THE IN VITRO UPTAKE OF HEXAVALENT CHROMIUM BY ERYTHROCYTES, LIVER AND KIDNEY TISSUE OF THE TURTLE CHRYSEMYS PICTA

Thesis for the Degree of Ph. D.

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Jack Russell Hoffert

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This is to certify that the

thesis entitled

The In Vitro Uptake
of Hexavalent Chromium by Erythrocytes,
Liver and Kidney Tissue
of the Turtle Chrysemys Picta

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By

Jack Russell Hoffert

AN ABSTRACT OF A THESIS

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ABSTRACT

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Blood samples from the turtle Chrysemys picta were taken by direct cannulation of the left aortic arch. The packed heparinized cells were washed three times with isotonic Ringer-phosphate-glucose buffer (pH 7.4). The uptake of chromium by cells was rapid initially, probably related to the amount of surface binding; this was followed by uptake at an exponential rate but without reaching an asymptotic value by 4 hours. The exponential uptake is related to the rate of diffusion through the cell membrane and to binding (either enzymatic or physical) to the cytoplasmic proteins. Cell homogenates showed a linear uptake over a 4 hour incubation period. The binding of chromium to the cell surface or to the cytoplasmic proteins does not appear to be dependent upon metabolic activity since experiments using NaF, NaCN, Na azide and $NaAsO_2$ (10^{-3}M) had no effect on uptake of hexavalent chromium by the erythrocytes or liver and kidney slices. Nucleated erythrocytes when placed in 20 p.p.m. chromium for 1/2 hour at 20°C. showed a significant

decrease in their electrophoretic mobility. This indicates a reduction in the zeta potential due to binding of chromium at the cell surface. Fractionation and isolation of the erythrocyte components has shown that chromium binds to the haemoglobin as well as to the cell ghosts and nuclei.

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INTRODUCTION

It is generally believed that pure metallic chromium is biologically inert and exerts no harmful effects on living tissues. Nearly all of the data available indicate that the chromium III (trivalent) compounds probably do not produce any serious damage to body tissues. In contrast, chromium VI (hexavalent) compounds exert an extremely irritating, corrosive and, under some circumstances, toxic action on body tissues. The harmful effects may be due to the oxidizing ability of the compound or general properties similar to those associated with heavy metals.

The factors involved in chromium accumulation and excretion have been described as has work on the transport and labeling of non-nucleated erythrocytes with hexavalent chromium-51. The application of the tagged cell technique in clinical practice is well documented. Measurements of total cell volume, blood volumes, plasma volumes and red cell life spans have all been accomplished using different forms of chromium-51.

Basic differences exist between the nucleated and non-nucleated erythrocytes. The nucleus is indirectly indicated as the controller of the oxidative processes of the cell.

Upon losing the nucleus in the normoblastic phase of hematopoiesis the non-nucleated red blood cell also loses its mitochondria. Approximately 80-85% of the dry weight of nucleated and 95% of the dry weight of the non-nucleated red blood cells is made up of metabolically inert haemoglobin (Ponder, 1948). For this reason it would be predicated that the red cell might have a different total metabolic scheme than that reported for somatic cells. In general, the Q_{O2} of the erythrocyte is about 20 times lower than that of somatic tissue. The avian nucleated red blood cell has a Q_{O2} some 10 times that reported for the non-nucleated red blood cell.

In general, the permeability of red cells to anions like Cl^- is extremely high in comparison with general ionic permeability. Most of the energy for active ion transport by non-nucleated red cells is derived from anaerobic glycolysis, whereas energy for active transport in nucleated erythrocytes is derived mainly from aerobic metabolism involving the tricarboxylic acid cycle.

Because of the many differences in the permeability and metabolic activity of the two types of cells the possibility of tagging nucleated erythrocytes with chromium was investigated and the results compared with data for

non-nucleated red cells. The establishment of a suitable procedure for tagging nucleated RBC's will permit the investigation of many physiological parameters of the blood and blood vascular systems of lower vertebrates. The *in vitro* binding of chromium to the nucleated red cell of the painted turtle was compared with the chromium-51 distribution in the liver and kidney following *in vitro* incubation. These studies have given some insight into the possible routes of excretion as well as areas of concentration of chromium in body tissues.

LITERATURE REVIEW

Inorganic Chemistry of Chromium

Chromium has been known only since 1766 when Legmann first isolated "crocoite," the natural lead chromate ($PbCrO_4$), from the Plomb range de Siberie. L. N. Vanquelin in 1797 proposed that this ore from Siberia was in actuality a new element and he named it "chroma," derived from the Greek word for color. The metal itself has no color, but most of its salts are colored. Chromium, atomic number 24, is a transition metal of the first long period, group VI of the periodic table and is characterized by its ability to function both as a nonmetal or metal. The chemical atomic weight is 52.01. Four isotopes of chromium occur naturally and have the following mass numbers and relative occurrence in nature: 50 (4.31%), 52 (83.76%), 53 (9.55%) and 54 (2.38%). The atomic radius of the chromium atom is 1.24 to 1.35 Å.

Chromium has many points of similarity with vanadium, molybdenum, manganese and tungsten and shows discontinuous changes in several properties which occur at temperatures of 37°C., Young's modulus, internal friction, restivity and coefficient of thermal expansion (Sully, 1954).

Radioactive chromium, Cr^{51}, is usually dispensed for routine clinical use as sodium chromate, $Na_2Cr^{51}O_4$. Chromium-51 has a half life of 27.8 days, disintegrating primarily by K capture with transmutation to vanadium with an 8% emission of 0.32 mev gamma. There is no alpha or beta emission (Beierwaltes, Johnson and Solari, 1957).

Chromium complexes and multivalent compounds complicate both the qualitative and quantitative analytical procedures. The oxidation of chromium III to chromium VI can be effected by relatively weak oxidants, such as the ferric ion, iodine and oxygen. Anhydrous chromium trichloride is insoluble in water but has a flaky structure with high surface area on which contaminating O_2 and H_2O vapor are readily absorbed. $CrCl_3$ is readily oxidized in air by:

$$4CrCl_3 + O_2 \longrightarrow 2Cr_2O_3 + 6Cl_2$$

Marshall (1960) showed that absorption of molecular oxygen by chromous salts provides one of the most efficient methods of producing anaerobic conditions. In the hexavalent state the salts of chromium may be considered as derived from the hypothetical chromic acid H_2CrO_4.

Hopkins (1942) has presented a basic outline of the major chemical forms of this element as follows:

Valence	Class	Reaction	Representative Salt	Ionic Nature
2	Chromous	Basic	$CrCl_2$	Cation
3	Chromic	Weakly basic	$CrCl_3$	Cation
3	Chromite	Weakly acidic	$NaCrO_2$	Anion
6	Chromate	Acidic	Na_2CrO_4	Anion
6	Dichromate	Acidic	Na_2CrO_7	Anion

Udy (1956) showed that H_2CrO_4 is an acid and dissociates in two steps as follows

$$H_2CrO_4 \longrightarrow H^+ + HCrO_4^- \quad (1)$$

$$HCrO_4^- \rightleftharpoons H^+ + CrO_4^= \quad (2)$$

In dilute solutions reaction #1 is practically complete to the right. Reaction #2 is reversible and the ionization constant is 6×10^{-7}. Solid dichromate when dissolved in H_2O gives:

$$Cr_2O_7^= + H_2O \longrightarrow 2H_2CrO_4$$

Usable methods of volumetric determination depend almost entirely upon the reduction of chromium valence from VI to III and reverse oxidation from a valence of III to VI as follows:

$$HCrO_4^- + 7H^+ + 3e \longrightarrow Cr^{+++} + 4\ H_2O$$

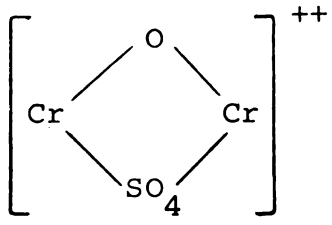
$$Cr_2O_4^= + 14H^+ + 3e \longrightarrow 2Cr^{+++} + 7\ H_2O$$

According to Udy (1956) the usual method of oxidation in a basic medium is to use H_2O_2 or permanganate. In an acid solution the oxidation is by persulfate or perchloric acid. Reduction is accomplished by ferrous sulfate or potassium iodide.

Of the many forms of chromium encountered in the above reactions, only the trivalent and hexavalent states show enough stability to exist in biological systems. Trivalent chromium is found in solutions at pH values below 7, while the hexavalent form is found in pure solution in much more alkaline media. Trivalent chromium, depending on the pH, exists as pure solutions, complex colloidal suspensions or flocculent precipitates.

Metal-Protein Compounds

All proteins are capable of binding metal ions. The stability and extent of the binding of the metal-protein compounds is varied greatly by the nature of the metal ion, the nature of the protein and the net charge on the protein. According to Gustavson (1958), the transition group elements generally possess the greatest affinity for proteins and therefore form the most stable compounds. Positively charged polynuclear chromium complexes such as



are held very firmly to protein and little if any dissociation is detectable. The chromium complexes are potent cross linking agents and in this way lead to formation of stabilized protein structures. The binding of basic chromium sulphate by collagen is important in the tanning of leather. Chromed hide resists the action of boiling water and proteolytic enzymes. Gustavson (1956) has assembled evidence which points to the carboxylate groups of collagen as the principal ligand groups to coordinate with Cr III. The anionic chromium complexes are attached to the ϵ-amino groups of the lysine residues and the arginine residues but the complexes formed are not as stable as the normal carbohydrate type. Cationic chromium fixation to collagen causes its isoelectric point to shift towards higher pH values (pH 5 to pH 7.5) due to partial inactivation of carboxyl ions of the zwitter ionic protein. Non-ionic chrome fixation doesn't affect the isoelectric point of the protein. Gustavson (1958) blocked 90% of the carboxylate groups in the collagen by esterification with methanol and 0.1N HCl.

The esterification caused a decrease in the degree of Cr III binding to protein.

Gurd and Wilcox (1956) point out that after the primary binding of Cr III, presumably further coordination occurs on aging by which other protein groups are brought into linkage with the metal, and perhaps further hydrolysis of the metal complexes will lead to larger aggregates of metal atoms. The protein chains are tied together in highly stable chelate structures. The chelate structures which are formed by Cr III have donor atoms of oxygen. Such complexes exchange their donor groups with great reluctance. The bonds to the metal are considered to be largely covalent.

Metallic cations are found intimately associated with proteins throughout biological systems. Most metallic cations which are found in living cells are found in bound form. Gurd and Wilcox (1956) point out that the trivalent metal ions, Al^{+++}, V^{+++}, Cr^{+++} and Fe^{+++} all bind tightly to oxygen in preference to nitrogen and have a strong tendency to form polynuclear complexes at low pH values. The side-chain groups such as carboxyl, imidazole, or sulfhydryl are usually of much more importance in binding the metal ions than the terminal amino and carboxyl groups. The positive and negative charges normally found on

proteins form a net oriented field which will hinder or accelerate the approach of the metal ion.

Naismith (1958) believes that anionic chromium complex (potassium dioxalatohydroxoaquochromiate) forms cross-linkages between the electronegative carboxyl side chains, with coordination links being involved with collagen and the chromium complex. This has not been proven as yet because of the denaturation of the protein during attempts to identify its reactive groups.

