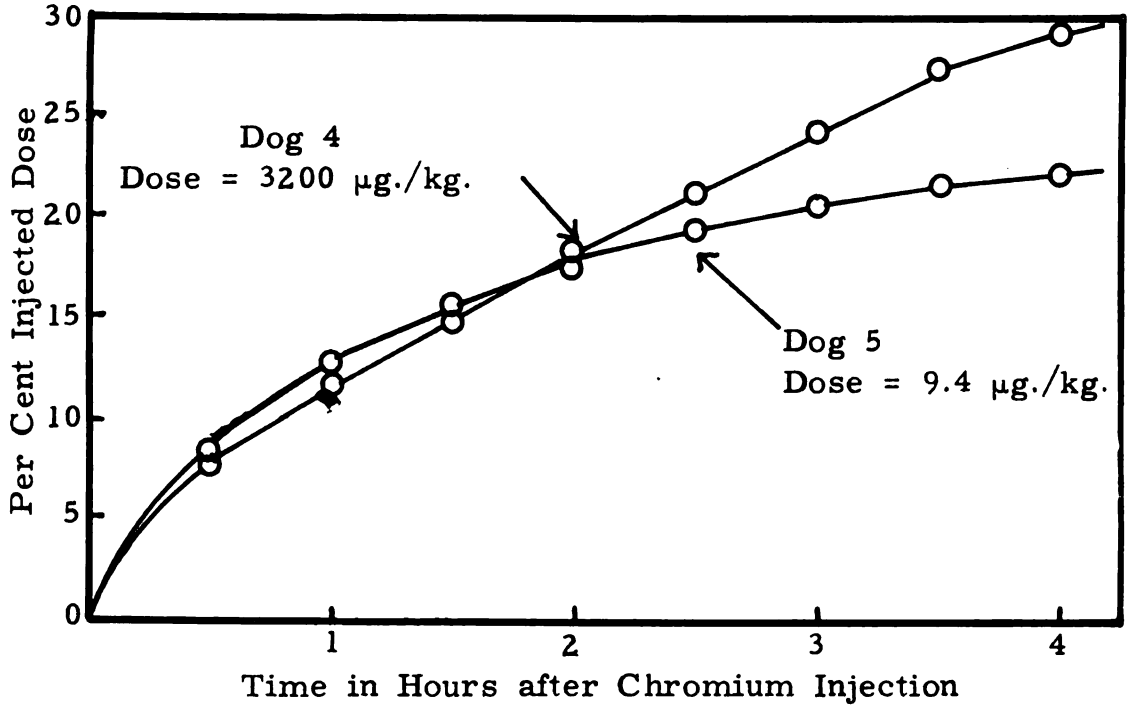


Figure 5. Accumulated excretion of trivalent chromium in bile.

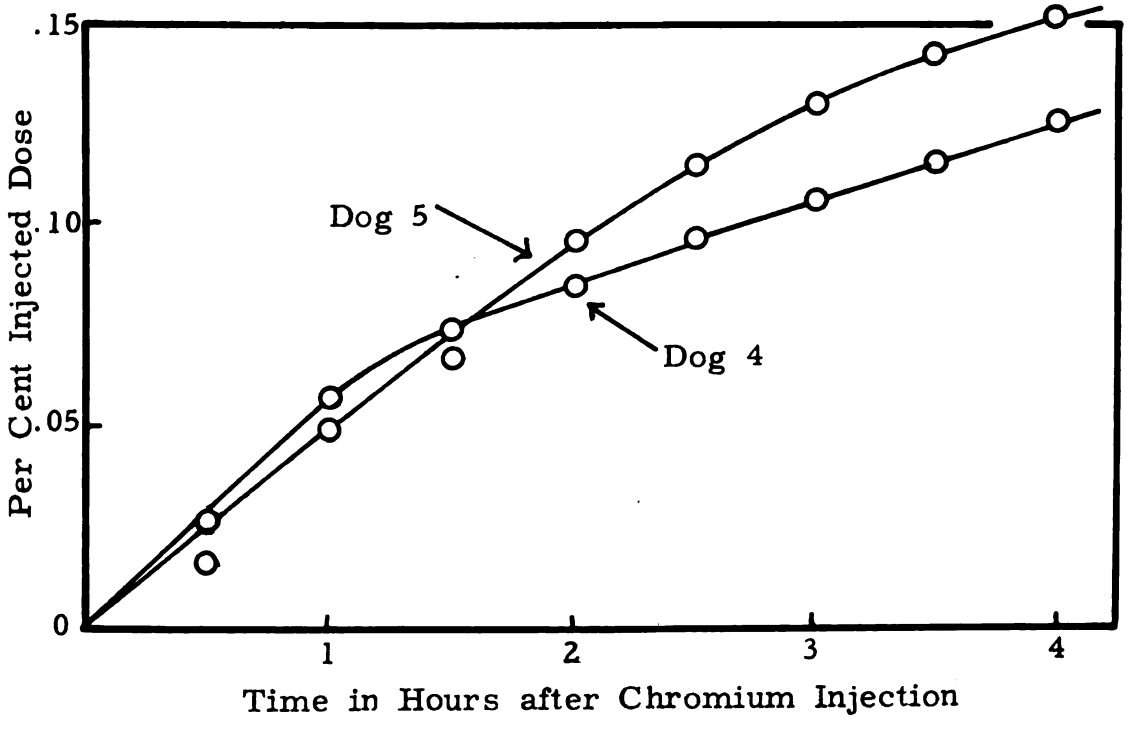
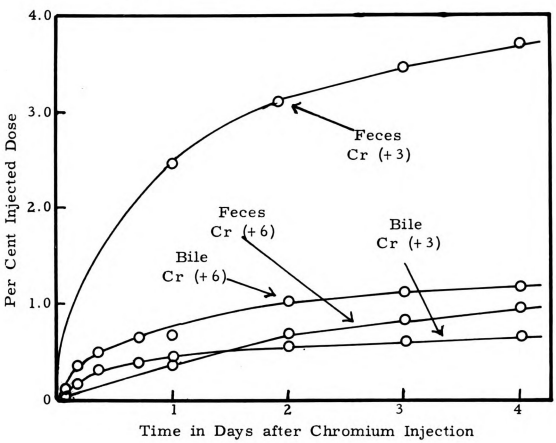
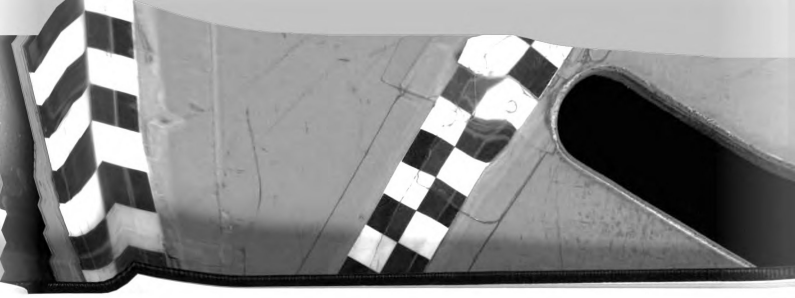


Figure 8. Comparison of four day excretion values of hexavalent and trivalent chromium in bile and feces.





quantity of chromium from the previous injection was found in urine and bile samples.

II. Mechanisms of Renal Excretion of Chromium

Table 1 shows the renal clearance determinations on eleven dogs used to evaluate renal mechanisms involved in chromium excretion. Creatinine and PAH clearances were measured to establish the normal range of renal function in each dog as well as to detect any depression in this function induced by intravenous injection of chromium solutions. Data used for calculating chromium clearances ($C_{chr.}$) and dialyzable chromium clearances ($C_{d\text{-}chr.}$) by formula 2 of Appendix C are given in Appendix A. In addition the ratios of clearances of creatinine to PAH (also called filtration fraction or F.F.), dialyzable chromium to creatinine ($C_{d\text{-}chr.}/C_{creat.}$), and dialyzable chromium to PAH ($C_{d\text{-}chr.}/C_{PAH}$) are presented. Chromium clearances in the first nine dogs were measured after a single intravenous injection of a solution of $Cr^{51}Cl_3$. In the last six of these, dialyzable chromium clearances were also measured. In dogs 17 and 18 both $C_{chr.}$ and $C_{d\text{-}chr.}$ were determined over a period of several hours during intravenous infusion of $Cr^{51}Cl_3$ in solution.

Figures 9 and 10 graphically illustrate the decrease in chromium clearances with time after injection. Figure 9 gives individual

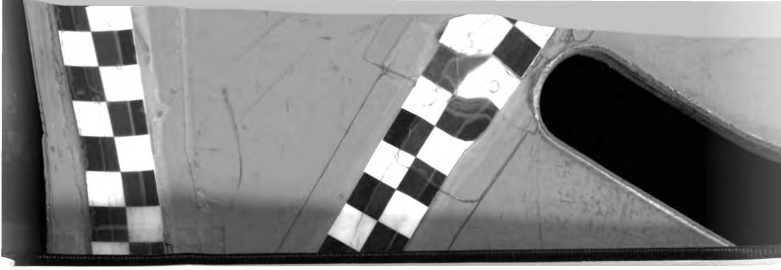


TABLE 1

RENAL CLEARANCE VALUES

Description	Clearance No.	Time after Inj. (min.)	$C_{creat.}$[a]	C_{PAH}	$C_{chr.}$	$C_{d\text{-}chr.}$
		Dog No. 8				
13.5 kg. female	1	78	109.8	233.9	2.86	-
75 ml./kg. water[b]	2	98	104.1	196.0	2.50	-
0.53 sq. meter	3	122	122.6	194.8	2.21	-
5.03 µg./kg. Cr	4	141	107.2	229.1	2.08	-
1 CPM=35.09 µµg.[c]	5	166	110.2	223.1	1.84	-
		Dog No. 9				
15 kg. female	1	66	98.9	240.6	2.91	-
75 ml./kg. water	2	82	97.5	244.2	2.37	-
0.66 sq. meter	3	106	97.2	238.0	2.36	-
294 µg./kg. Cr	4	127	119.3	273.8	2.25	-
1 CPM=551.0 µµg.	5	148	119.7	297.2	2.32	-
		Dog No. 10				
22 kg. male	1	117	72.3	275.0	2.60	-
No water	2	147	77.8	243.8	2.09	-
0.87 sq. meter	3	218	78.2	202.5	1.50	-
250 µg./kg. Cr	4	240	69.7	187.2	1.37	-
1 CPM=440.0 µµg.	5	321	77.0	205.2	0.86	-
	6	350	69.4	167.8	0.86	-
	7	430	94.4	287.3	0.70	-
	8	450	98.4	291.0	0.63	-

[a] Units are milliliters per minute per square meter.

[b] Water given orally.

[c] 1 CPM = one count per minute.