Gustavson (1956) looks at the tanning process as an electroneutralization of the charges of the carboxyl groups into the chromium complex, which results in the formation of coordinate-covalent bonds of great stability between the carboxyl and the chromium atoms of the complex.

In an excellent review of interaction of Cr III and Cr VI with proteins Grogan and Oppenheimer (1955) state that much of the "protein binding" as determined by dialysis techniques may represent the formation of chromium olate complexes that are practically nondiffusible and approach the colloidal state. This is particularly true of the chromic chloride. The $CrCl_3$ when injected into rats and rabbits was distributed in a pattern similar to that of small sized, slow clearing colloids.

The Interaction of Chromium Compounds with Biological Fluids

Hexavalent chromium remains soluble, whereas trivalent chromium is relatively insoluble at normal body pH. Michael (1939) reported that anionic hexavalent chromium precipitated plasma proteins on the acid side of their isoelectric point and cationic trivalent chromium on the alkaline side. This precipitation is reversible and proteins are not changed. The denaturation of proteins by chromium salts on the acid side of their isoelectric point has a marked temperature coefficient, proceeding rapidly at body temperature and therefore may have physiological significance. Quantitative estimations of the protein-chromium precipitation showed that the protein combines in a definite stoichiometric proportion. Cr VI is reported to be reduced to Cr III by contact with beef plasma albumin at pH 7 (Michael, 1939).

In spite of the fact that traces of chromium are almost universally present in plants and animals there is no evidence at present to indicate what role, if any, chromium plays in either plant or animal physiology.

Gordon and Thompson (1936) studied the connection between tanning action and opsonization by using washed guinea pig leucocytes and staphylococci in saline. $CrCl_3$, chrome

alum and potassium bichromate all acted as opsonins in inducing phagocytosis. The tanning reactions described above will occur on the surface of the organisms and bring about changes which allow for phayocytosis by the leucocytes (Gordon and Thompson, 1937).

Studies by Horecker, Statz and Hogness (1939) showed that aluminum ions in very small concentrations accelerated the aerobic oxidation of succinate (succinic dehydrogenase cytochrome system). Trivalent chromium can be substituted for aluminum in accelerating the aerobic oxidation. The action of aluminum and chromium can not be satisfactorily interpreted.

Titanium with an atomic number of 22 and vanadium with an atomic number of 23 have been shown to have varying actions on certain biological oxidations. Bernheim and Bernheim (1939) investigated the effects of elements with numbers from 24 to 28 by addition of their salts to a suspension of rat liver. With the exception of manganese they had very little effect on O_2 uptake. Mn (M/20,000) caused definite inhibition of O_2 uptake of rat liver but not of kidney and brain. In a study by Meldrum and Roughton (1934) the properties of carbonic anhydrase were not affected by $CrCl_3$.

The activation of phosphoglucomutase by metal ions was reported by Stickland (1949). The greatest activity of phosphoglucomutase was found only in the simultaneous presence of three active ingredients: hexosediphosphate, Mg^{++}, and a second metal (Al^{+++}, Cr^{+++}, Pb^{+++}, Fe^{+++} or Ce^{+++}). Consideration of the affinities then suggested that if the two-metal activation has any physiological importance then Cr^{+++} and Mg^{+++} are the metals concerned.

Perlman (1945) added $K_2Cr_2O_7$ to Aerobacter aerogenes and found that levels of 10 μg./l. resulted in an increased synthesis of pantothenic acid. Chromium alone increased the synthesis of biotin.

Herrman and Speck (1954) found that the chromate-treated homogenates showed decreased extractibility of nucleic acids. The effect of chromium is specific insofar as treatment of tissues with other acids such as trichloracetic acid, metaphosphoric acid, picric acid or Bouin's solution doesn't decrease extractibility of the nucleic acids. It seems probable that a small portion of chromate is reduced as is evidenced by the greenish color of the residue and in this state it forms complexes with nucleic acids or nucleoproteins, which are more resistant to the decomposition of trichloracetic acids. Both RNA and DNA

at pH 7 are precipitated immediately by $CrCl_3$. Two moles of chromium and 1 mole of acid react. Cr VI compounds at pH 7 had no precipitating effect.

Wacker and Vallee (1959) recently isolated from beef liver a ribonucleoprotein containing 0.1% chromium. A 20,000X increase in chromium concentration occurred in the ribonucleoprotein fraction as compared to the whole liver homogenate. The chromium may play some part in the function of the nucleic acids either in maintenance of structure or specific sites of binding between proteins and nucleic acids. The high chromium content of liver was first noted in connection with glutamic dehydrogenase fraction from beef liver. Investigation into a wide variety of phyla and species (Wacker and Vallee, 1959) showed that all of them contain large amounts of some metal ions in the nucleic acids.

Curran and Azarnoff (1961) presented data that showed that manganese markedly increases the incorporation of radioactivity (C^{14} labeled acetate) into cholesterol and slightly into fatty acids. Chromium greatly stimulates incorporation into both cholesterol and fatty acids.

Schwarz and Mertz (1961) showed that chromium III is necessary for the maintenance of a normal glucose tolerance,

which appears to indicate that it may be a bioelement essential for mammalian organisms. As such, the impairment of glucose tolerance due to Cr III deficiency does not constitute a very serious danger to animals. However, the phenomenon resembles the disturbances of tolerance in diabetes and other diseases. Mertz, Roginski and Schwarz (1960), using fat bodies of rats, showed that at low levels of insulin the supplementation of chromium greatly increased the uptake of glucose.

Toxicology of Chromium

Hexavalent chromium is a very strong oxidizing agent and for this reason it is difficult to believe that it could exist in the living cell as such. In the presence of organic matter, whether in the living cell, the plasma or alimentary canal reduction of Cr VI is inevitable. It is possible that hexavalent chromium might upset the fine balance of the oxidation-reduction enzymes and hydrogen carriers, but such an effect needs investigation (Water Research News, 1960).

The most striking chronic and acute toxic effects have been noted largely with the Cr VI compounds and have been attributed to the high solubility, diffusibility under physiological conditions, escharotic, and oxidizing

properties (Grogan and Oppenheimer, 1955). The apparent innocuousness of Cr III compounds is probably due to their insolubility in biological fluids.

The lack of activity of chromium in animal metabolism has become an asset to workers studying such varied subjects as feed utilization, blood volume, and tissue half life. Chromium compounds may well prove to be valuable tools in the study of animal and plant physiology.

Udy (1956) reports only a slight stimulatory response in plants with low levels of chromium and therefore the lack of evidence for nutritional requirement of this element would suggest that there is little justification for incorporation of special chromium containing materials in fertilizers.

The latest standard for drinking water (U. S. Public Health Service, 1946) specifies a maximum of 0.05 p.p.m. by weight of hexavalent chromium. No standards for trivalent chromium compounds in food or water have been published. Partington (1950) cites many cases of suicidal poisonings with oral doses of potassium dichromate. Acute gastro-enteritis, blood and albumin in the urine, vomiting, and gastric hemorrhage all occur within 10 minutes after the poisoning.

The retention of dichromate and H_2SO_4 by glassware, as studied by Henry and Smith (1946) revealed as much as 0.01 μg./ml. chromium after 10 rinsings in distilled water. A 95% inhibition of enzyme urease is reported for a 1-10 μg./ml. chromium solution. In synthetic media 10 μg./ml. chromium is very toxic to the growth of Staphylococcus aureus. A ten-fold increase in the chromium concentration is needed to obtain equivalent inhibition when the organism is cultured in a nutrient broth. This is undoubtedly due to binding of heavy metal ions by the nutrient broth. Because of the extreme difficulty of ridding glassware of dichromate after cleaning in cleaning solution, Henry and Smith (1946) deemed it advisable to use another method for cleaning all glassware that was to be used in experiments with living cells or enzyme isolations.

Richards (1936) found that only 50% of Amblystoma hatched normal larvae when the bichromate concentration was 0.001 μg./ml. A diatom Raphidium was injured by 1.0 μg./ml. of bichromate. 0.1 μg./ml. was sufficiently toxic to invalidate experiments made with yeast cells Oscillatoria.

Cann, Webster and Johnson (1932) found milk contained up to 0.5 p.p.m. chromium from storage tanks. They found that chromium in the food even to the extent of 100 p.p.m.

had no deleterious effects on the general health and reproduction capacity of the rat. Orally administered chromium is not retained by the rat but is promptly and completely eliminated by the gastro-intestinal tract.

Akatsuka and Fairhall (1934) reported chromic salts such as chromic carbonate and chromic phosphate are not poisonous to cats whether introduced through the digestive tract or the respiratory system. The derivatives of chromic acid (lead chromate) certainly exhibit toxic properties.

Samitz and Pomerantz (1958) showed that chromates can be absorbed by the skin of guinea pigs; and no evidence that nickel salts were absorbed. Sodium lauryl sulfate produced local irritation on the skin. MacKenzie et al. (1958) administered Cr VI and Cr III in the drinking water of rats. The Cr VI was absorbed about 9X more than the Cr III. Drinking water containing 25 p.p.m. chromium produced no toxic symptoms in one year. No differences were found between the controls as to water intake, food consumption, weight gain or blood picture.

Oral ingestion of Cr VI compounds may lead to intense irritation of the gastro-intestinal tract resulting in violent epigastric pain, nausea, vomiting, severe diarrhea, and hemorrhages. Severe poisoning can be produced in

animals by intravenous and subcutaneous injections of these compounds.

Gross and Heller (1946) found that when potassium and zinc chromate were added to the feed or drinking water of animals, the chromium was excreted in the feces in an insoluble complex form, possibly bound with proteins. Potassium dichromate was reported to be more readily absorbed than $CrCl_3$ when injected subcutaneously in animals.

Kidney lesions in man and animals have been reported more frequently than any other type of systemic damage. The nephritis involves chiefly the tubules, with destruction of the epithelium. Singh and Singh (1946) experimented with $CrCl_3$ *in vitro* and indicated that the toxic doses resulted in tonic contraction of unstriated muscle, while lowered concentration led to inhibition.

MacNider (1924) stated that the chromate induced renal injury is primary tubular and affects particularly the epithelium of the convoluted tubules. Late in such a nephritis the glomeruli participate in the injury. A rapid reduction in urine formation occurred along with the inability of the kidney to eliminate phenolsulphonphthalein, and a retention of chlorides, blood urea, non-protein nitrogen and creatinine. The chromate nephritis

is associated with the development of an acid intoxication which is the result of the retention phenomenon.

Hunter and Raberts (1933) reported the following order as experimental nephrotoxins: uranium nitrate, mercuric chloride and potassium bichromate. Because of the local irritating and corrosive properties and violent gastro-intestinal manifestations, bichromate is not too good. They believe that chromium is a pure tubular poison, of the first and middle division of the proximal convoluted tubules in the monkey. Ophuls (1911) reported sediment in the uriniferous tubules, with hyperemic kidney, albuminuria and necrosis of epithelium following subcutaneous injections of potassium bichromate in the guinea pig. He also found that the rabbit was more susceptible than the guinea pig to experimental nephritis produced by subcutaneous injections of chromates.

Palmieri and Mele (1960-1961) reported that injections of 10-20 mg./kg. doses of $Cr_2(SO_4)_3$ into the dog produces conspicuous lowering of the blood pressure. The dose of 100 mg. caused a rapid and marked hypotension which, following convulsions, terminated in the death of the animal. In all the animals there was a diminution of the

amplitude of cardiac contraction. The reduction of amplitude is probably from an action directed on the myocardial fibers similar to action of mercury, cobalt and vanadium.

The most common effects of chromium compounds on industrial workers are those which result from direct contact of the skin with the chromates or with chromic acid. The skin reactions which are of two different types--chrome ulcers and chrome dermatitis--have been described frequently in the medical literature. No cases of cancer of the skin have been reported as a result of exposure to chromium or any of its compounds. Common effects which result from the inhalation of chromate dust or chromic acid mist are ulceration and perforation of the nasal septum. Bronchogenic carcinoma is believed by most authorities to be attributed to the carcinogenic action of hexavalent chromium. Some believe the carcenogenic property of the chromate dust is due to its irritating effect. Others believe that the chromates are not carcinogenic per se, but that they act on some of the organic substances in the body, leading to the production of a carcinogenic agent. Amounts of chromium needed for cancer production are not known and negative results on attempts to produce lung cancer in animals with chromium have been reported many times.

Hueper (1958) states that the carcinogenic effect of chromium might depend upon an adequate and prolonged release of biologically more active hexavalent chromium from pulmonary deposits of inhaled biologically rather inert trivalent chromium compounds. The total available epidemiologic, chemical and experimental evidence, nevertheless, favors the concept that chromium represents the carcinogenic agent. It has been demonstrated by Baetjer, Damron and Budacz (1959) that men employed in the chromate chemical manufacturing industry have higher rates of bronchogenic carcinoma than those in a comparable control group.

Since the mechanism of cancerization is thought to involve sublethal genetic transformation or mutations, the site of such changes should be in the cell nucleus, producing direct action of these agents on the genetic nucleoprotein materials of the cell. Grogan (1958) showed that repeated intravenous administration of Cr VI resulted in penetration of chromium into the platelets and leukocytes as well as the erythrocytes. Repeated I.V. doses also resulted in Cr VI appearing in the nucleus of erythrocytes.

Fairhall (1957) stated that the death rate due to cancer of the respiratory system among exposed employees

was 21.8%. This is 16X the expected rate.

Distribution and Retention of Chromium in the Organism

Baetjer, Damron and Budacz (1959) in an extensive paper described the distribution and retention of chromium in men and animals. I.P. injections of Cr VI in the guinea pig results in more chromium being excreted and more appeared in the blood than with the intratracheal route. With the trivalent form the higher blood level was due to a greater plasma value. No chromium was found in the bones one to three days after the intratracheal injection of chromic chloride or up to 90 days after injection with sodium chromate. The lungs retained trivalent chromium not only to a greater extent than the hexavalent form but also for a much longer period. When water-soluble chromates were injected intratracheally, only about 15% of the injected dose was recovered from the lungs. Twenty per cent was found in the blood and another 5% in the liver, kidneys and spleen altogether. Slices of human lung bind Cr VI to a lesser extent than lung tissue of guinea pigs. Both lung and liver show the same binding for $CrCl_3$ with no species differences. Lungs do not contain any components which

bind chromium to a greater extent than other tissues and lung of man does not bind Cr III differently than lungs of other animals tested.

Wennesland et al. (1957a) reported a slight accumulation of Cr^{51} in the spleen and lung tissues of the rabbit. Wennesland et al. (1957b) found no evidence of Cr^{51} accumulation in spleens of dogs killed with sodium pentobarbital. The longer the storage time of the tagged RBC the greater will be the excess of Cr^{51} found in the lung, liver and spleen, particularly if the cells have been stored in saline. This is believed to be a function of the reticuloendothelial system. Sequestration of cells in the lung may be analogous to trapping in the organ of leukocytes or macromolecules. The pulmonary circulation was found capable of removing and delivering vast quantities of leukocytes from the blood, as well as storing and destroying them. Bierman et al. (1956) also reported that the cells may go through the pulmonary epithelium into the alveoli and be lost via sputum or G.I. tract if the sputum is swallowed. They may be destroyed here (lungs) and form granulocytes "plate-like bodies." The effects of temperature on the uptake of colloidal $CrPO_4$ by isolated rat liver (Brauer, Leong and Halloway, 1957) has a Q_{10} for the

overall reaction rate equal to 1.92 between 20°C. and 38°C. The uptake can be represented as an irreversible reaction between the colloid micelles and an effectively unlimited fixed active phase, presumably the surface of the Kupffer cells.

Knoll and Fromm (1960) found that in rainbow trout exposed to 2.5 mg. Cr/l. in tap water accumulation of chromium in concentrations exceeding the environment in the spleen, posterior gut, pyloric caece, stomach, and kidney. All these organs were believed to be correlated with excretion. The major route of entry of hexavalent chromium into the trout is probably the gills. They stated that the nucleated fish erythrocytes behave differently than the mammalian red blood cells in that they showed little uptake of Cr VI.

Ingrand (1961) concluded that the distribution of Cr^{51} in the mouse after I.V. injection of radioactive sodium chromate obtained by autoradiography showed a localization in the liver, in the spleen and in the skeleton at the level of the zones of osseus repair.

Ehrlich mouse ascites carcinoma cells were labeled with radiochromate and the valence state of the chromium and the intracellular distribution studied by Rajam and Jackson

(1958). Seventy-one per cent of the total intracellular Cr^{51} was associated with the soluble cytoplasmic fraction; 52% was associated with the ethanol precipitated protein fraction. Hexavalent chromium appeared to be intracellularly reduced to the trivalent state. The cell membrane of the tumor cell is impermeable to intracellular Cr III ions. The stability of this intracellular label is not dependent on protein binding alone.

Gray and Sterling (1950a, 1950b), in their classical papers on the methodology of chromium tagging of red cells and plasma proteins of the human red cells, stated that: (1) Anionic hexavalent form of the isotope labeled red cells while the cationic trivalent form bound firmly to the plasma proteins. Ninety per cent uptake occurred by human cell in two hours when done <u>in vitro</u> in saline. (2) The site of tagging of the erythrocytes with Cr VI appears to be on the globin portion of the haemoglobin. (3) Haemoglobin demonstrated a significantly greater capacity for Cr III than for Cr VI. (4) It was suggested that the anionic hexavalent chromium diffuses through the red cell membrane and is bound by the haemoglobin. (5) There is probably a reduction of Cr VI to Cr III before this binding takes place.

The preceding discussion of the pharmacology of chromium-51 makes it clear that chromium forms a stable and nontoxic label for red blood cells if anionic hexavalent chromium is used. Cationic trivalent chromium is a good label for plasma proteins. The rationale used for determining plasma volume, hematocrit, total blood volume or total red blood cell mass is presented by Beirwalters, Johnson and Solari (1957). The Council of Pharmacy and Chemistry of the A.M.A. (1955) stated that sodium radio chromate (Cr^{51}) has not been shown to produce any significantly deleterious effects on normal erythrocytes either radioactive or metallic. The maximum dose at any one time was set at 390 μc. per person.

Ebaugh et al. (1953) reported a 90% uptake of Cr VI by human cells at 39°C. in 5 minutes using an ACD incubation solution. ACD solution was given as trisodium citrate = 1.65 gm., citric acid = 0.60 gm., dextrose = 1.84 gm., diluted to 75 ml. A 50% uptake occurred in five minutes at 26°C. compared to only a 10% uptake at 1.8°C. in five minutes with human RBC. They found no change in concentration of chromium salt ranging from 0.25 to 9.5 μgm. salt/ml. whole blood. At lower pHs higher uptake of Cr^{51} was reported. Mollison and Veall (1955) pointed out

the possibility that some Cr^{51} might be bound to the small amount of plasma protein adhering to the surface of the red cell. The uptake is better with packed cells in ACD than with whole blood mixed with ACD.

Von Ehrenstein and Zacharias (1958) reported that heparin decreases Cr^{51} uptake and that for human cells ACD was the best medium. Ca^{++} ions diminish the uptake of chromate. Ca^{++} ions also diminish the binding of Cr by haemoglobin *in vitro*. It is believed that the Ca^{++} is affecting other structures in addition to the cell membrane.

Direct measurement of the rate of elution of chromium from red cells has been done by Smith and Krivit (1960). The average elution per day *in vitro* of normal mature red cells was 0.2%. This low rate of elution was said to be due to the use of a phosphate-glucose-buffer solution in place of the normal saline solution. The addition of glucose decreased the rate of autochemolysis.

Davson and Reiner (1942) stated that the permeability of the RBC to anions (Cl^{-}) is extremely rapid in comparison to general ionic permeability. Possibly the specialization of the membrane has occurred to permit the rapid diffusion of the negative ions. The predominantly lipid membrane represents an effective and selective barrier to

diffusion so that the cell may maintain an internal environment different from its surrounding medium.

Excretion of intravenously injected chromium in the dog is by way of the urine (Collins, 1958). Excretion in bile and feces is negligible. Glomerular filtration and tubular reabsorption are the two mechanisms involved in the renal handling of unbound chromium. Collins also found evidence for tubular excretion of dialyzable chromium, but stated this was probably of minor importance. In vivo reduction occurs after intravenous injection of Cr VI because no Cr VI is found in the urine. Results of this work indicate that chromium is excreted at least in part in organic combinations.

Nucleated Erythrocytes

Lyman (1945) showed that there was an anti-hemolytic action of calcium in the blood of the snapping turtle and that the blood can not be oxalated without inducing complete hemolysis. Dilution with NaCl also induces hemolysis. In other species of turtles Ca^{++} ions are of less importance as an antihemolytic agent. The calcium ion and other ions can alter the permeability of the cell membranes. For example, chromium acts by precipitating the protein of the cell wall

and the action is not comparable to that of calcium. Hamkid and Ferguson (1940) found hemolytic action of fluorides on certain dogfish nucleated erythrocytes. Oxalates and arsenates produced similar effects. All of the above chemicals removed free magnesium and calcium which in turn altered the permeability of the RBC. Maizels (1956) reported that the African tortoise has erythrocytes which transport Na^+ and K^+ and the energy for this transport is derived from oxidative metabolism. They cannot maintain their relative impermeability to Na^+ and K^+, unless calcium is present in the external medium. Metabolism and cation transport in human erythrocytes are based on glycolysis.

MATERIALS AND METHODS

Experimental Animal

The common painted turtle (Chrysemys picta) was selected as an experimental animal for the following reasons: (1) their morphology, gross anatomy and physiology have been explored, (2) sexual differences are relatively simple, (3) they are readily available during any time of the year, and (4) they are easily kept under laboratory conditions.

The animals were obtained from a commercial supply house and stored at 12°C. in stainless steel tanks containing water to a depth of 5 inches. One week prior to use as experimental animals they were kept at 26° C. in 26 gallon glass aquaria, each of which contained a wire platform just above the water level. Fresh tap water was placed in the tanks once every week just after feeding. Chrysemys picta were observed to feed only under water. The painted turtle is reported as omniverous in its natural environment but under the experimental condition they would eat only meat scraps. Leafy green vegetables were never consumed.

The turtles were all at least 4-5 inches long and therefore considered to be mature. Only those turtles that were healthy and showed no signs of pathology other than that noted below were used in this study.