TABLE 1 (Continued)

Description	Clearance No.	Time after Inj. (min.)	$C_{creat.}$	C_{PAH}	$C_{chr.}$	$C_{d\text{-}chr.}$
		Dog No. 11				
18 kg. male	1	60	108.3	285.4	3.11	37.9
No water	2	72	110.8	336.2	2.75	38.6
0.74 sq. meter	3	183	123.9	248.3	2.10	37.1
5.22 μg./kg. Cr	4	206	134.1	270.1	2.12	47.2
1 CPM=13.2 μμg.	5	297	164.1	307.2	1.74	44.3
	6	312	161.7	330.5	1.65	43.4
	7	457	174.4	357.7	1.21	45.9
	8	482	172.8	360.7	1.18	50.0
		Dog No. 12				
14.5 kg. female	1	122	115.6	217.7	2.23	25.6
50 ml./kg. water	2	148	118.9	231.3	2.21	25.7
0.62 sq. meter	3	342	134.4	380.0	1.60	30.5
6.50 μg./kg. Cr	4	2880	141.4	-	0.28	34.7
1 CPM=15.5 μμg.	5	2940	114.2	-	0.25	31.3
		Dog No. 13				
19 kg. male	1	152	85.8	244.9	2.48	28.3
50 mg./kg. water	2	266	79.5	279.0	2.07	28.0
0.88 sq. meter	3	385	78.4	279.4	1.37	26.7
4.94 μg./kg. Cr	4	512	77.4	269.7	0.98	26.4
1 CPM=17.5 μμg.	5	633	76.2	243.0	0.98	28.7
		Dog No. 14				
22 kg. male	1	63	84.0	291.8	3.05	30.0
50 ml./kg. water	2	135	80.3	222.8	2.28	30.6
0.87 sq. meter	3	222	98.0	286.3	2.14	37.7
91.0 μg./kg. Cr	4	305	105.8	314.1	1.20	25.6
1 CPM=80.5 μμg	5	393	105.3	344.0	1.15	32.8
	6	453	102.6	337.9	0.99	40.0

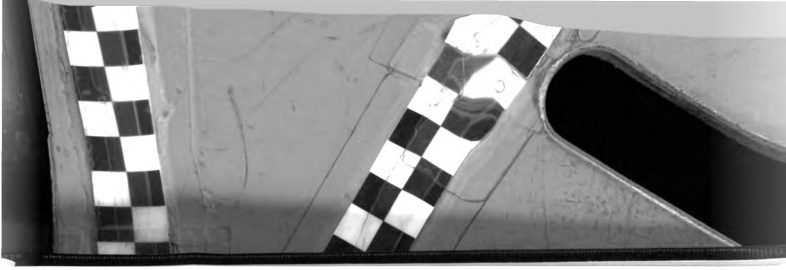
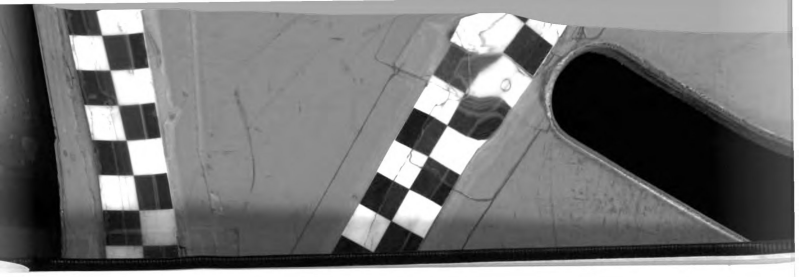


TABLE 1 (Continued)

Description	Clearance	Time after Inj. (min.)	$C_{creat.}$	C_{PAH}	$C_{chr.}$	$C_{d\text{-}chr.}$
		Dog No. 15				
23 kg. female	1	74	88.4	299.3	2.72	29.2
50 ml./kg. water	2	194	92.1	302.4	2.11	34.3
0.97 sq. meter	3	281	96.8	286.6	1.66	33.7
86.2 µg./kg. Cr	4	374	99.0	325.9	1.24	35.1
1 CPM=221 µµg.	5	496	98.3	314.6	0.86	35.5
		Dog No. 16				
19 kg. male	1	79	58.3	164.9	1.69	33.4
50 ml./kg. water	2	169	56.3	185.9	1.18	36.6
0.86 sq. meter	3	259	62.9	172.1	0.80	36.6
1900 µg./kg. Cr	4	377	69.4	191.7	0.59	31.8
1 CPM=303 µµg.	5	445	63.7	200.2	0.49	32.2
		Dog No. 17				
25 kg. male	1	103	111.3	246.0	11.24	50.8
50 ml./kg. water	2	133	118.3	265.4	11.67	54.9
1.09 sq. meter	3	258	136.3	262.0	7.65	67.1
97.0 µg./ml. at	4	350	137.2	173.9	6.65	67.5
2.43 ml./min.	5	409	136.0	290.7	3.72	67.0
1 CPM=502 µµg.	6	444	132.9	319.9	3.32	69.7
		Dog No. 18				
22 kg. male	1	71	70.4	203.6	2.98	24.3
50 ml./kg. water	2	185	77.1	188.0	1.80	25.8
.087 µg./ml. at	3	309	96.8	228.2	1.32	30.0
1.95 ml./min.	4	385	98.5	228.4	1.20	33.8
1 CPM=3.2 µµg.	5	468	116.7	233.8	0.84	34.1
Number[d]			11	11		8
Mean			101.7	259.9		36.6
Standard deviation			24.34	40.13		11.58
Coefficient of variation			0.239	0.154		0.316

[d]Each observation is average of five to eight determinations.



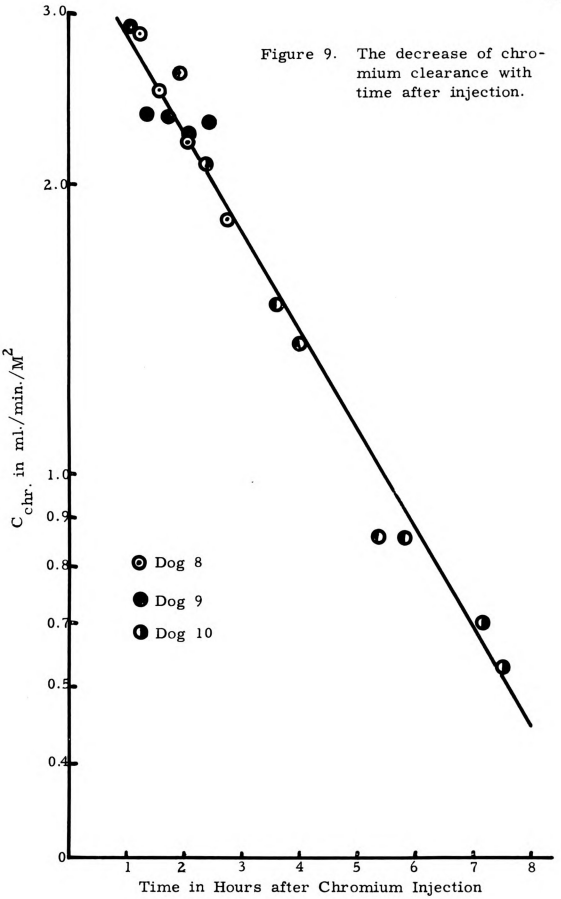
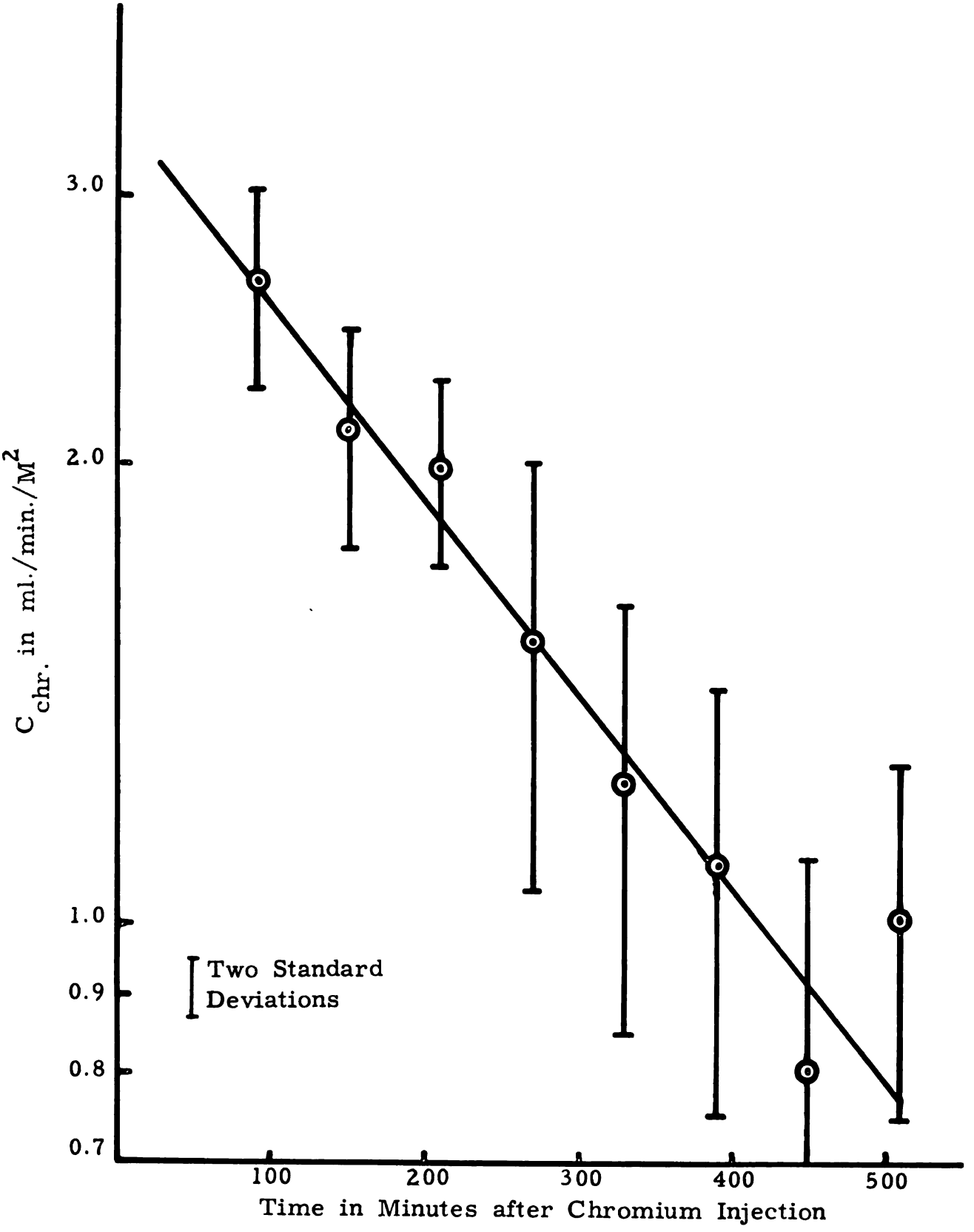
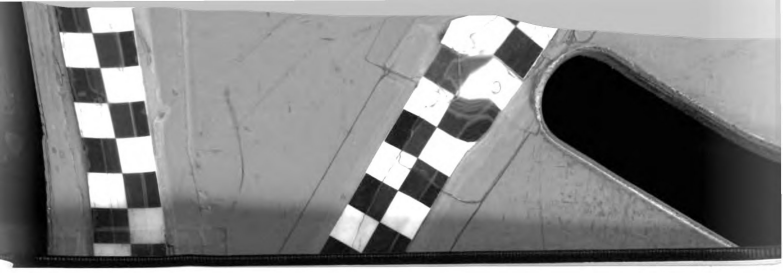


Figure 9. The decrease of chromium clearance with time after injection.

Figure 10. The decrease in chromium clearances of Dogs 8 through 17 with time after Cr^{51} injection.





clearances for the first three dogs, while in Figure 10 chromium clearances measured in a given hour after the $Cr^{51}Cl_3$ injection were averaged for all dogs receiving a single dose of chromium chloride.