The general condition of the turtles was not affected by a slight infestation of leeches which were found clinging to the shell and skin. Like many of the lower vertebrates, turtles are parasitized by all of the major classes of protozoans but the relationships between these parasites and definite turtle diseases are not known. Many times during histological examination, examples of vascular parasitism were observed (see figure H), however, there was no indication of any tissue or organ pathology. Of interest was the high degree of pigmentation found in the liver. At first the pigment appeared to be hemosiderin, but by histochemical tests using Turnbull's Blue Method for hemosiderin (Gridley, 1960), negative results were obtained (see figure C). The technique using the bleaching procedure by Gridley (1960) revealed the pigment to be melanin (see figure D). In a general review of the literature it was found that melanin pigmentation of organs occurs very frequently in lower animals. The liver appeared, on gross examination, to be light red-brown, often pale brown. Histochemical tests revealed slight infiltration of adipose tissue and the presence of glycogen. In many cases the urinary bladder of the turtle was observed to contain considerable sediments. This has been reported to be without symptomatology (Kaplan, 1957).

One bacterial infection was encountered but was felt not to be of serious consequence. The infection first showed up as a white-brown film or paste around the head and the eyes. This, in advanced stages, spread over the neck and, in very advanced cases, was observed on the front limbs. In severe cases, before treatment, complete closure of the eyes resulted from build-up of pus and exudate.

A routine bacteriological examination revealed that the animals were infected with Aeromonas hydrophila. This organism was found resistant to Bacitracin, Erythromycin and Penicillin and susceptible to Aureomycin, Neomycin, Terramycin tetracycline etc. The infection was treated by adding, to the water in the holding tanks, injectable Liquamycin manufactured by Pfizer Laboratories. Liquamycin contains 10 mg./ml. oxytetracycline, of which 50 ml. were added to 150 liters of water once a week until the infection was cleared up.

Breed, Murray and Smith (1957) stated that A. hydrophila was a 3 μ motile red-shaped pathogenic bacterium found in frogs, salamanders, fish, mice, guinea pigs, rabbits and snakes. It causes hemorrhagic septicemia. The source probably was the holding tanks, kept in the same room, used for the storage of frogs which were infected with red leg.

Optimum temperature for this organism was reported at 10° C.-15°C. which included the holding temperature. Periodic treatment in sunlight (ultraviolet) light has been reported by Kaplan (1957) to aid in combating this type of bacterial infection.

A few animals were found with tumors located on the neck. The pathology report from the Department of Pathology stated the tumor was a chronic, multiple abscess (figure E). Predominate tissue changes revealed that this lesion is characteristic of an abscess of long duration. A well developed connective tissue capsule around the abscess was found and some bacteria were also present.

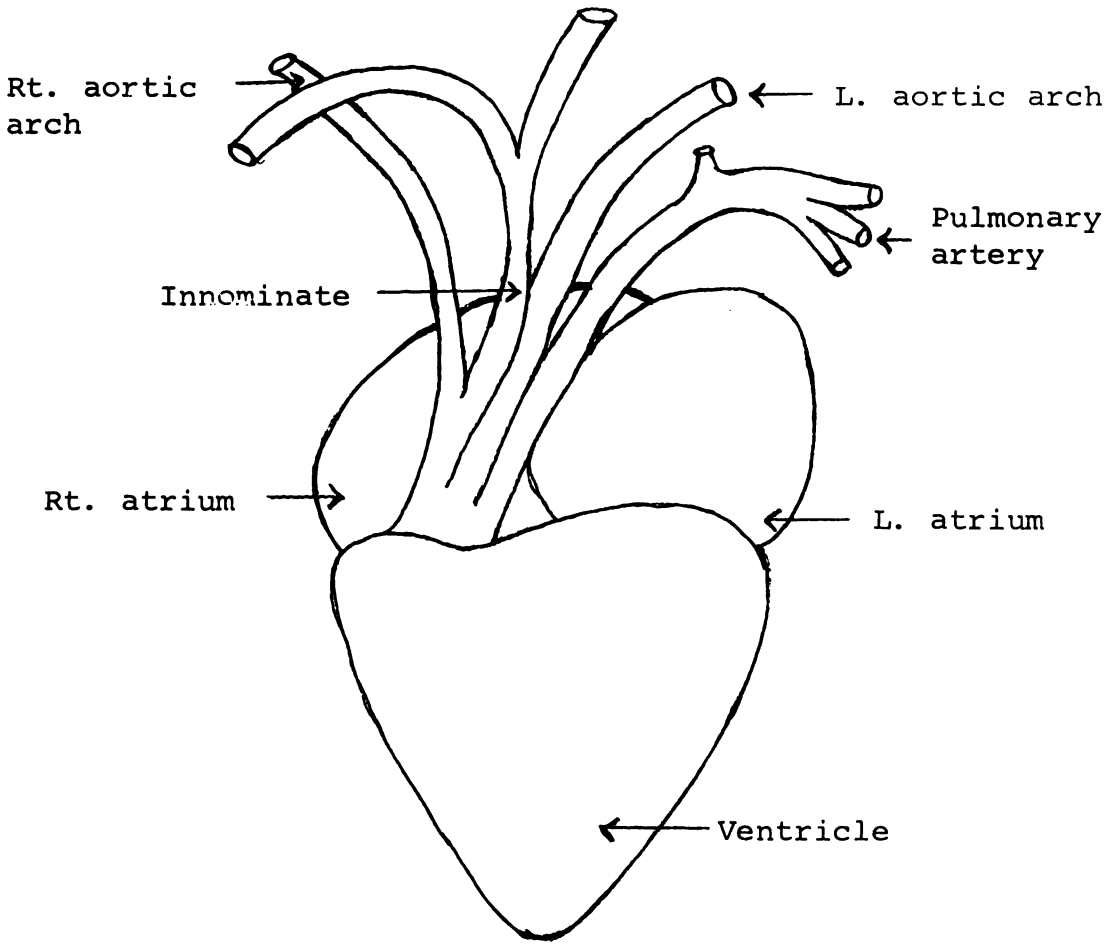
Figures F-J show additional histological sections from the turtle.

Tissue Preparation

The animals were killed with a heavy blow to the head, the plastron removed, and blood withdrawn by direct canulation of the left aortic arch (see figure 1). After removal of the pericardium a "bull-dog" clamp was placed as far distal as possible but still able to clamp the right aortic arch, innominate, left aortic arch and the pulmonary arteries, thus diverting the blood from the somatic circulation.

Figure 1

Ventral View of the Turtle Heart


Rt. aortic arch
L. aortic arch
Pulmonary artery
Innominate
Rt. atrium
L. atrium
Ventricle

A 22 gauge needle attached to a 6 inch piece of tygon tubing served as a cannula and blood was allowed to flow into a graduated centrifuge tube containing heparin as an anticoagulant.

An average sample of 7 ml. whole blood was centrifuged for 10 minutes at 2000 r.p.m. and the straw-yellow plasma removed using a micro-suction apparatus. The standard tests for bilirubin (Method of Malloy and Evelyn, from Hawk, Oser and Summerson, 1947) were negative; therefore, it was concluded the yellow color was not caused by excessive destruction of erythrocytes or hepatic biliary malfunction. The plasma pigment was not identified.

The cells were washed three times by resuspending them in 15 ml. Ringer's phosphate solution (see below), mixed and centrifuged. The washed cells were then diluted to the desired concentration.

Ringer's Solution (cold blooded)

NaCl	6.5 gm.
KCl	0.1 gm.
$CaCl_2$*	0.2 gm.

Add glass distilled water to 1000 ml.

*Dissolved first and then added to salt solution.

Phosphate Buffer (pH 7.4)

80.4 ml. M/15 Na_2HPO_4

19.6 ml. M/15 KH_2PO_4

Ringer Phosphate

Ten volumes of Ringer's solution are added to 1 volume of M/15 phosphate buffer, pH 7.4

The tissue was removed and rinsed three times in Ringer's phosphate solution and tissue slices 0.5 mm. thick were used in all *in vitro* studies. Wet weights were to the nearest 0.1 mg.

Radioactive Isotope Methodology

Chromium-51 as $Cr^{51}Cl_3$ in HCl solution was obtained from Oak Ridge National Laboratory in shipments of 15 mc. each. A representative shipment would have a concentration of about 80^{+}_{-} 10% mc./ml. with a specific activity of 355707 mc./gm., total chromium content of 0.226 mg./ml. and the normality of the HCl solvent = 1.95.

Conversion to hexavalent chromium was by the method as given by Schiffman, 1957. Approximately 50 µg. of chromium-51 are made basic with 2 ml. of 6N NaOH and 1 ml. of 3% H_2O_2 added. The solution is then heated to 110°C. for 5 minutes, then boiled for 1 hour, thus allowing for the peroxide to be driven off. After 1 hour, 0.2 ml. 6N NaOH were added while the solution was still hot. If bubbles were given off this indicated that the peroxide had not all

been boiled off, therefore additional boiling was carried out until no excess H_2O_2 was present. The solution was next neutralized by addition of approximately 2.0 ml. 6N HCl. It was cooled and the hexavalent Na_2CrO_4 was ready to use. It was found that if during the neutralization that back titration was necessary, the chromium-51 might be precipitated out of solution due to the high ionic strength of the solvent.

The detection apparatus consisted of the following components: a two-inch thallium treated NaI scintillation detector assembly, Model DS5; a radiation analyzer Model 1810; a Model 183 scaling unit and a well counter--all manufactured by the Nuclear Instrument and Chemical Corporation, Chicago, Illinois.

In all cases each individual experiment was done at constant geometry which was adjusted for best possible resolution. Counting errors were at the 5% level or lower. The activity in counts per minute (c.p.m.) was expressed as per unit mass. No attempt was made to analyze the chromium content of the tissue in order to calculate specific activities. In most cases the chromium content approached the minimal detectible concentration using the microchemical analytical methods.

Because essentially only gamma radiation is counted by a scintillation tube, no correction for self-absorption was found necessary. Absorption of Cr-51 was found by Knoll (1959) to be insignificant under the geomentry as used in this work.

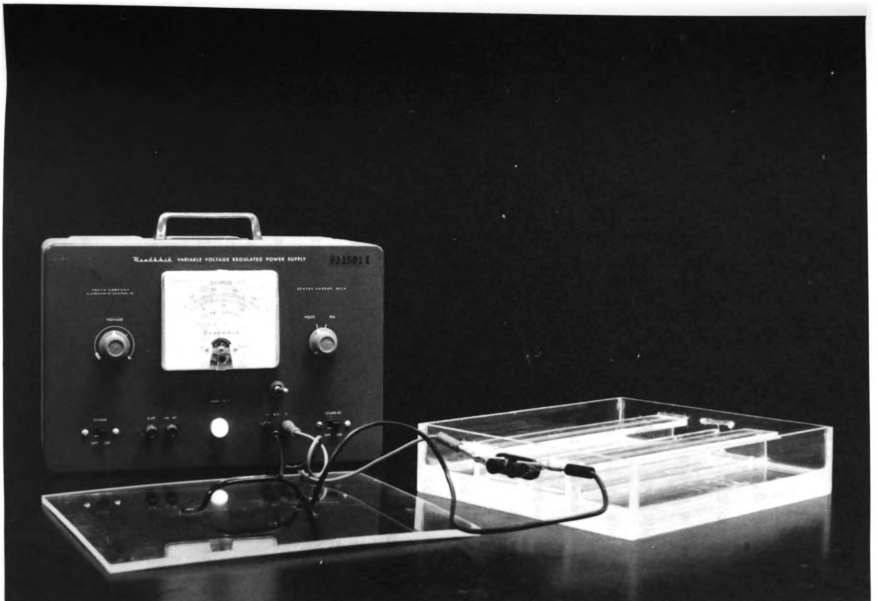
Electrophoresis

Figure 2 shows the apparatus for electrophoresis designed by the author and made of 1/4 inch plastic. It is versatile enough to perform regular filter paper electrophoresis, starch gel electrophoresis and micro-electrophoresis with Oxoid Cellulose Acetate Electrophoresis Strips obtained from Consolidated Laboratories Inc., Chicago Heights, Illinois.