Trivalent chromium in plasma is largely bound to plasma proteins but some exists in the "free" state (the term "free" in quotation marks is used only to indicate chromium that is not bound to plasma proteins). Since the "free" form should be more readily excreted by the kidney, its measurement could clarify some of the renal processes involved in chromium excretion. Figure 11 shows the per cent dialyzable ("free") chromium in the plasma of an 18.5 kg. dog after the intravenous injection of 650 μg. chromium chloride per kg. body weight. The per cent dialyzable chromium in the plasma of six dogs each given a single injection of trivalent chromium is indicated in Figure 12.

III. Some Physical and Chemical Properties of Chromium in Biological Fluids

Equilibrium dialysis of urine samples gave the results shown in Table 2. Duplicate determinations were made on five urine samples collected from each of two dogs after the intravenous injection of chromic chloride. The results are expressed as per cent of total chromium that is dialyzable.

Figure 11. The decrease in per cent dialyzable chromium in plasma with time after injection.

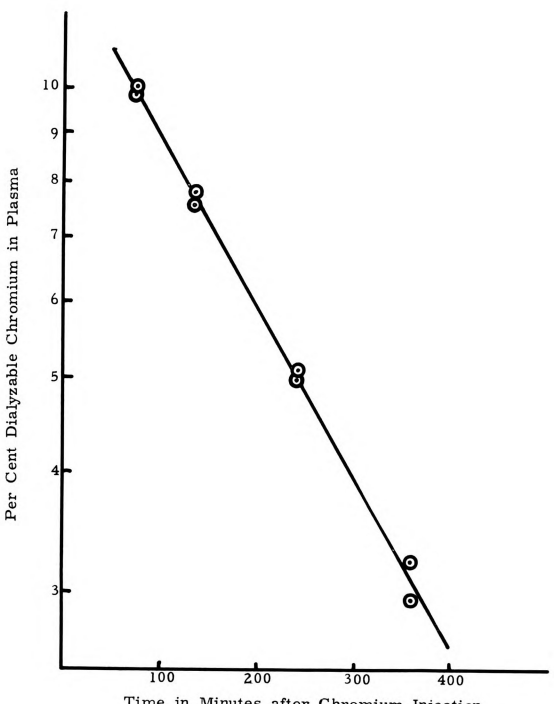


Figure 12. The decrease in per cent dialyzable chromium in plasma of six dogs with time after injection.

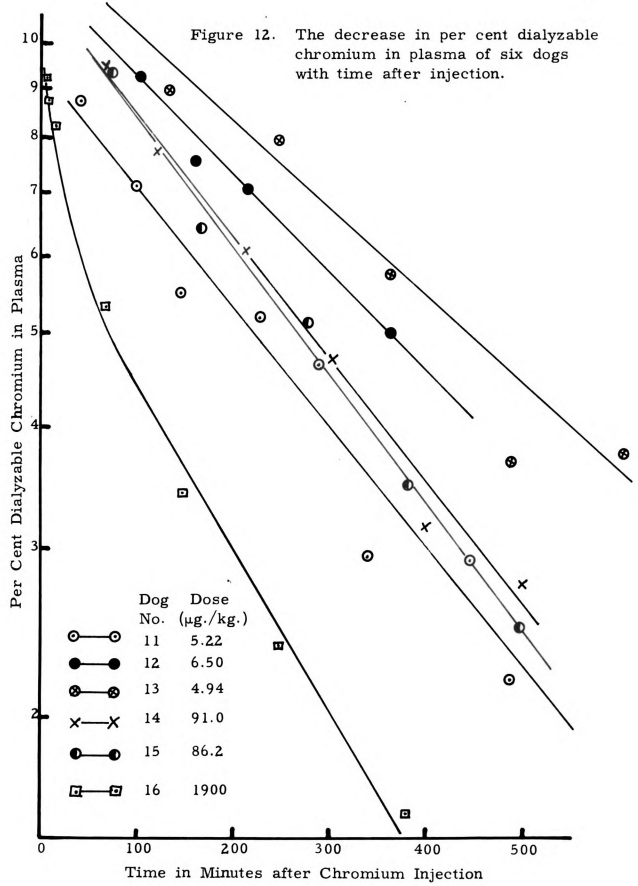


TABLE 2

PER CENT DIALYZABLE CHROMIUM IN URINE

Urine Collection Period	Dog 14	Dog 15
1	98.5	86.2
	92.5	76.6
2	105.1	69.3
	-	80.2
3	103.2	85.6
	-	86.6
4	104.3	89.9
	-	88.2
5	98.5	81.2
	99.3	83.2
Number	5	5
Mean	101.4	82.7
Standard deviation	4.1	5.4
Coefficient of variation	0.04	0.06

Figures 13 and 14 graphically present results of the rate dialysis procedure. In these figures the rate of diffusion through a dialysis bag of Cr^{51} excreted in urine after intravenous injection of $Na_2Cr^{51}O_4$ is compared to that of $Na_2Cr^{51}O_4$ added to saline or urine.

Table 3 shows the results of chemical reduction when hexavalent chromium was added to stored (previously frozen) or fresh urine. Urine of one dog injected with hexavalent chromium was also tested qualitatively for presence of chromate ion and quantitatively for total chromium content.

Ion exchange resins were used in investigating the ionic charge of chromium in various fluids from two dogs (Table 4). Samples from dog 4 were run through anion exchange resin after passage through cation exchange resin, while in dog 18 fresh samples were used for each resin. Results are expressed as per cent Cr^{51} removed by the resin.

The last two experiments in this group were designed specifically to gain information on possible binding of chromium to a dialyzable component of urine and plasma. Table 5 shows the chromatographic R_f values of chromium and ninhydrin-positive substances present in urine and a plasma dialyzate. While the presence of Cr^{51} and ninhydrin-positive substances at the same place on paper

Figure 13. Dialysis of Cr^{51} in saline and urine of a 10.5 kg. dog after intravenous injection of $Na_2Cr^{51}O_4$.

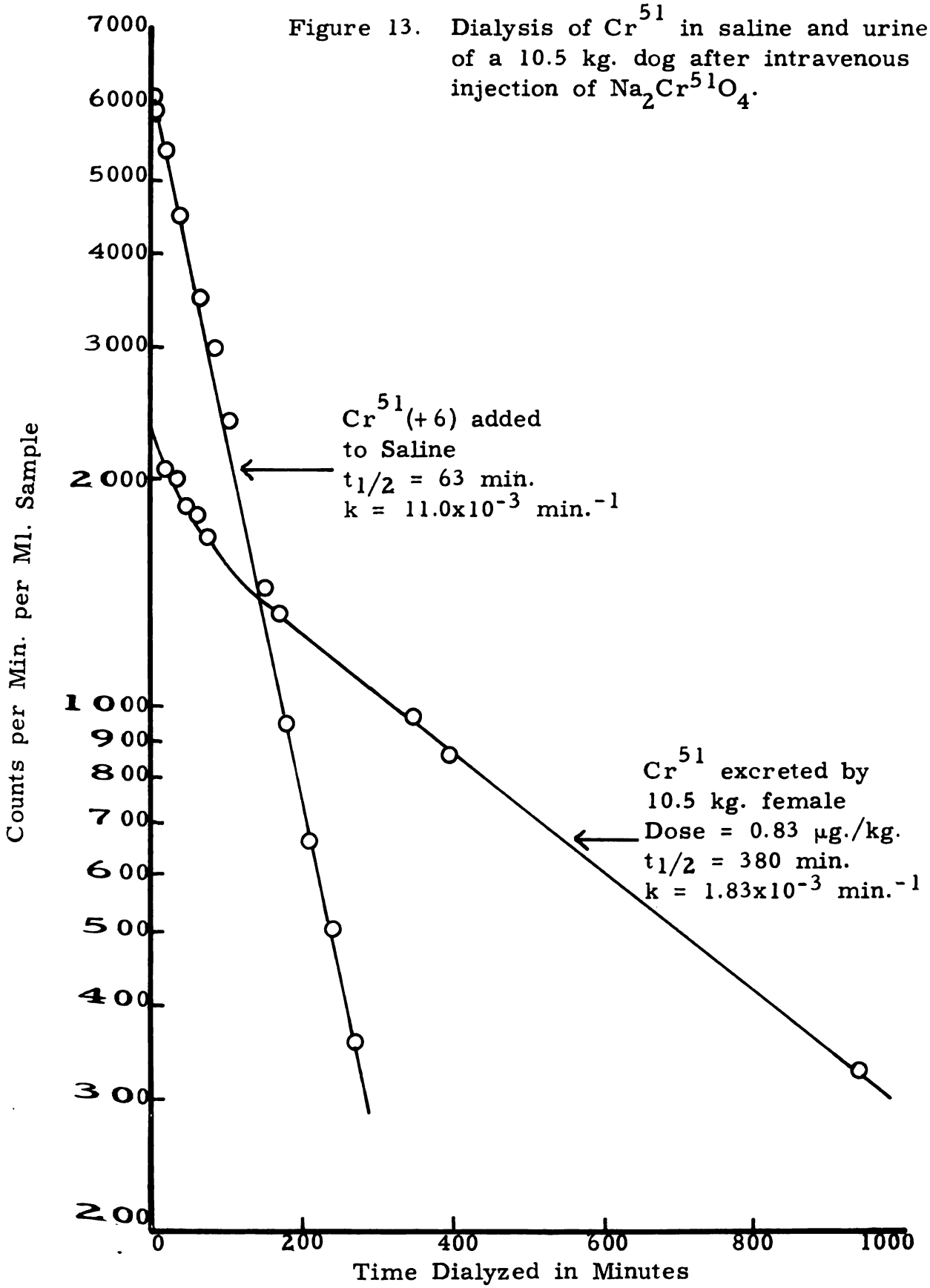
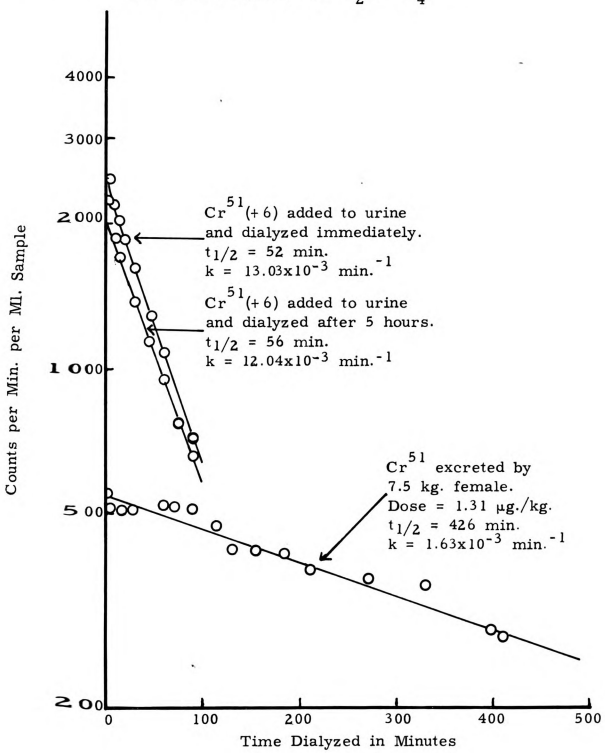


Figure 14. Dialysis of Cr^{51} added to urine in vitro and urine after intravenous injection of $Na_2Cr^{51}O_4$.



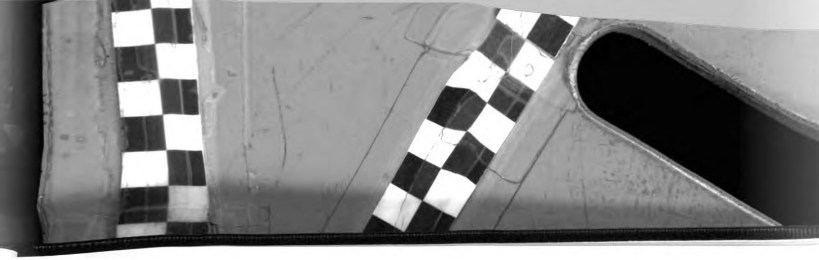


TABLE 3

REDUCTION OF HEXAVALENT CHROMIUM IN VIVO AND IN VITRO

Procedure	Time (minutes)	Total Chromium (μg./5 ml.)	Hexavalent Chromium (qual. test)
100 μg. hexavalent chromium added to stored urine	2	5	+++
	10	5	+++
	60	5	+++
	120	5	+++
	360	5	+++
100 μg. hexavalent chromium added to fresh urine	5	5	-
	10	5	-
	15	5	-
200 μg. hexavalent chromium added to fresh urine	5	10	+
	10	10	±
	30	10	-
	60	10	-
Cr^{51} excreted by dog after injection of 40 mg. $Na_2Cr^{51}O_4$	0-6[a]	125	-
	6-18	168	-
	18-30	119	-

[a] Indicates time interval in which sample was collected.

TABLE 4

IONIC PROPERTIES OF CHROMIUM IN BIOLOGICAL FLUIDS

Sample	Dog 18		Dog 4	
	Per Cent Removed	Avg. Pct. Removed	Per Cent Removed	Avg. Pct. Removed
	Cation Exchange Resin			
Cr infusion or injection solution[a]	48.42 48.09	48.25	99.85 99.95	99.90
Pooled urine	0.56 -0.28	0.14	6.74 5.24	5.99
Plasma dialyzate	0.73 -2.30	-0.79	5.86 2.29	4.07
Pooled bile	- -		-2.41 10.67	4.13
	Anion Exchange Resin			
Cr infusion or injection solution	40.56 -	40.56	87.48 89.20	88.33
Pooled urine	98.64 -	98.64	95.97 96.06	96.01
Plasma dialyzate	97.81 -	97.81	89.71 89.52	89.61
Pooled bile	- -		27.59 27.22	27.40

[a] Dog 18 was infused with $Cr^{51}Cl_3$; dog 4 received a single injection of $Cr^{51}Cl_3$.

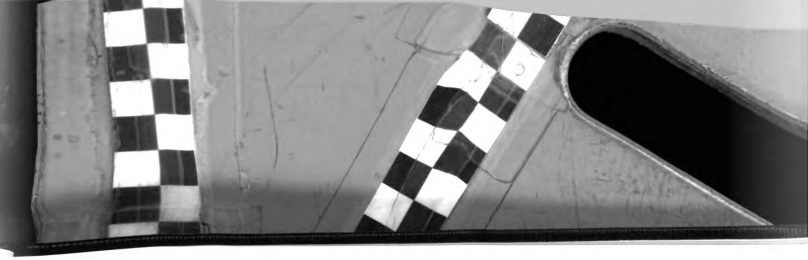
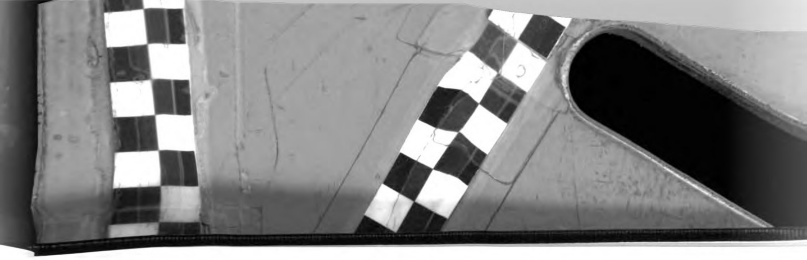


TABLE 5

THE R_f VALUES OF Cr^{51}, NINHYDRIN-POSITIVE SUBSTANCE, CREATININE, AND PAH IN SAMPLES TAKEN FROM DOG 12

Sample	Creatinine	PAH	Cr^{51}	Ninhydrin-positive Substance		
Infusion solution .	.16	.38	.06			
	.17	.37	.07			
Plasma dialyzate.	.13	-[a]	.05	.06	.39	
	.15	-	.04	.07	.35	
Urine	.23	.34	.06	.03	.06	.09
	.27	.24	.05	.03	.06	.08
PAH -		.41				
Creatinine	.22					

[a] Indicates none could be found in sample.



chromatograms does not prove that they are bound together, their location at the same spot would be expected if binding occurred. Per cents of Cr^{51} precipitated from urine and a plasma dialyzate by ammonium hydroxide are shown in Table 6. The addition of $Cr^{51}Cl_3$ to normal urine and plasma dialyzate in vitro served as controls to demonstrate that Cr^{51} in these fluids would precipitate with ammonium hydroxide.

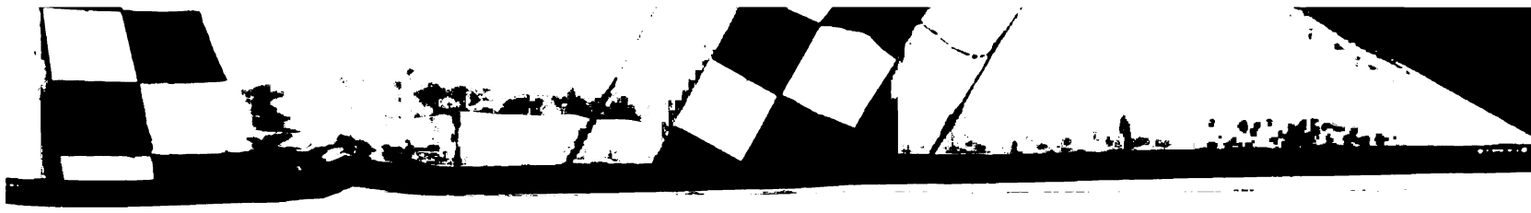
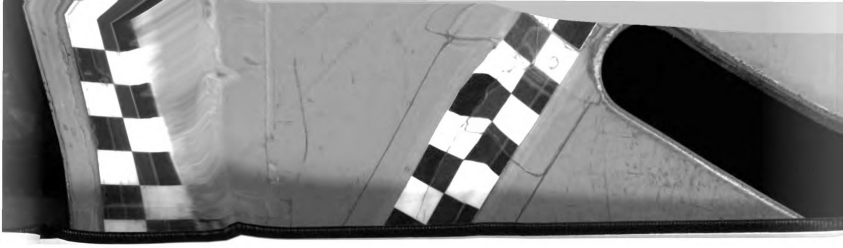


TABLE 6

PRECIPITATION OF CHROMIUM FROM BIOLOGICAL FLUIDS USING AMMONIUM HYDROXIDE

Sample	Per Cent Precipitated	Average Per Cent Precipitated
Urine in vivo[a]	28.23	26.93
	25.63	
Urine in vitro	77.47	77.82
	78.18	
Plasma dialyzate in vivo	30.34	31.73
	33.13	
Plasma dialyzate in vitro	97.11	97.16
	97.21	

[a] 19 kg. Dog injected intravenously, with 705 μg $Cr^{51}Cl_3$ and urine used.



DISCUSSION

I. Routes of Chromium Excretion

Data summarized in Figures 2 through 6 compare excretion of chromium in urine and bile over a four-hour period after intravenous injection of chromium. Over 94 per cent of Cr^{51} excreted after administration of $Na_2Cr^{51}O_4$ appeared in urine, while 99.5 per cent of $Cr^{51}Cl_3$ was excreted by this route. These Figures also show that about three times more trivalent than hexavalent chromium was excreted four hours after injection. Three different dosage levels of hexavalent and two of trivalent chromium were administered to these dogs. In all cases the larger doses resulted in a greater per cent of the injected dose being excreted. These findings were also confirmed with $Na_2Cr^{51}O_4$ over a four-day period. A possible explanation for these findings is that a smaller per cent of large doses is bound to erythrocytes or plasma proteins (as Gray and Sterling, 1950, showed in vitro), leaving more "free" for urinary excretion. However, biliary excretion of either valence state showed no such dosing effect.

Bile fistula dogs were used to measure chromium excretion in feces as well as urine and bile over a four-day period (Figures 7 and 8). Figure 7 indicates that Cr^{51} of $Cr^{51}Cl_3$ or $Na_2Cr^{51}O_4$ was excreted rapidly immediately following injection but that the rate of excretion decreased with time. With both valences, over 80 per cent of the four-day excretion value was found in urine collected the first day. The liver is one of the major organs of uptake of Cr^{51} after $Cr^{51}Cl_3$ or $Na_2Cr^{51}O_4$ injection (Visek et al., 1953), so that a relatively large excretion of chromium in bile would perhaps be expected. However, less than 5 per cent of excreted Cr^{51} was present in bile. After $Na_2Cr^{51}O_4$ injection, the amount of Cr^{51} found in feces was also negligible and only slightly greater after dosing with $Cr^{51}Cl_3$.

Data on Cr^{51} excretion, after administration of $Cr^{51}Cl_3$, are in good agreement with those of Kraintz and Talmage (1952). They found 40 per cent of Cr^{51} in urine and none in feces of rats one day after the intravenous injection of $Cr^{51}Cl_3$ in acetate buffer. The one-day excretion values in dog 6 (Figures 7 and 8) were 41.5 and 2.5 per cent of the injected $Cr^{51}Cl_3$ in urine and feces, respectively. On the other hand, the four-day excretion values of Cr^{51} after intravenous administration of $Cr^{51}Cl_3$ to rats reported by Visek et al. (1953) do not agree with those found here. They reported 15 per cent in urine and 20 per cent in feces, while the corresponding values

here in dog 6 were 49.3 per cent in urine and 4.4 per cent in feces plus bile. Discrepancies in excretion of Cr^{51} after giving $Na_2Cr^{51}O_4$ were also large. Their four-day excretion values for this valence were 35 and 17 per cent in urine and feces, while the average results obtained here for dogs 6 and 7 were 20.5 per cent in urine and 2.0 per cent in feces plus bile. The results reported by Sutherland and McCall (1955) are intermediate between those of Visek et al. and the data reported here. They found that urine contained 35 per cent and feces a negligible amount of Cr^{51} after the intravenous administration of $Na_2Cr^{51}O_4$ to humans.

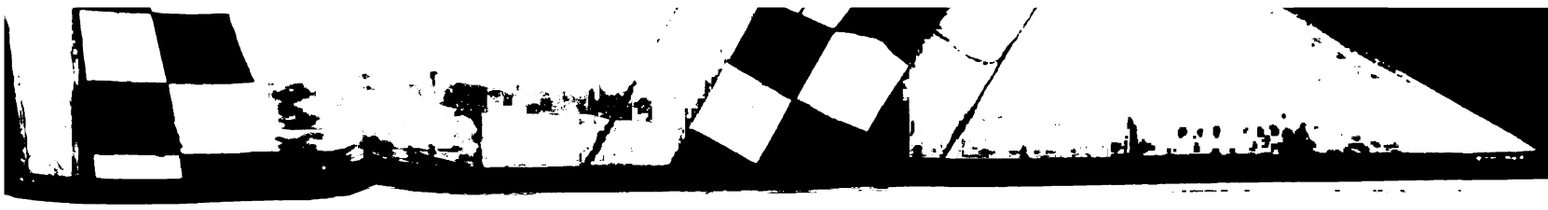
The inconsistencies in results reported for chromium excretion can probably be attributed to differences in concentration, pH, and presence of other substances in the dosing solutions as well as species differences in the animals used.