The base of the tank is divided into four buffer compartments. The two central ones, contained the electrodes of platinum wire. The two corresponding outer chambers are connected to the inner chambers by several holes drilled through the buffer baffles. Strips are suspended across the tank and filter paper wicks then complete the circuit into the buffer solutions. Strip holders are short rectangular plastic bars. A tight fitting cover insures efficient vapor saturation of the apparatus. The whole apparatus is placed in a refrigerator at 4^{o}C. during a run.

Figure 2

Apparatus for Electrophoresis


VARIABLE VOLTAGE REGULATED POWER SUPPLY

For protein separation the cellulose acetate strips were saturated with barbitone buffer, pH 6.8

Sodium diethyl barbitone	10.0 gm.
Sodium acetate (hydrated) diluted to 1 liter	6.5 gm.

A Hamilton Microliter Syringe is used to apply a sample to the strip, already impregnated with buffer, and then the strip is placed in the apparatus. At least 150 V. should be applied and the current should not exceed 0.4 ma. per cm. width. The D.C. power supply used was a constant voltage Heath Kit #PS-3.

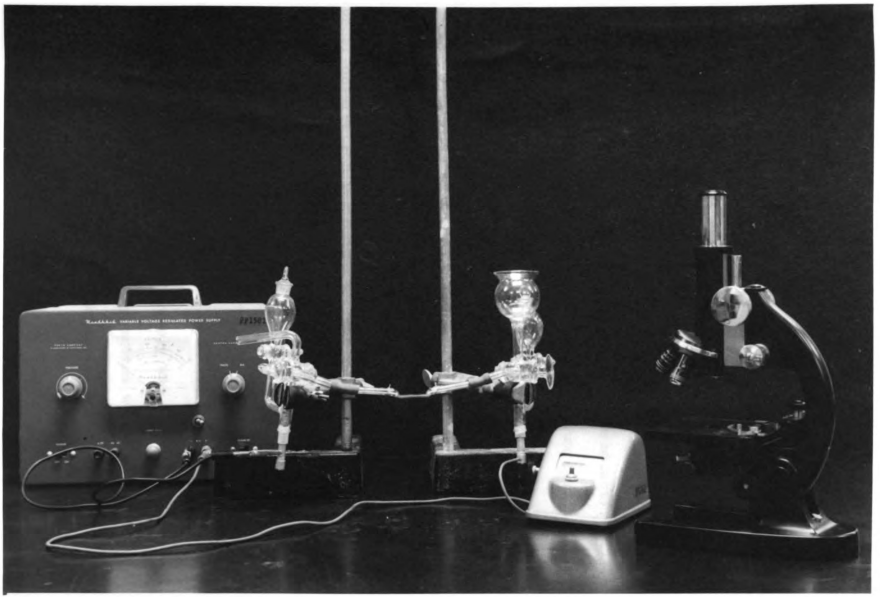
After an appropriate time interval the strips are removed and dried by being suspended in a hot air oven for 10-30 minutes at 80°C.-100°C. They are next stained by floating on the surface of a 0.2% Ponceau-S stain in 3% trichloracetic acid for 10 minutes. In the case of poor penetration of the stain a few drops of methanol are used to wet the back of the strip, which is then restained. Destaining was accomplished by washing in several changes of 5% aqueous acetic acid.

Cataphoresis Apparatus

The general phenomenon, called electrokinetics, resides in the motion of two electrically charged surfaces or bodies relative to each other. It is important to note

Figure 3

Northrup-Kunitz Cataphoresis Apparatus



that electric mobility is due to charges at or near the surface of the particles or molecules. It is a function of the surface charge density, i.e., the number of charges or charged groups per unit area of the surface. Velocity of motion of particles in an electric field is, among other factors, constant; a function of (1) the particle's own charge, (2) current strength (potential or gradient), and (3) resistance to motion offered by the medium (viscosity). The net charge on a protein membrane is due essentially to ionization of carboxyl and hydroxyl groups. The magnitude of the charge depends upon the pH of the solution.

Electrophoretic mobility, μ, is defined experimentally, as the distance (in microns) transversed by the particle under observation per μ in time (sec.) with a voltage gradient (v./cm.). It follows that μ has the dimension of μ/sec./v./cm.

The apparatus used was the Northrup-Kunitz Cataphoresis Apparatus (figure 3), purchased from the Arthur H. Thomas Company, Philadelphia. Power supply was the Heath Kit PS-3. Procedure for calibrating the apparatus was taken from Technological Service, A. H. Thomas Company, 1955.

Paper Chromatograms

A simplified polychromatic technique as developed by Moffat and Lytle (1959) was used to study the amino acid composition of protein hydrolysates. In all cases the solvent system used was butyl alcohol-acetic acid-water (4:1:5). Whatman No. I filter paper in strips 2.5 cm. wide were used in unidimensional descending chromatographic chambers. Strips were developed for 10-16 hours, the time needed for solvent front to migrate 20 or more cm. The strips were then removed, dried in an oven for 5 minutes at 104°C.-110°C, sprayed with the ninhydrin-cupric nitrate indicator, and placed in an oven for 1.5 to 2 minutes at 105°C. The N-CN indicator consisted of two solutions I + II. Solution I contains 0.2% ninhydrin (anhydrous 1,2,3 triketohydrindene) in 50 ml. of absolute ethyl alcohol, 10 ml. of glacial acetic acid, and 2 ml. of 2,4,6 collidine. Solution II was a 1.0% solution of cupric nitrate trihydrate in absolute alcohol. Solutions I + II are combined in a ratio of 25 to 1.5 just before using.

Preparation of Hemoglobin and Globin

A stock solution of HbO_2 is prepared from red cells washed as stated above. White, Beaven and Ellis (1956)

give the following method for preparation of hemoglobin solutions. The washed cells are mixed with one volume of water and repeatedly frozen and thawed until laked. The stroma is separated by high-speed centrifugation at 15-20X 10^3 G for one hour at 5°C. This concentrated solution (approximately 10 g./100 ml.) keeps well at 5°C. in small full bottles.

Lemberg and Legge (1949) formulated the method used in the preparation of globin. Washed, laked corpuscles (HbO_2 solution from above) was cooled to 0°C. and then added gradually to a ten-fold volume of acetone containing 1% hydrochloric acid also cooled to 0°C. The mixture is allowed to stand for two or three minutes and then is filtered; the acid mixture is washed several times with suction and allowed to dry. All operations are carried out at low temperatures.

Block and Bolloing's (1951) article on the preparation of hydrolysate of proteins gives the following methods. A sample of protein containing 1.6 mg. of nitrogen is hydrolysed under reflux with 10 ml. of 1N HCl for 20 hours. The excess HCl is removed by evaporation to dryness in vacuo at 35°C. or on the stream bath, and the resulting thin film of amino acid hydrochlorides is placed in a vacuum desiccator

over soda lime for 24 hours or longer. The hydrolysate is then taken up in warm water, filtered, again evaporated to dryness, and finally taken up in exactly 1 ml. 10% isopropanol. This solvent is used because it is an effective preservative and yet does not cause esterification under these conditions.

Autoradiography

Histoautoradiographs were prepared in hopes of localizing chromium-51 within cells and organ systems. The excellent book by Boyd (1955) gave many ideas on how to accomplish this end.

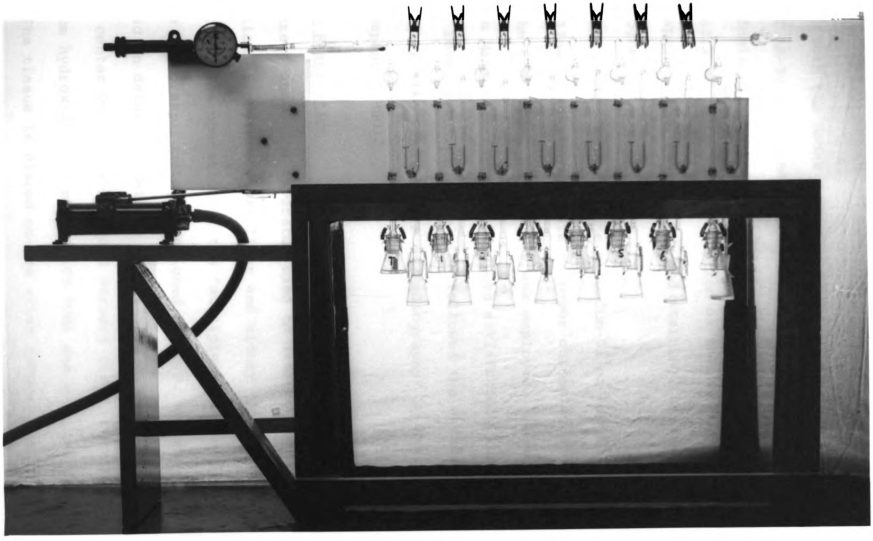
Chromium-51 has as its only major radiation a weak gamma ray of 0.32 mev energy. For this reason a fairly sensitive film must be used. Also a film of fine, closely packed grains is necessary for the fine resolution needed with histoautoradiography.

For the histological sections the most sensitive of the Kodak nuclear tracking emulsions (NTB-3) was first utilized. Unfortunately this material combines its sensitivity to gamma radiation with its sensitivity to virtually all other types of radiation; thus it was observed to pick up undesirable background too rapidly.

Figure 4

Multiple-unit Constant-pressure Microrespirometer.

Photograph Courtesy of E. P. Reineke.



Next, both the Kodak Autoradiographic Stripping Plates AR-10 and AR-50 were used (available from Kodak Limited, London, England), but only the AR-50 proved satisfactory. See Appendix for detailed description of the techniques used in application, development and staining the histo-autoradiograms.

For the localization of the chromium-51 on the filter paper following electrophoresis or paper chromatography, one of the high speed x-ray films was employed. Kodak No-Screen Medical X-ray Film produced satisfactory resolution. Development of the plates followed the directions set forth in "The Fundamentals of Radiography" by Eastman-Kodak Company (1960).

Cellular Respiration

Figure 4 shows the apparatus used to measure the oxygen consumption of erythrocytes, liver and kidney tissues of the turtle. The operation of the "multiple-unit constant-pressure microrespirometer," designed by E. P. Reineke, is described in detail by Reineke (1961).

The center well of a standard Warberg flask contains potassium hydroxide (0.1 ml. of 10% KOH) and a filter paper wick. The tissue is placed on ice after removal from the

animal. One hundred mgms. (wet weight) of tissue slices are placed in 2 ml. of Ringer's phosphate with 50 mg./ml. glucose contained in the main flask. The tissue was sliced to 0.5 mm. thickness with a Stadie Riggs hand microtome. Samples of each tissue were dried to constant weight at 95^{o}C. to determine the percentage dry matter content. Erythrocytes were counted with a hemocytometer and the metabolic results based on oxygen consumed per 10^{9} cells.