II. Mechanisms of Renal Excretion of Chromium

The concept of the renal excretion of plasma dialyzable chromium did not originate until after chromium clearances ($C_{chr.}$) were measured in several dogs. Chromium clearances on the first two dogs (Table 1, dogs 8 and 9), measured on consecutive urine samples over a relatively short period of time, varied from 2.91 to 1.84 ml. per minute per sq. meter surface area. This range of variation

might be expected in $C_{chr.}$; however, a tendency for the clearances to decrease systematically with time after chromium dosing was unexpected. Clearances on a third dog, No. 10, were determined at several time intervals over a six-hour period. Data from this dog (from 2.60 to 0.63 ml. per minute per sq. meter surface area) clearly showed that $C_{chr.}$ decreased with time after the $Cr^{51}Cl_3$ injection. A plot of $C_{chr.}$ in Figure 9 established that chromium clearances decrease exponentially with time after $Cr^{51}Cl_3$ administration. Since GFR and ERPF determinations indicated normal renal function, some aspect of chromium excretion was producing a reduction of $C_{chr.}$; in any case, chromium clearances alone did not clarify renal mechanisms employed in chromium excretion.

Trivalent chromium in plasma is largely bound to the plasma proteins, but some exists in the "free" state. Since the "free" form would be filterable at the glomerulus, its measurement should shed some light on renal mechanisms involved in chromium excretion. Figure 11 shows the per cent dialyzable ("free") chromium in the plasma of an 18.5 kg. dog after the intravenous injection of 650 µg. per kg. body weight of trivalent chromium. The finding that the per cent dialyzable chromium in plasma also decreased exponentially with time after the $Cr^{51}Cl_3$ injection offered an explanation of the reduction of $C_{chr.}$ observed with time. If each of the chromium



clearances were multiplied by the reciprocal of the decimal equivalent of the per cent plasma dialyzable chromium at a time midway in the urine collection period, the answer would be in terms of a clearance of plasma dialyzable chromium ($C_{d\text{-}chr.}$). For example, the mid-point of the urine collection period for clearance 1 of dog 8 (Table 1) was 78 minutes after the $Cr^{51}Cl_3$ injection. At this time, Figure 11 shows that 9.7 per cent of the plasma chromium was dialyzable. The factor used to multiply $C_{chr.}$ by was 1/0.097, or 10.3. Then 10.3 times the $C_{chr.}$ of 2.86 gives a dialyzable chromium clearance of 29.5 ml. per minute per sq. meter surface area. This means that 29.5 ml. of plasma are completely cleared of dialyzable chromium each minute for each square meter surface area of the dog. The dialyzable chromium clearances calculated in this manner for the first three dogs are shown in Table 7.

This method of determining $C_{d\text{-}chr.}$ depends on the supposition that per cent plasma dialyzable chromium at any one time after $Cr^{51}Cl_3$ injection is the same in different dogs and at different doses. Figure 12, showing the per cent plasma dialyzable chromium of dogs 11 through 16, demonstrates that this is not the case. There is wide individual variation in the per cent chromium unbound in plasma of different dogs at the same time after injection, although the per cent dialyzable chromium in any one dog a short time after injection

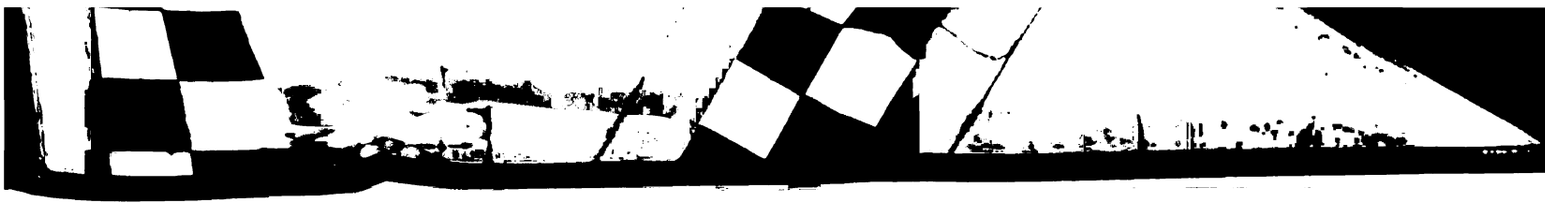


TABLE 7

DIALYZABLE CHROMIUM CLEARANCES IN MILLILITERS PER MINUTE PER SQUARE METER SURFACE AREA AS CALCULATED FROM PER CENT "FREE" CHROMIUM IN PLASMA

Clearance	Dog Number		
	8	9	10
1	29.5	28.5	31.3
2	27.8	24.9	28.4
3	27.4	27.4	27.3
4	27.7	29.5	28.5
5	27.1	31.8	23.7
6			26.6
7			29.0
8			30.5

shows a uniform exponential decrease. The relation between dose of chromium and the rate constant, k (where $k = .693/t_{1/2}$), for decreasing plasma dialyzable chromium is shown in Table 8. The coefficient of linear correlation, r, between dose and k is 0.75, which is significant at the 1 per cent level. This statistic means that 56 (0.75^2) per cent of the variation in k can be attributed to the dose. With respect to the dose-k relationship, it was surprising that larger doses resulted in more rapid decline in per cent plasma dialyzable chromium.

Figure 12 (dog 16) shows that the per cent plasma dialyzable chromium decreases more rapidly immediately after the chromium injection than at a later time. In three other dogs a rapid component was also observed. The cause for this rapid fall is not clear. In determining percentage of plasma dialyzable chromium, the amount dialyzable is divided by total chromium content of plasma. Any factor decreasing the amount dialyzable without greatly affecting the total will result in a greater per cent bound. The rapid rate of decrease in per cent plasma dialyzable chromium is probably due to "free" chromium diffusing out of the plasma space, leaving a larger part of that remaining in the bound form. In vitro tagging of dog plasma proteins with Cr^{51} indicates that only two-thirds of trivalent chromium becomes bound (Gray and Sterling, 1950, found

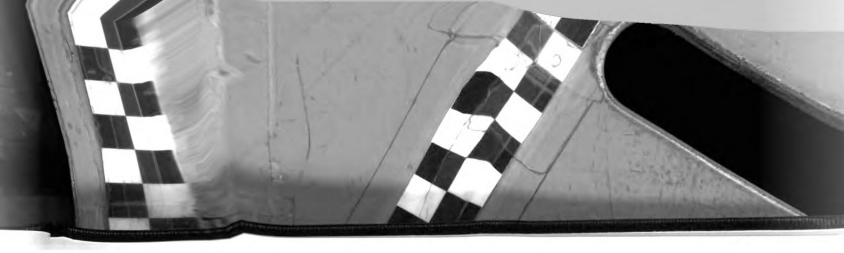


TABLE 8

RELATIONSHIP BETWEEN THE DOSE OF CHROMIUM INJECTED AND THE RATE CONSTANT (k) FOR THE DECREASE IN PER CENT DIALYZABLE CHROMIUM IN PLASMA[a]

Dose (μg./kg.)	$t_{1/2}$ (min.)	k (per min.)
1900	178	.00395
650	170	.00408
91.0	235	.00295
82.2	225	.00308
6.50	295	.00245
5.22	242	.00287
4.94	330	.00211

[a]Coefficient of linear correlation (r) = 0.75.

about 66 per cent bound with bovine albumin in vitro), while in vivo tagging of dog plasma shows 85 to 90 per cent bound a few minutes after injection. The difference between these two figures represents "free" chromium that left the plasma.

The slow rate of decrease in per cent dialyzable chromium extending over many hours after injection is visualized as a net rate equal to the decrease caused by renal excretion minus the return of dialyzable chromium to the plasma space from extravascular sites. Another factor affecting the rate of decrease would be chromium freed from plasma proteins by elution or degradation. This aspect of chromium metabolism deserves further study.

$C_{d\text{-}chr.}$ in dogs 10 through 18 was determined by the standard clearance formula using the plasma concentration of dialyzable chromium. The value for $C_{d\text{-}chr.}$ in eight dogs was 36.6 ± 11.6 ml. per minute per sq. meter surface area. Five to eight measurements were made on each animal at periods of 1 to 10-1/2 hours after the chromium injection. Two clearances measured on one dog at 48 and 49 hours after the chromium injection were within the normal range but the Cr^{51} activity of the plasma dialyzate was so low (an average of 0.95 count above background for four determinations) that the results are questionable. The last two animals used in this study received chromium by infusion over a 7 to 8 hour period instead of

a single injection. One of these dogs had a high $C_{d\text{-}chr.}$ but otherwise the clearances were similar to those of other dogs.

The mean and standard deviation for creatinine clearances in the eleven dogs in this study was 101.7 ± 24.3 ml. per minute per sq. meter surface area. This value is somewhat higher than that of Houck (84 ± 19.1 ml. per min.) or Russo (95 ml. per min.) possibly because of saline contained in the creatinine-PAH infusion solution. Saline has been shown to increase GFR and ERPF (Baldwin et al., 1949; Hare et al., 1944), and at the infusion rates employed (1-3 ml. per min.) some dogs received as much as 1.5 liters of 0.85 per cent sodium chloride. The value for PAH clearances of 259.9 ± 40.1 was in good agreement with those of Houck (266 ± 66) and Russo (238), all expressed as ml. per minute per sq. meter surface area.

A small quantity of blood proteins is excreted by the normal kidney (Waterhouse and Holler, 1948; McGeachin and Hargan, 1957), and there is evidence that hemoglobin reabsorption from the glomerular filtrate amounts to about 3 per cent of GFR (Monke and Yuile, 1940). These processes could be involved in chromium excretion; however, the amount of protein (as measured by Albumtest tablets) in clearance urine samples was too small to account for more than a few per cent of the urine chromium. Also, the finding that nearly

all of the Cr^{51} excreted in urine was dialyzable (Table 2) rules out excretion of protein-bound chromium. The alternate possibility, that plasma proteins containing bound chromium are filtered and the chromium eluted into glomerular fluid prior to protein reabsorption, is extremely unlikely because of the firmness of the chromium-protein bond (Gray and Sterling, 1950). The finding that $C_{chr.}$ is dependent on time after the chromium injection (Figure 10), while $C_{d\text{-}chr.}$ is not (Figure 15; F values not significant), argues against excretion of chromium in this manner.

On the other hand, plasma dialyzable chromium is certainly filterable at the glomerulus (the average pore diameter of the dialysis tubing was 24 Å, while the pore size of the glomerulus [lamina densa] may be on the order of 100 Å [Hall, 1954]) and must exist in glomerular filtrate in the same concentration as in plasma water. The amount of dialyzable chromium filtered is equal to glomerular filtration rate times plasma concentration. If only glomerular filtration were involved in chromium excretion this quantity should appear in the urine. The finding that less chromium is present in urine than is filtered indicates some chromium is reabsorbed from tubular fluid. Tubular reabsorption amounts to 63 per cent ($1 - C_{d\text{-}chr.}/C_{creat.} \times 100$) of the amount filtered in the eight dogs in which it was measured.