All tissues were run at 37^{o}C. with constant agitation. Liver and kidney tissue maintained their oxygen consumption for over 1.5 hours. The erythrocytes held a constant metabolic rate for over 3 hours. After a normal control value for Q_{O2} was obtained for each flask, a known amount of chromate was added from the side arm. Q_{O2} measurements continued and calculated values compared with the control values.

RESULTS

In Vitro Chromium Accumulation

A number of physical factors have been reported to affect the in vitro rate and total amount of chromium-51 uptake by the non-nucleated erythrocytes. Within the physiological temperature range, an increase in temperature will increase the uptake rate.

Some anticoagulants are reported to affect chromium accumulation and an ACD solution (trisodium citrate, citric acid and dextrose) is the recommended medium for chromium tagging of human cells. The undesirable reducing effects of glucose on hexavalent chromium would make it seem desirable to keep the level of glucose as low as possible. It is also reported that glucose is needed for maximum uptake of hexavalent chromium by the non-nucleated erythrocytes. Heparin was the most effective anticoagulant to use on turtle blood and it did not interfere with the uptake of chromium by the nucleated erythrocyte.

Concentrations of chromium-51 ranging from 0.25 to 9.5 μg./ml. have been shown not to affect the uptake rate by non-nucleated erythrocytes.

The effects of different media on the accumulation of hexavalent chromium ($Na_2Cr^{51}O_4$) by the nucleated erythrocytes

TABLE 1

EFFECT OF DIFFERENT MEDIA ON UPTAKE OF $Na_2Cr^{51}O_4$ BY NUCLEATED ERYTHROCYTES

Treatment	% Uptake[a]
0.6% NaCl	12.9
0.6% NaCl + 0.5 mg. heparin/10 ml.	13.5
0.6% NaCl + 0.5 mg. heparin/10 ml. + 1 mg. Ca^{++}/ml.	25.3[b]
0.6% NaCl + 1 mg. Ca^{++}/ml.	25.5[b]
Ringer's solution + 0.5 mg. heparin/10 ml.	16.2

[a] 2 hours; 21.5°C; phosphate buffer pH 7.4

[b] Rapid agglutination, slight hemolysis

were investigated. Washed cells were added to 0.6% NaCl and the uptake at the end of two hours at 21.5°C. was determined and compared with the total amount of activity in the media. The media were modified by addition of reagents as shown in table 1. After the two hour incubation period the cells were removed and washed 3 times with 0.6% NaCl solution. Washed, tagged cells were then placed in plastic containers for counting in the well scintillation counter.

In both cases where Ca^{++} ions were added (table 1) to the media, very evident agglutination occurred within 25 minutes. Statistical analysis revealed that cells in Ringer's solution had a greater uptake than those in NaCl alone. Heparin had no significant effect on the chromium-51 uptake.

Figure 5 summarizes the results of comparison of the basic Ringer phosphate buffer media with ACD and Ringer phosphate glucose media.

In both experiments (#1 and #2) addition of glucose at a concentration of 50 mg.% (normal blood level for the turtle), no statistically significant effect was observed at the 5% level. Use of the recommended ACD solution, adjusted to the osmotic concentration of turtle erythrocytes resulted

TABLE 2

ELECTROPHORETIC MOBILITY OF NUCLEATED ERYTHROCYTES WHEN TAGGED WITH Na_2CrO_4

Animal	No.	Chromium Concentration[a] (mg. Cr VI/l.)	Mobility (μ/sec./cm./volt)	
			Control	Treated
Turtle	24	20	2.507 ± 0.387	1.765 ± 0.484[d]
Turtle[b]	24	20	1.668 ± 0.564	1.892 ± 0.407
Turtle	23	20	2.079 ± 0.326	1.578 ± 0.305[d]
Turtle	23	20	1.566 ± 0.254	1.383 ± 0.213[d]
Turtle	23	20	1.280 ± 0.248	1.123 ± 0.181[c]
Turtle	23	20	1.606 ± 0.444	1.209 ± 0.275[d]
Turtle	23	10		2.446 ± 0.385[c]
Turtle	23	20		1.968 ± 0.333[c]
Turtle	23	40		1.734 ± 0.265[c]

[a] Incubated for 30 minutes at 20°C; then washed.

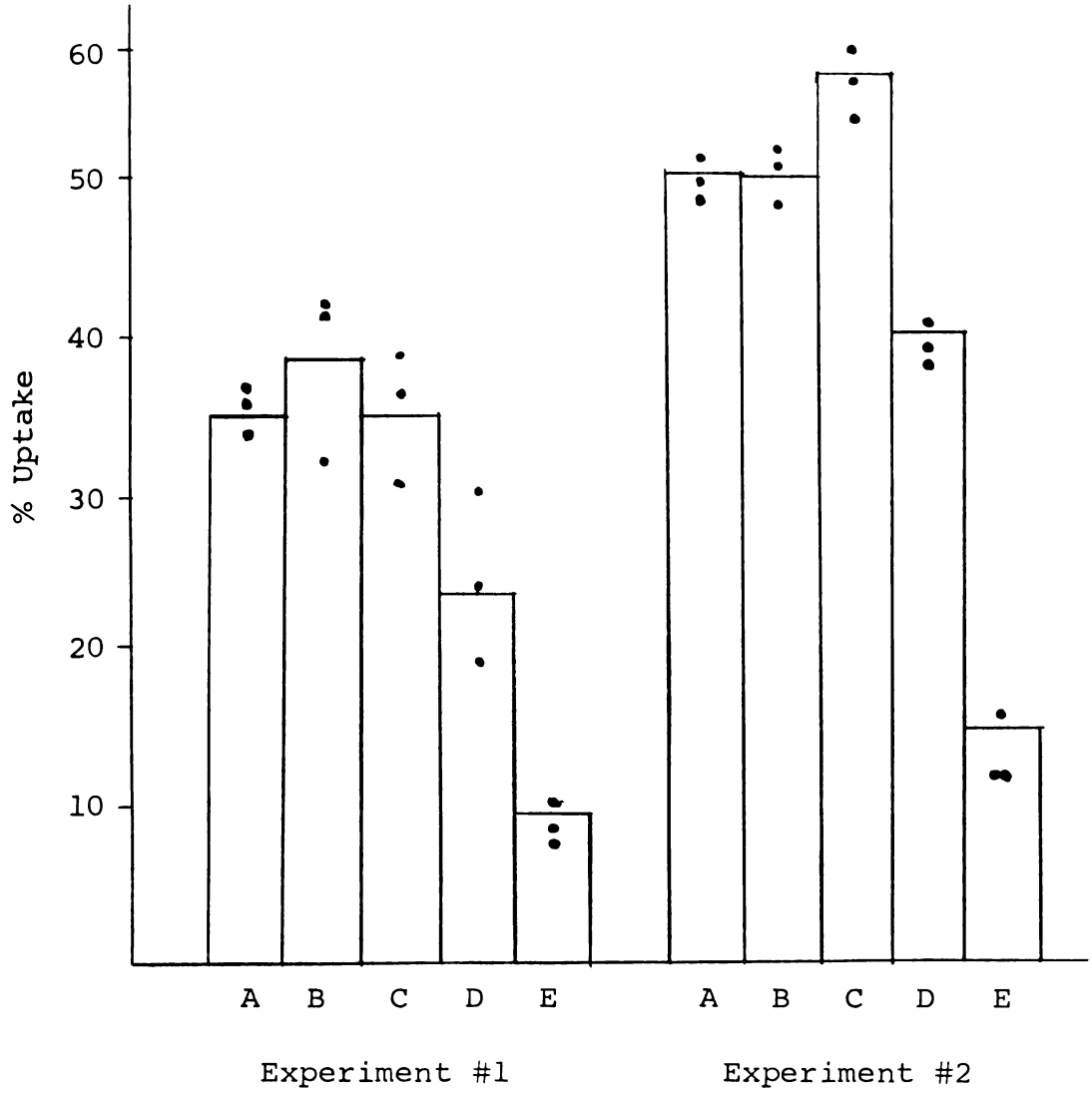
[b] Stored in Ringer phosphate glucose for 24 hrs. at 2°C., before using.

[c] 5% significance.

[d] 1% significance.

Figure 5

Uptake of $Na_2Cr^{51}O_4$ by Nucleated Erythrocytes



A = Ringer phosphate + 50 mg./100 cc. glucose
B = Ringer phosphate
C = Ringer phosphate + glucose + 10^{-3}M NaF
D = Ringer phosphate + glucose + 10^{-3}M NaCN
E = ACD

in a statistically significant decrease in chromium accumulation compared to uptake from the Ringer phosphate glucose media.

Two of the common metabolic inhibitors were added to the incubation media at a concentration of 10^{-3}M. At this concentration complete inhibition of enzyme systems affected would normally be expected. NaF (10^{-3}M) had no effect on chromium accumulation in either experiment. NaCN, on the other hand, did cause a significant decrease in chromium-51 accumulation. In the tubes containing NaCN, modification of the cell wall took place because of the accumulation of free hemoglobin indicating hemolysis.

At the end of the 2 hour incubation period cells in the Ringer's phosphate media contained a higher percentage protein-bound chromium than those in the ACD media.

Electrophoretic Mobility

The electrophoretic mobility of nucleated erytyrocytes was measured at a temperature of 21°C. in Ringer phosphate glucose buffer at pH 7.4 (table 2). Values are expressed as μ/sec./cm./volt and were all made at the level of 0.201 times the depth of the cell. At this level automatic correction for electroendosmotic flow is made.

Control values indicate that the erythrocyte's membrane has a net negative charge or zeta potential. Incubation of the cells with Na_2CrO_4 for 30 minutes at 21°C. followed by washing, then resuspension in fresh buffer, always caused a significant decrease in the electrophoretic mobility.

The nucleated erythrocytes, being more dense (i.e., higher specific gravity), tend to settle or sink in the Ringer phosphate buffer much faster than human erythrocytes. For this reason one must be very careful to measure the velocity of only those cells that are at the proper level in the electrophoresis cell. Many artificial methods of increasing the viscosity of the medium were tried, including addition of sucrose, albumin, gum acacia etc., but no satisfactory media could be found. In most cases when the viscosity was high enough to significantly slow the settling of the cells the osmotic balance of the media was upset, causing hemolysis.

Temperature

Results of the effects of temperature on the uptake rate are presented in table 3. It was found that the slope of the lines, fitted by the method of least squares, have

values that are not significantly different from zero for temperatures of 2°C. and 54°C. The coefficient of correlation also was not significantly different from zero for these temperatures. The values for "a" (i.e. the Y intercept at time zero) show a generally increasing value with increasing temperature.

The Q_{10} (temperature coefficient) was calculated using the equation:

$$\log Q_{10} = (10/t_1-t_2)(\log k_1/k_2)$$

where

k_1 is the rate at temperature 1

k_2 is the rate at temperature 2

Distribution of $Na_2Cr^{51}O_4$ in the Nucleated Erythrocytes

The procedure for this section was a modification of that presented by Rajam and Jackson (1958). Cells prepared as presented above were incubated at 37°C. and samples removed and cells washed after 4, 60 and 120 minutes.