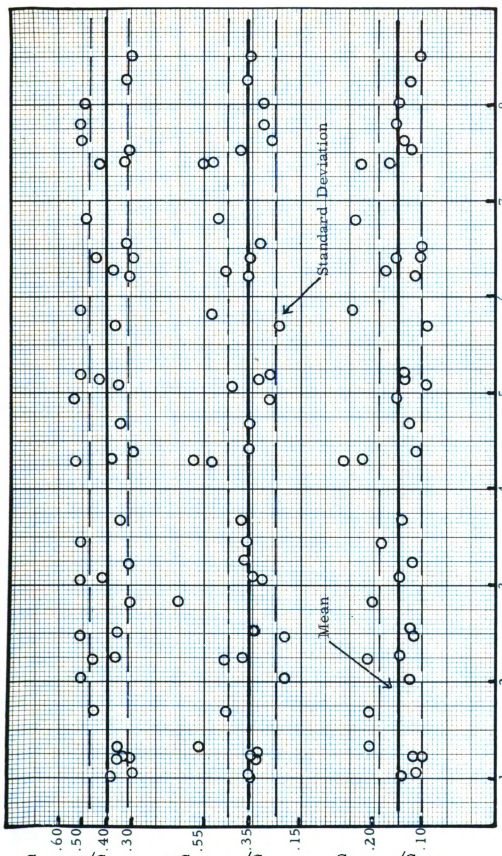


Figure 15. Variation in clearance ratios with time after chromium injection.

Unequivocal proof that a substance is excreted by the tubules is extremely difficult unless its clearance is greater than GFR. However, tubular excretion in every instance where it has been adequately examined is limited by a maximal rate of tubular transport (Smith, 1951). At plasma levels above those necessary to saturate the tubular excretory mechanisms the clearance declines. Figure 16 shows the dialyzable chromium clearance ratios plotted against the plasma level of chromium. There is over an 800 fold difference in the dialyzable chromium concentration in plasma without a reduction in the clearance ratios. Either the peak plasma level of dialyzable chromium was not high enough to saturate tubular mechanisms or tubular excretion of dialyzable chromium plays little part in chromium excretion. Tubular maximum (T_M) measurements for chromium are necessary to clarify the role of the tubules in chromium excretion.

The ratios, $C_{d\text{-}chr.}/C_{PAH}$ and $C_{d\text{-}chr.}/C_{creat.}$, for dialyzable chromium at high plasma levels increased significantly ("t" tests) above those at medium or low plasma levels. Reasons for this are not known.

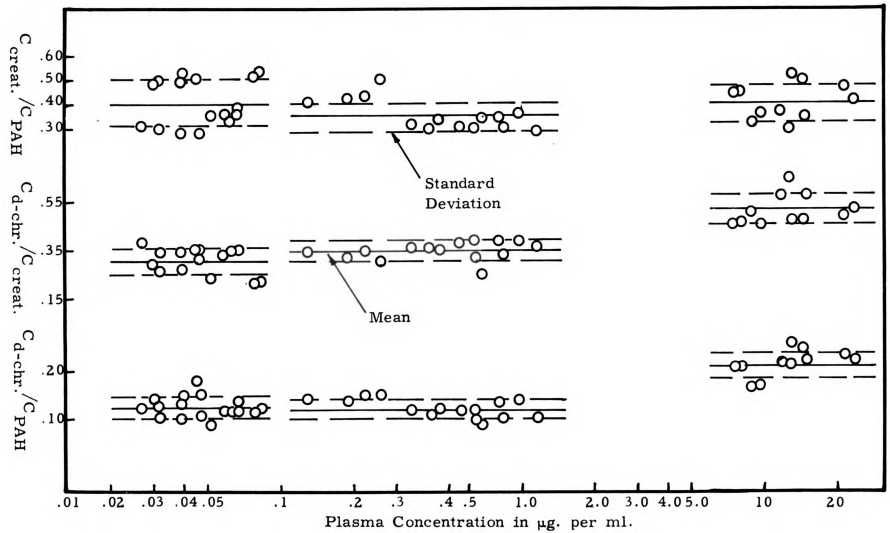
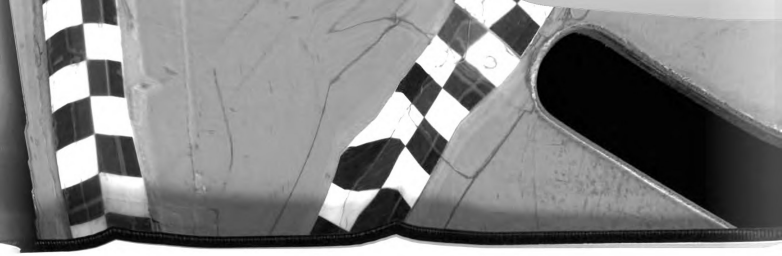


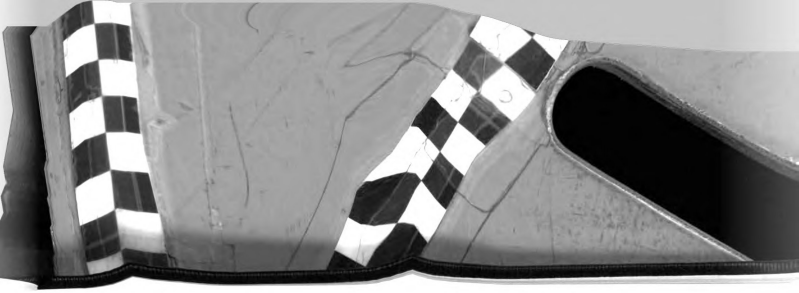
Figure 16. Variation in clearance ratios with plasma concentration of chromium.



III. Some Physical and Chemical Properties of Chromium in Biological Fluids

Rate dialysis (Figures 13 and 14) of Cr^{51} excreted in urine conclusively demonstrated that this chromium diffused more slowly and presumably existed as larger molecules than the $Na_2Cr^{51}O_4$ injected into the animal. The finding that intravenously injected hexavalent chromium was excreted in the trivalent state (Table 3) offers an explanation to this decreased diffusion rate of excreted Cr^{51}. Since the diffusion rate of $Cr^{51}Cl_3$ was not measured, it is not known if reduction of $Na_2Cr^{51}O_4$ would account for the large decreases in diffusion rates observed.

The finding by ion exchange absorption that trivalent chromium excreted in urine or present in a plasma dialyzate existed in the anionic form could be interpreted as being due to the binding of this chromium to organic material to form negatively charged complexes. However, in interpreting these results it is essential to remember that hydrolysis of $CrCl_3$ readily occurs to form anionic complexes with water when no organic material is present. This is well illustrated in the infusion solution of dog 18. A small quantity of $Cr^{51}Cl_3$ was added to 1300 ml. of creatinine-PAH infusion solution at a pH of 5.2, and only 48.2 per cent of the Cr^{51} was removed by the cation exchange resin, while 40.6 per cent was absorbed by the



anion exchange resin. The difference of 11.2 per cent was presumably uncharged. In contrast, the injection solution for dog 4 had a pH of 3.8, and 99.9 per cent of the Cr^{51} existed in the cation form as shown by absorption with Amberlite 120. One factor in favor of the organic complexing concept is that chromium in urine or a plasma dialyzate is soluble, while $CrCl_3$ in a solution adjusted to the same pH precipitates.

Paper chromatograms of chromium excreted in urine or present in plasma dialyzate resulted in a small but consistent (R_f = 0.04 to 0.06) movement of the Cr^{51} from the origin. In the solvent mixture used, cationic chromium was reported to have an R_f value of 0.65 (Pollard and McOmie, 1953). The difference in the R_f value found here and those reported previously may have been due to the use of acetate complexed chromium in determining the value reported by Pollard and McOmie. By developing the chromatograms with ninhydrin, definitive proof that Cr^{51} was not bound to amino-containing substances could result since this reaction gives a positive test with proteins, peptones, peptides, amino acids, and other primary amines including ammonia (Hawk et al., 1954). The finding of ninhydrin-positive substances on the paper strips with R_f values about the same as those of Cr^{51} supports but does not prove that chromium is excreted in the bound form.

The best evidence that dialyzable chromium in plasma and urine is at least in part in organic combination was provided by the precipitation procedure. In this experiment the greater part of anionic $Cr^{51}Cl_3$ added to normal urine and plasma dialyzate was precipitated by ammonium hydroxide, while less than one-third of intravenously injected chromium present in these fluids was precipitated.

SUMMARY AND CONCLUSIONS

1. In the dog the major route of excretion of intravenously injected chromium is in the urine; excretion in bile and feces is negligible. In acute experiments on anesthetized dogs about 9 per cent of the injected dose of hexavalent and 25 per cent of trivalent chromium were excreted in the urine in four hours. Less than 0.5 per cent of either valence was present in bile collected over a four-hour period after intravenous injection of $Cr^{51}Cl_3$ or $Na_2Cr^{51}O_4$. The four-day excretion values in urine, bile, and feces in one bile fistula dog were 50, 0.5, and 3.7 per cent, respectively, of injected $Cr^{51}Cl_3$. In two dogs hexavalent chromium average excretion values were 20, 0.9, and 1.2 per cent of the injected dose in urine, bile, and feces, respectively.

2. After a single intravenous injection renal clearances of $Cr^{51}Cl_3$ decrease exponentially with time from 2.5 or 3 ml. per min. at one hour to less than 1 ml. per min. at eight hours after injection.

3. Measurement of dialyzable Cr^{51} in plasma showed that the per cent of chromium not bound to plasma proteins also decreased exponentially with time after injection and at a rate about equal to the decline in chromium clearance. Large doses of chromium were

found to result in a more rapid decline in the percentage of chromium not bound to plasma proteins. Possible reasons for the reduction in per cent dialyzable chromium in plasma are discussed.

4. A dialyzable chromium clearance with a mean and standard deviation of 36.6 ± 11.6 ml. per minute per sq. meter surface area was determined. This clearance showed that glomerular filtration and tubular reabsorption are two mechanisms involved in the renal handling of unbound chromium. Tubular excretion of dialyzable chromium may occur. However, no reduction in the clearance at high plasma chromium levels favors the conclusion that excretion by the tubules is of minor importance.

5. Simultaneous creatinine and PAH clearances were normal, demonstrating that renal function was not impaired over a large range of plasma chromium concentration.

6. No hexavalent chromium could be found in urine after intravenous injection of sodium chromate, an indication that in vivo reduction occurs. Freshly collected urine was found to reduce hexavalent chromium while stored urine did not.

7. Chromium in urine, bile, and plasma dialyzate is anionic, as shown by ion exchange absorption after the intravenous administration of cationic chromic chloride.

8. Results of dialysis, ion exchange absorption, paper chromatography, and precipitation studies indicate, but do not prove conconclusively, that chromium is excreted at least in part in organic combination.