The cells were hemolyzed by freezing and thawing. After hemolysis a sample was centrifuged, the cell walls and nuclei removed, and their chromium content determined. It was found that on hemolysis the soluble cytoplasmic proteins were released and the cell wall then closed in around the

TABLE 3

INFLUENCE OF TEMPERATURE ON THE UPTAKE OF $Na_2Cr^{51}O_4$ BY NUCLEATED ERYTHROCYTES

Temp. °C.	No.	a[1]	b[1]	r_{xy}[2]	Q_{10}	
5	5	3234	73*	0.68*		
14	5	3392	174	0.96	(2-14)	1.21
20	5	4495	241	0.96	(14-20)	1.17
37	5	2970	783	0.99	(20-37)	1.20
54	5	6630	36*	0.20*		

[1] $Y = a + bx$

[2] Coefficient of correlation

* Not significantly different from zero

nucleus. The soluble proteins were precipitated by addition of ethanol (final concentration = 50%). The precipitated proteins in each case were separated by centrifugation at 900 G for 15 minutes and the chromium-51 content determined as before.

The protein-free supernatant was counted to determine the total free (non-protein bound) chromium. A portion of this final non-protein supernatant was used for the purpose of determining the valance state of the intracellular chromium. To the supernatant 1 ml. of a 5% $CrCl_3$ solution (non-radioactive) was added and mixed. The Cr^{+++} and the Cr^{51+++} ions reach equilibrium and both ions are precipitated with ammonium hydroxide. This precipitate was washed with distilled water, removed by centrifugation and counted.

The data are summarized in figure 6. Values reported for cell walls, bound chromium, and Cr III were obtained from 6 observations each. The other data were calculated by subtraction from an assumed value of 100% for the total activity. Statistical analysis revealed that the activity on the cell walls did not change over a 2 hour period. The amount of free Cr III decreased and the amount of bound Cr increased, both at the 5% level of significance.

Effects of metabolic Inhibitors on Uptake of $Na_2Cr^{51}O_4$ by Turtle Tissue

Various inhibitors were added to a Ringer phosphate medium containing cells and chromium and their effects on the hexavalent chromium uptake were determined. Five observations were made over a period of eight hours. Table 4 gives a summary of the effects of these treatments on the slopes of the fitted lines. Those treatments having a significant effect are so indicated in the table.

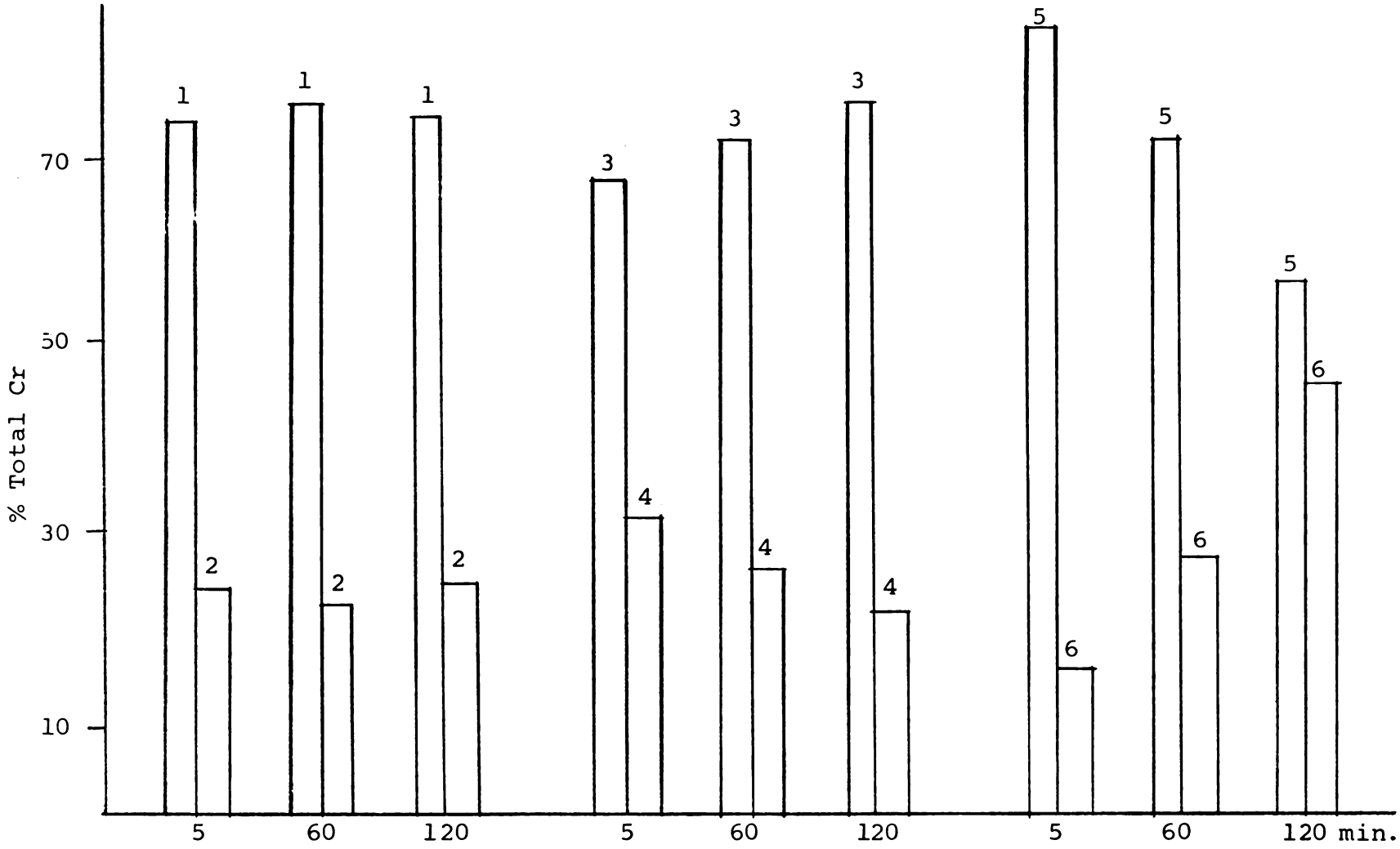
The NaCN (10^{-3}M) treatment caused hemolysis and agglutination of the erythrocytes. A concentration of 10^{-3}M is considered to be high enough to inhibit the major percentage of the affected enzyme systems.

Electrophoresis and Paper Chromatograms

In figure 7 several of the filter paper electrophoresis strips of hexavalent and trivalent chromium with a radioautograph superimposed are shown. The dark bands located on strips #5 and #6 are caused by the exposure of the overlaying x-ray plate. All other strips are either $CrCl_3$ or Na_2CrO_4 samples. The non-radioactive chromium ions were present in high enough concentrations to be detected by their own natural colors. Strips #2, #3, #4 and #7 are all of trivalent chromium. Strips #1, #6 and #9 were of

Figure 6

Distribution of $Na_2Cr^{51}O_4$ in the Nucleated Erythrocyte



(1) Cell Wall vs. (2) Cytoplasmic (3) Bound Cr vs. (4) Free Cr (5) Cr III vs. (6) Cr VI

TABLE 4

EFFECT OF METABOLIC INHIBITORS ON UPTAKE OF $Na_2Cr^{51}O_4$ BY TISSUE

Tissue	Inhibitors	Media	Effect on Slope
Kidney[1]		R.P.	Control
Kidney		R.P.G.	N.E.[2]
Kidney	Boiled (4 min.)	R.P.G.	Decrease**
Liver[1]		R.P.	Control
Liver		R.P.G.	N.E.
Liver	Boiled (4 min.)	R.P.G.	Decrease**
Erythrocytes		R.P.M.	Control
Erythrocytes	Saponin	R.P.G.	N.E.
Erythrocytes	HgCl (10^{-3}M)	R.P.G.	N.E.
Erythrocytes	NaAzide (10^{-3}M)	R.P.G.	N.E.
Erythrocytes	NaCN (10^{-3}M)	R.P.G.	Decrease*

[1] 0.5 mm. tissue slices at 37°C.

[2] N.E. - no significant effect

**1% level of significance

*5% level of significance

Figure 7

Filter Paper Electrophoresis with Superimposed Radioautograph. For explanation see text.

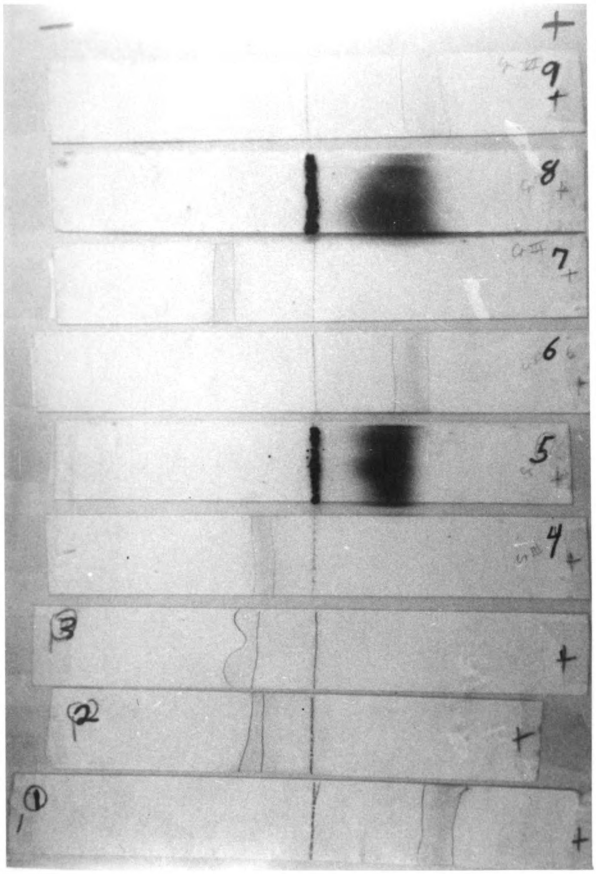
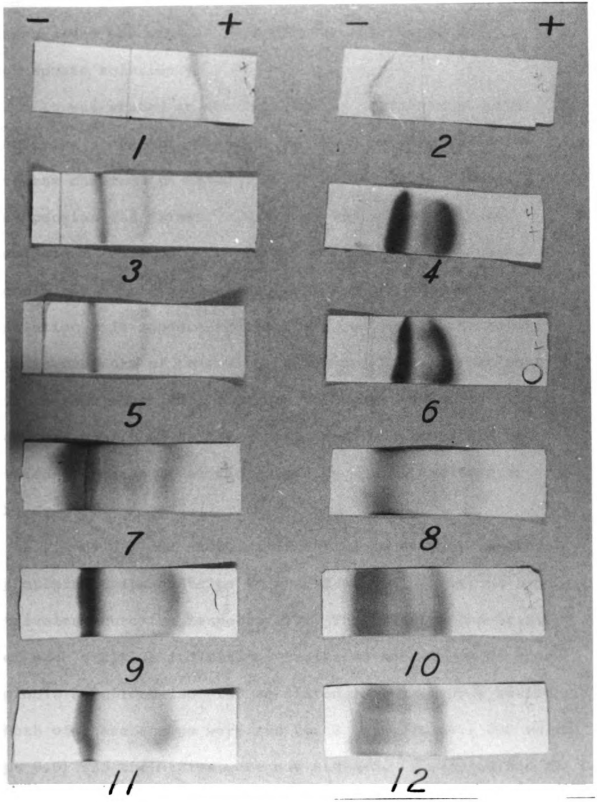

−
+
9
+
8
+
7
+
6
+
5
+
4
+
3
+
2
+
1
+

Figure 8

Cellulose Acetate Electrophoresis Strips.