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APPENDIXES

APPENDIX A

FOUR-HOUR EXCRETION VALUES OF CHROMIUM IN ACUTE ANESTHETIZED DOGS, FOUR-DAY EXCRETION VALUES OF CHROMIUM IN BILE FISTULA DOGS, AND DATA USED TO CALCULATE CHROMIUM CLEARANCES

FOUR-HOUR EXCRETION VALUES OF CHROMIUM IN ACUTE ANESTHETIZED DOGS
(in μg. per collection period)

Collection Period	Dog[a] 1	2	3	4	5
			Urine		
0-30 minutes . . .	.0275	2.797	1276.0	2499	8.924
30-60 minutes . .	.0358	1.104	692.0	1243	4.858
60-90 minutes . .	.0370	0.975	668.0	1011	2.846
90-120 minutes . .	.0340	1.325	412.0	1108	2.076
120-150 minutes .	.0201	0.672	272.0	962.8	1.787
150-180 minutes .	.0186	0.754	236.0	995.0	1.530
180-210 minutes .	.0231	0.580	288.0	940.2	0.888
210-240 minutes .	.0278	0.598	296.0	579.6	0.674
			Bile		
0-30 minutes . . .	.0007	0.0442	3.200	9.016	0.0171
30-60 minutes . .	.0027	0.0736	8.800	9.016	0.0342
60-90 minutes . .	.0012	0.0524	7.600	3.542	0.0278
90-120 minutes . .	.0013	0.1113	25.60	5.796	0.0235
120-150 minutes .	.0017	0.0442	29.60	3.864	0.0193
150-180 minutes .	.0018	0.0414	28.80	2.898	0.0171
180-210 minutes .	.0017	0.0883	27.60	2.899	0.0128
210-240 minutes .	.0018	0.1049	29.60	2.897	0.0096

[a]Dog 1: weight, 7.5 kg.; dose, .395 μg./kg.; valence, +6.
Dog 2: weight, 12.5 kg.; dose, 7.28 μg./kg.; valence, +6.
Dog 3: weight, 10.0 kg.; dose, 4,000 μg./kg.; valence, +6.
Dog 4: weight, 10.0 kg.; dose, 3,200 μg./kg.; valence, +3.
Dog 5: weight, 11.0 kg.; dose, 9.40 μg./kg.; valence, +3.

FOUR-DAY EXCRETION VALUES OF CHROMIUM IN BILE FISTULA DOGS

(in μg. per collection period)

Collection Period	Dog[a] 6	7	6
Urine			
0-2 hours	1375	1.665	6.214
2-4 hours	939.3	0.6773	2.312
4-8 hours	937.5	0.6365	2.910
8-16 hours	505.8	0.5141	3.500
16-24 hours	567.2	0.3754	3.229
0-1 day	4325	3.868	18.165
1-2 days	417.3	0.4366	1.844
2-3 days	390.2	0.2203	0.9046
3-4 days	231.2	0.1306	0.6468
Bile			
0-2 hours	10.84	0.1306	0.0481
2-4 hours	48.59	0.0290	0.0297
4-8 hours	35.40	0.0139	0.0546
8-16 hours	50.22	0.0187	0.0336
16-24 hours	7.948	0.0094	0.0315
0-1 day	1530	0.2016	0.1975
1-2 days	59.43	0.1469	0.0367
2-3 days	22.04	0.0261	0.0271
3-4 days	16.44	0.0078	0.0236
Feces			
0-1 day	52.20	0.1783	1.071
1-2 days	62.50	0.1199	0.2827
2-3 days	24.75	0.0620	0.1512
3-4 days	11.74	0.0622	0.1084

[a] Dog 6: weight, 11 kg.; dose, 1,640 μg./kg.; valence, +6.
Dog 7: weight, 15 kg.; dose, 2.72 μg./kg.; valence, +6.
Dog 6: weight, 11 kg.; dose, 4.02 μg./kg.; valence, +3.

DATA USED TO CALCULATE CHROMIUM CLEARANCES

Clearance No.	Time after Inj. (min.)	Urine Collection Period (min.)	Urine Vol. (ml.)	Urine Flow (ml./min.)	Urine Conc. (μg./ml.)
		Dog No. 8			
1	78	20.5	200	3.12	0.0099
2	98	24.5	200	4.92	0.0099
3	122	19.5	200	4.32	0.0067
4	141	24.7	200	2.93	0.0077
5	166	15.5	200	2.64	0.0041
		Dog No. 9			
1	66	17.5	200	5.27	0.2640
2	82	21.0	200	6.56	0.2450
3	106	19.0	200	7.09	0.2070
4	127	21.7	200	6.02	0.2150
5	148	20.5	200	3.63	0.1960
		Dog No. 10			
1	117	32.0	200	1.82	0.4530
2	147	28.0	200	3.00	0.3000
3	218	24.0	200	3.56	0.1580
4	240	20.5	200	3.60	0.1200
5	321	25.0	200	3.99	0.0863
6	350	33.0	200	3.39	0.1130
7	430	20.0	200	3.62	0.0529
8	450	66.5	200	3.50	0.0881

DATA USED TO CALCULATE CHROMIUM CLEARANCES (Continued)

Plasma Conc. (μg./ml.)	Dial. Plasma Conc. (μg./ml.)	F.F.	$\frac{C_{d\text{-}chr}}{C_{PAH}}$	$\frac{C_{d\text{-}chr}}{C_{creat.}}$
		Dog No. 8		
0.0634	-	.47	-	-
0.0607	-	.53	-	-
0.0585	-	.63	-	-
0.0560	-	.47	-	-
0.0538	-	.49	-	-
		Dog No. 9		
1.570	-	.41	-	-
1.493	-	.40	-	-
1.396	-	.41	-	-
1.335	-	.44	-	-
1.250	-	.40	-	-
		Dog No. 10		
1.250	-	.26	-	-
1.180	-	.32	-	-
1.010	-	.39	-	-
0.980	-	.37	-	-
0.920	-	.38	-	-
0.910	-	.41	-	-
0.870	-	.33	-	-
0.850	-	.34	-	-

DATA USED TO CALCULATE CHROMIUM CLEARANCES (Continued)

Clearance No.	Time after Inj. (min.)	Urine Collection Period (min.)	Urine Vol. (ml.)	Urine Flow (ml./min.)	Urine Conc. (μg./ml.)
		Dog No. 11			
1	60	24.0	200	0.37	0.0185
2	72	21.0	200	0.43	0.0135
3	183	22.0	200	1.11	0.0080
4	206	24.5	200	1.56	0.0087
5	297	13.5	200	2.25	0.0035
6	312	18.0	200	2.34	0.0043
7	457	26.5	200	2.50	0.0038
8	482	23.5	200	2.59	0.0031
		Dog No. 12			
1	122	27.7	200	1.75	0.0161
2	148	23.7	200	2.85	0.0126
3	342	40.0	200	1.12	0.0104
4	2880	68.0	200	0.48	0.0009
5	2940	70.0	100	0.22	0.0017
		Dog No. 13			
1	152	40.5	200	2.67	0.0262
2	266	41.0	200	1.12	0.0178
3	385	42.5	200	1.07	0.0100
4	512	43.5	200	0.48	0.0061
5	633	52.5	200	0.48	0.0061
		Dog No. 14			
1	63	39	380	9.23	0.3149
2	135	31.5	95	1.75	0.6391
3	220	31.5	104	2.35	0.4492
4	305	29.0	112	2.48	0.1843
5	393	26.0	92	2.00	0.1812
6	453	43.0	101	1.42	0.2327

DATA USED TO CALCULATE CHROMIUM CLEARANCES (Continued)

Plasma Conc. (μg./ml.)	Dial. Plasma Conc. (μg./ml.)	F.F.	$\frac{C_{d\text{-}chr}}{C_{PAH}}$	$\frac{C_{d\text{-}chr}}{C_{creat.}}$
		Dog No. 11		
0.0670	0.0055	.38	.133	.350
0.0631	0.0045	.33	.115	.348
0.0470	0.0023	.50	.149	.299
0.0452	0.0020	.50	.175	.352
0.0402	0.0017	.53	.144	.270
0.0394	0.0015	.49	.131	.268
0.0318	0.0008	.49	.128	.263
0.0304	0.0007	.48	.139	.289
		Dog No. 12		
0.0840	0.0073	.53	.118	.221
0.0780	0.0062	.51	.111	.216
0.0525	0.0028	.35	.083	.227
0.0153	0.0001	-	-	.245
0.0153	0.0001	-	-	.274
		Dog No. 13		
0.0593	0.0052	.35	.116	.330
0.0475	0.0035	.28	.104	.352
0.0390	0.0020	.28	.096	.341
0.0322	0.0012	.29	.098	.341
0.0270	0.0009	.31	.118	.377
		Dog No. 14		
1.155	0.1175	.29	.103	.357
0.973	0.0725	.36	.137	.381
0.795	0.0452	.34	.132	.385
0.680	0.0320	.34	.082	.242
0.640	0.0225	.31	.095	.311
0.632	0.0152	.30	.118	.390

DATA USED TO CALCULATE CHROMIUM CLEARANCES (Continued)

Clearance No.	Time after Inj. (min.)	Urine Collection Period (min.)	Urine Vol. (ml.)	Urine Flow (ml./min.)	Urine Conc. (μg./ml.)
		Dog No. 15			
1	74	33.5	132	2.75	0.5480
2	194	35.0	147	3.05	0.2704
3	281	35.0	103	1.80	0.2489
4	374	34.0	91.5	0.93	0.1858
5	496	35.0	71.0	0.31	0.1442
		Dog No. 16			
1	79	35.0	186	3.60	4.052
2	169	39.0	102	1.08	4.936
3	259	39.0	114	1.38	2.769
4	377	36.0	96	1.00	1.847
5	445	55.0	96	0.65	2.141
		Dog No. 17			
1	103	28.5	244	5.75	10.73
2	133	30.5	253	6.98	12.27
3	258	37.5	290	6.67	14.01
4	350	27.5	211	5.49	13.71
5	409	35.0	176	3.31	17.42
6	444	37.0	187	3.43	16.96
		Dog No. 18			
1	71	36.0	250	5.83	0.0250
2	185	44.0	162	2.32	0.0554
3	309	33.0	113	1.61	0.0636
4	385	33.5	164	3.10	0.0478
5	468	40.0	140	2.00	0.0543
Number[a]					
Mean					
Standard deviation					
Coefficient of variation					

[a]Each observation is an average of five to eight determinations.

DATA USED TO CALCULATE CHROMIUM CLEARANCES (Continued)

Plasma Conc. (μg./ml.)	Dial. Plasma Conc. (μg./ml.)	F.F.	$\frac{C_{d\text{-}chr}}{C_{PAH}}$	$\frac{C_{d\text{-}chr}}{C_{creat.}}$
		Dog No. 15		
0.8170	0.0076	.30	.098	.330
0.5550	0.0023	.30	.113	.372
0.4540	0.0022	.34	.118	.348
0.4170	0.0015	.30	.108	.355
0.3500	0.0009	.31	.113	.361
		Dog No. 16		
14.80	0.7500	.35	.203	.573
12.70	0.4100	.30	.197	.650
11.70	0.2570	.37	.213	.582
9.70	0.1800	.36	.166	.458
8.90	0.1350	.32	.161	.505
		Dog No. 17		
7.50	1.660	.45	.207	.456
8.00	1.700	.45	.207	.464
13.0	1.480	.52	.256	.492
14.5	1.430	.50	.246	.492
21.6	1.200	.47	.230	.493
23.7	1.130	.42	.218	.524
		Dog No. 18		
0.0670	0.0082	.35	.119	.345
0.1300	0.0091	.41	.137	.335
0.1890	0.0084	.42	.131	.310
0.2240	0.0080	.43	.148	.343
0.2600	0.0064	.50	.146	.292
		11	8	8
		0.397	0.140	0.369
		0.075	0.045	0.102
		0.189	0.320	0.276

APPENDIX B

DETAILED PROCEDURES FOR PAH, CREATININE, AND CHROMIUM ANALYSES, AND FOR OXIDATION OF $Cr^{51}Cl_3$ TO $Na_2Cr^{51}O_4$

DETAILED PROCEDURES FOR PAH, CREATININE, AND CHROMIUM ANALYSES, AND FOR OXIDATION OF $Cr^{51}Cl_3$ TO $Na_2Cr^{51}O_4$

Protein-free Plasma Filtrate

1. Centrifuge the heparinized blood to obtain plasma.
2. Pipette 2 ml. plasma into 20 ml. water in a 50 ml. centrifuge tube.
3. Add 8 ml. 11.2 per cent trichloro-acetic acid solution.
4. Stopper, shake well, and let stand 10 minutes, shaking at least once.
5. Centrifuge for 10 minutes at 2500 r.p.m. and use clear supernatant for chemical analysis.

Urine Creatinine

1. Pipette 3 ml. of the final diluted urine into a 10 ml. test tube in duplicate.
2. Add 1 ml. 0.04 M picric acid[1] solution.
3. Add 1 ml. 0.75 N NaOH and mix.
4. Let stand exactly 20 minutes and read in a colorimeter at 540 mμ.

Reagent blank: Three ml. distilled water in place of diluted urine.

Other reagents are the same.

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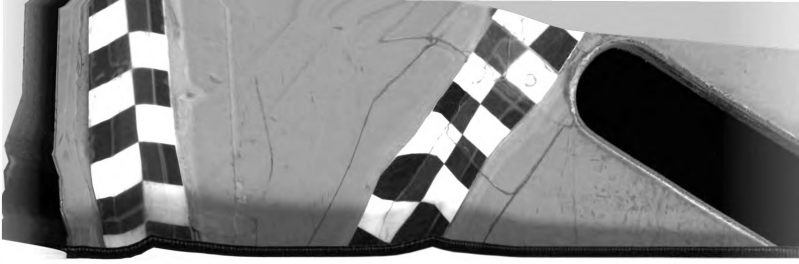
[1] Made by diluting saturated picric acid solution with water and standardizing to 0.04 M with 0.1 N NaOH and phenolphthalein.



Standards: Three tubes containing 20, 40, and 60 μg. creatinine in 3 ml. distilled water. This is done in duplicate. A standard curve is then prepared by plotting the per cent transmission (B and L Spectronic 20 colorimeter was used) against content of creatinine on semilogarithmic paper.

Plasma Creatinine

Pipette in duplicate 3 ml. of protein-free filtrate into two 10 ml. test tubes and proceed as for urine creatinine. The creatinine standards and reagent blank are the same as for urine creatinine.

Urine PAH

1. Pipette 3 ml. of the final diluted urine into a 30 ml. test tube containing 7 ml. water. This is done in duplicate.
2. Add 2 ml. 1.2 N HCl and mix.
3. Add 1 ml. $NaNO_2$ and mix. The $NaNO_2$ (100 mg. per cent) is stored in the refrigerator and prepared fresh every three days.
4. After standing not less than three nor more than five minutes add 1 ml. ammonium sulfamate and mix. Ammonium sulfamate (500 mg. per cent) is stored in the refrigerator and prepared fresh every three weeks.

5. After standing not less than two nor more than five minutes add 1 ml. N (1-napthyl) ethylenediamine dihydrochloride (100 mg. per cent) and mix well. This reagent will keep indefinitely if stored in a dark bottle in the refrigerator.
6. Let stand 20 minutes and read in the colorimeter at 540 mμ. The color is stable indefinitely.

Reagent blank: Ten ml. distilled water is used.

Standards: Three tubes containing 10, 20, and 50 μg. PAH in 10 ml. distilled water are used. This is done in duplicate. A standard curve is prepared as described for creatinine determination.

Plasma PAH

Pipette, in duplicate, 5 ml. of protein-free filtrate into two 30 ml. test tubes containing 5 ml. distilled water and proceed as for urine creatinine. Reagent blank and standards are the same as in the urine PAH determination.

Saltzman Procedure for Chromium

1. Each sample prepared so as to contain 4 to 15 μg. chromium in 1 to 10 ml. is ashed in 125 ml. Phillips beakers by adding 0.5 ml. concentrated HNO_3 (redistilled) and 0.25 ml. 40 per cent

sodium bisulfate, and evaporating on a Lindberg hotplate. If organic material is present the ashing procedure should be repeated. Duplicates are run on all samples.

2. Ten ml. 0.5 N H_2SO_4 is added to redissolve the sample, 0.5 ml. 0.1 N $KMnO_4$ added, and the beaker heated for 20 minutes at 100° C.
3. Five per cent sodium azide added to the hot samples at a rate of 1 drop every 10 seconds with swirling between drops, is used to decolorize the sample. Three to 5 drops are usually sufficient.
4. The decolorized sample is immediately cooled in a tray of cold water and transferred quantitatively to a 25 ml. volumetric flask.
5. Color is developed by adding 1 ml. s-diphenylcarbazide reagent.[1]
6. One minute after color development, 2.5 ml. 4 M NaH_2PO_4 is added as a buffer and the flask diluted to the mark with distilled water.
7. The pink color is read at 540 mμ. within 30 minutes.

Reagent blank: Water is used instead of a sample and carried through the entire procedure.

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[1] Prepared by dissolving 10 gms. phthalic anhydride in 175 ml. redistilled 95 per cent ethanol (warmed for solution) and adding 0.625 gms. s-diphenylcarbazide dissolved in 50 ml. ethanol, the combination being made up to 250 ml. with ethanol.

Standards: 5, 10, and 15 μg. quantities of Na_2CrO_4 prepared in duplicate are added to Phillips beakers and carried through with the samples. A standard curve is prepared as described for urine creatinine.

Oxidation of $Cr^{51}Cl_3$ to $Na_2Cr^{51}O_4$

1. Place sample to be oxidized in 125 ml. Phillips beaker and make basic with 2 ml. 6 N NaOH.
2. Add 1 ml. 3 per cent hydrogen peroxide and boil for 1 hour. Add distilled water as necessary to keep from drying out.
3. Add 0.2 ml. more 6 N NaOH while still hot. If any bubbles appear, boil for 15 min. longer and again add 0.2 ml. NaOH.
4. Make neutral with 2 ml. 6 N HCl.

APPENDIX C

RENAL CLEARANCE AND STATISTICAL FORMULAS

RENAL CLEARANCE FORMULAS

The basic formula used in renal clearance calculations is

$$C_x = (U_x \cdot V)/(P_x) \quad (1)$$

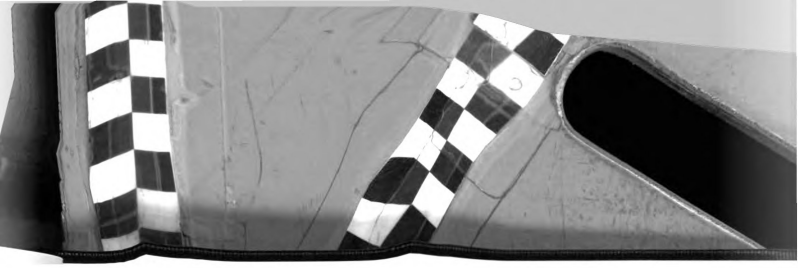
where C_x is the renal clearance of substance x in milliliters per minute, U_x is the urine concentration of substance x in undiluted urine, P_x is the average plasma level of the substance during the urine collection period, and V is the rate of urine flow in milliliters per minute.

A more workable formula is

$$C = (U \cdot V)/(P \cdot T \cdot SA) \quad (2)$$

where C is the renal clearance in milliliters per minute per square meter surface area, U is the urine concentration including all washings, V is the urine volume including all washings over the entire urine collection period, P is the plasma concentration at a point three minutes before the mid-point of the urine collection period, and SA is the animal's surface area in square meters. This formula is easier to use in calculations because less effort is required to get the values into the proper units for substitution. In addition, it is felt that this formula provides a more accurate estimate of renal clearance than formula 1. In clearance measurements the rate of urine flow (V in formula 1) is subject to the largest error

because it depends on the volume of fluid minus washings obtained during the urine collection period. If, for example, a small volume of the last wash fluid is left in the bladder at low rates of urine flow during a creatinine measurement, a large error in V of formula 1 would result but only a small decrease in the value U·V in formula 2 would be noted because the last wash fluid would contain little creatinine.



CORRELATION COEFFICIENT (r)[1]

$$r = \Sigma xy \Big/ \sqrt{\Sigma x^2 \cdot \Sigma y^2}$$

where:

$$\Sigma xy = \Sigma XY - [(\Sigma X)(\Sigma Y)]/N$$

$$\Sigma x^2 = \Sigma X^2 - (\Sigma X)^2/N$$

$$\Sigma y^2 = \Sigma Y^2 - (\Sigma Y)^2/N$$

N = number of observations.

Significance of r is determined by referring to a table of percentile values of r when $\rho = 0$.

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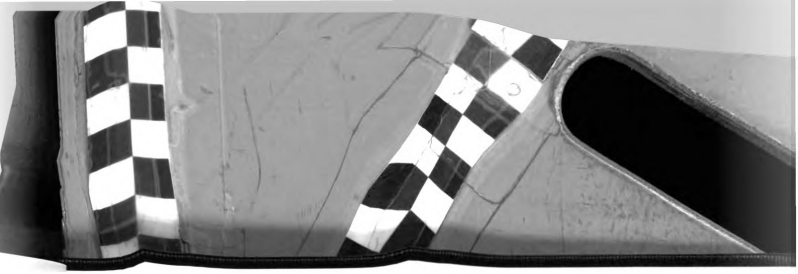
[1]Statistical formulas, pages 108-10, based on Walker and Lev, 1953.



F STATISTIC

The F statistic is used to determine if the slope of a plotted line is significantly different from 0.

$$F = [r^2(N-2)]/[1-r^2]$$

where:

F and r are the same as in the correlation coefficient.

The F statistic in this instance has 1 degree of freedom for the numerator and N-2 degrees of freedom in the denominator.

"t" TEST

$$t = (\bar{X}_1 - \bar{X}_2) / \sqrt{s^2_{X_1 - X_2}}$$

where:

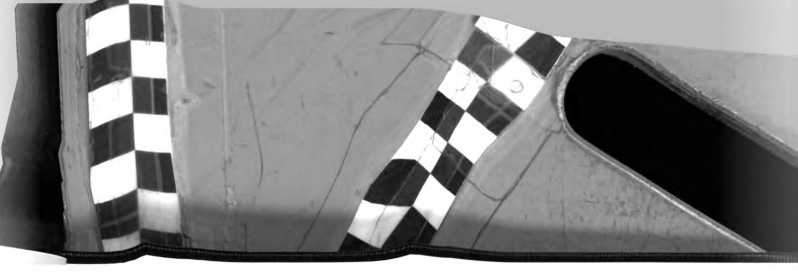
N_1 = number of observations in X_1

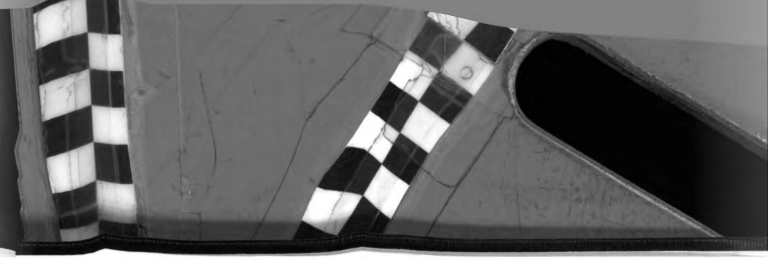
N_2 = number of observations in X_2

$s^2_{\bar{X}_1 - \bar{X}_2}$ = variance of difference between the means.

$$s^2_{X_1 - X_2} = \frac{\Sigma X_1^2 - (X_1)^2/N_1 + \Sigma X_2^2 - (\Sigma X_2)^2/N_2}{N_1 + N_2 - 2} \cdot (N_1 + N_2)/N_1 N_2$$

Degrees of freedom = $N_1 + N_2 - 2$.





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