See text for explanation.


−
+
−
+
1
2
3
4
5
6
7
8
9
10
11
12

hexavalent chromium. The buffer system was Ringer phosphate solution pH 7.4

It was stated in the methodology section that with addition of excess salts during the conversion of trivalent chromium to hexavalent chromium a colloidal suspension was formed. Strips #5 and #6 are of this colloidal solution. It was found that this suspension could not be redissolved in distilled water or Ringer's solution. It appears that a small portion of the activity is in the form of hexavalent chromium, but a larger portion is in some other form that has no charge at pH 7.4. In conversions in which the formation of a colloid had not taken place the total radioactivity was in the form of $Na_2Cr^{51}O_4$.

Figure 8 shows examples of cellulose acetate electrophoresis strips. Strips #1 and #2 are of hexavalent and trivalent chromium respectively. The polarity and origin of each strip is indicated. Strips #3 and #5 are of hemoglobin solutions isolated as stated in the methods sections. Both of these strips were run for 2 hours (6 ma.; 200 volts; pH 8.6) and the strips were not stained. Strips #4 and #6 are identical except they were stained. Two definite bands

were resolved and further examination revealed that the chromium-51 activity, determined by x-ray film, was located in both fractions.

Chemical separation of the globin protein from the haem portion of the hemoglobin molecule showed that the chromium-51 was all located on the globin.

Strips #7, #8, #9, #10, #11 and #12 are of normal turtle plasma. Staining procedure and buffers used have been described above. No tagging of plasma proteins was attempted.

Paper chromatograms were run on both the hydrolysates of globin and hemoglobin with intentions of locating the sites of chromium binding. Twenty amino acid standards were prepared as directed by Moffat (1959). Rf values were calculated for all standards which were run concurrently with hydrolysates.

The polychromatic stain fades in two weeks so immediate reading is necessary. Both globin and hemoglobin hydrolysates revealed 17 definite amino acids and the hemoglobin hydrolysates had several other spots that were not identifiable. These spots were probably associated with iron.

The chromatograms derived from hydrolysates of hemoglobin and globin, tagged with chromium-51, were placed on

x-ray film and exposed for varying periods of time. In all cases one major band of activity appeared which was not associated with any amino acids. Paper chromatograms of hexavalent and trivalent chromium gave Rf values identical to the Rf values of the heavy concentration of radioactivity observed in both hydrolysates.

Histoautoradiography

Figures K, M, N and O show the typical resolutions of the historadiograms made with AR50 film. AR50 has large grain size and this, coupled with the necessity of long exposure (over three months), results in a loss of resolution. The films were exposed during the summer months of 1961. The fallout levels in the state, caused by the Russian tests, were at the highest recorded level; therefore, the background activity was also very high.

The most successful autographs were of tissues removed 6 days following in vivo injection of $Na_2Cr^{51}O_4$. Figure N shows a typical erythrocyte. In most cases the activity was so high that the image of the red cell was very poor. Figure O gives a view of the cross section of the small intestine and shows no accumulation of chromium in any of the many tissue layers photographed. Figure L shows a

TABLE 5

EFFECT OF EXPOSURE TO Na_2CrO_4 ON THE Q_{O2} OF TURTLE TISSUE

Tissue[1]	No.	Cr VI (mg. Cr/l)	Q_{O2} (µl./hr. X 10^9 cells)
Erythrocyte	106	Control	20.88 ± 0.828 (S.E.)
Erythrocyte	21	20	20.30 ± 1.849*
Erythrocyte	19	200	10.79 ± 1.941**
			Q_{O2} (µl./hr. X mg. dry wt.)
Liver	93	Control	0.93 ± 0.069
Liver	46	20	0.94 ± 0.097
Liver	35	200	0.61 ± 0.039*

Note: Liver dry wt. = 23.0 ± 0.847% of the wet wt.

[1] 37°C.; Ringer's phosphate pH 7.4 + 50 mg./100 cc. glucose

* 5% significance of difference from zero

** 1% significance of difference from zero

normal H & E photomicrograph of the mesentery of the stomach area; a large artery and vein are very prominent. The radioautograph from the same block reveals a high chromium concentration located near the loose connective and lymphoid tissue below the artery.

Figure J of the normal turtle lung reveals the unusual fact that this tissue normally has much smooth muscle. Figure K is a radioautograph of lung tissue showing many areas of chromium accumulation. These high density spots were not found in the background areas; however, association of the activity with any type of tissue was not possible.

Effect of Exposure to Na_2CrO_4 on the Q_{O2} of Turtle Tissues

The procedure used to measure the oxygen consumption of turtle tissues has been described. The data for Q_{O2} for the red cells were based on µl. O_2 consumed per 10^9 cells. The Q_{O2} for liver tissue was based on mg. dry weight calculated from the percentage dry weight times the wet weight.

Liver dry weight was found to be 23.0 $\pm$ 0.8% of the wet weight. Of additional interest is the fact that the metabolically active tissue of the turtle represents 65.7% of the total body weight, the shell being 34.3 $\pm$ 0.9% of the

body weight. Table 5 presents the data on the effects of chromium VI on the Q_{O2}.

Distribution of Chromium Following Intraperitoneal Injection of $Na_2Cr^{51}O_4$

Five turtles were given chromium-51 as $Na_2Cr^{51}O_4$ intraperitoneally and six days later the animals were sacrificed; tissues and organs were removed and weighed. Tissue samples were washed in Ringer solution, placed in 10 ml. plastic counting flasks and counted in the well counter. Table 6 gives the percentage body weight for each of the organs collected.

The results of a comparison of organ chromium levels to the blood levels of chromium are presented in table 7. Only two tissues, the kidney and liver, have activities higher than the blood level. The total blood activity was divided so that 16% of the chromium was in the cells and 84% of the total chromium-51 was in the plasma.

Na^{22} was used in an attempt to measure the extracellular volume of the tissues removed in the study. Na^{22} was injected into the animal and one hour later tissues were removed and counted in the well counter. Calculation of "Na^{22} space" followed the example of Manery and Bale (1941).

TABLE 6

ORGAN-BODY WEIGHT RELATIONSHIPS IN *CHRYSEMYS PICTA*

Tissue	Per Cent Body Weight	S.D.
Liver	4.6%	±0.1
Stomach	1.8	1.2
Intestine	3.3	0.6
Lungs	1.1	0.1
Spleen	0.3	0.1
Heart	0.4	0.1
Testes	0.5	0.1
Oviduct	2.4	0.4
Pancreas	0.3	0.1
Kidney	0.4	0.1
Eggs	7.2	0.9

TABLE 7

DISTRIBUTION OF CHROMIUM IN VARIOUS TISSUES FOLLOWING INTRAPERITONEALLY INJECTED $Na_2Cr^{51}O_4$

Tissue	No.	$R = \frac{\text{organ c.p.m./gm.}}{\text{blood c.p.m./ml.}}$	Na^{22} "Space"
Blood[1]	5	1.00	2.80 ± 0.04 (S.D. cells)
Kidney	5	2.11	44.80 ± 5.10
Liver	5	1.88	27.19 ± 3.65
Lungs	5	0.52	56.60 ± 9.99
Testes	3	0.44	
Spleen	5	0.38	10.80 ± 8.06
Bile	5	0.37	
Pancreas	5	0.34	
Intestine	5	0.32	
Urine	4	0.30	
Oviduct	2	0.30	
Stomach	5	0.28	
Heart	5	0.25	47.90 ± 18.50
Muscle	5	0.20	20.93 ± 3.46

[1]Chromium location 6 days after injection

Blood cells = 16% of total blood activity
Plasma = 84% of total blood activity

Calculation of the Na^{22} space of the liver permitted calculation of the total cellular and extracellular chromium in the liver. On a per gram basis the hepatic cell did not seem to be able to accumulate chromium to a greater extent than the nucleated erythrocyte.

DISCUSSION

The nucleated erythrocyte, in many ways, was found to metabolize chromium differently from that predicted on the basis of previous work with human anucleated erythrocytes.

Human erythrocytes show a high uptake of hexavalent chromium when incubated in a buffered solution of tri-sodium citrate, citric acid and dextrose (ACD solution). The removal of Ca^{++} ions from the media seems to increase the uptake of hexavalent chromium by these cells. Addition of 1 mg./ml. of Ca^{++} ions will decrease uptake as much as 50%. It is well known that removal of Ca^{++} ions from whole blood (i.e., during treatment with oxalate or citrate for prevention of coagulation) results in no adverse changes in the human erythrocyte (Von Ehrenstein and Zucharias, 1956).

It is reported (Maizels, 1956) that the removal of Ca^{++} ions from blood of the African tortoise will lead to lysis. Lyman (1946) stated that one can not dilute turtle blood with NaCl or oxalate without inducing complete hemolysis; however, as little as 0.0017M Ca^{++} ion can prevent this hemolysis. Ferguson (1940) reported again that Ca^{++} ions were needed in the blood of the dogfish, turtle and snakes

in order to prevent hemolysis. Treatment with fluorides and arsenates produces hemolysis by removal of magnesium and calcium which in turn alters the permeability of the nucleated erythrocyte. In general, calcium ions will decrease the permeability of the cell membrane to cations. Other ions such as chromium were reported by Lyman (1945) to prevent hemolysis of nucleated red cells, in media of low Ca^{++} ions, by a process of precipitation of the proteins of the corpuscle walls, and therefore its action was not comparable to that of calcium.

Results from this work suggest that, in some way, the calcium ion is functional in controlling the permeability of the cell wall to hexavalent chromium. The small amount of Ca^{++} present in the Ringer's solution is sufficient to demonstrate this control.

At higher calcium ion concentrations the membrane is more drastically altered as shown by the marked agglutination. Associated with this agglutination is a marked increase in the uptake of hexavalent chromium. This uptake is probably a membrane phenomenon. Northrop and Freunad (1923) describe electrolyte control of agglutination.

Because removal of Ca^{++} ions is not a practical means of controlling coagulation of turtle blood the possible use of

heparin was investigated. Von Ehrenstein and Zacharias (1958) have reported that heparin decreased the uptake of chromium by 1/3 in the case of the anucleated cells. In the present study the accumulation of chromium by the turtle erythrocyte was shown to be unaffected by the presence of heparin, indicating differences in the mechanism of transport of cations and anions by the two types of red cells.

Under the conditions identical to those used in this study the human cell is reported to accumulate 90% of the available chromium in 30 minutes while turtle cells accumulated only 30%. The turtle cells show a very rapid uptake which is completed within 10 minutes, followed by a much slower uptake that is linear with time and shows little or no decrease after 13 hours. The first rapid phase has been associated with the accumulation and binding to the surface of the cells. The second phase has been associated with the accumulation of chromium in the cytoplasm, a slower process governed by transfer through the cell wall.

The accumulation of chromium by the nucleated erythrocyte of the turtle does not seem to be dependent on the presence of glucose. One would therefore suspect that the process might be one of simple diffusion, not requiring

metabolic energy. A similar situation is reported by Saltman, Kisken and Bellinger (1956). They have investigated the mechanism of iron transport in mammalian systems and found that the uptake of iron by the rat liver slice was independent of metabolic energy and yet could lead to the establishment of an apparent concentration gradient of the metal within the cell. Iron and chromium are metallic ion capable of forming chelated compounds and in some way this property is tied up with the unusual properties of their transport kinetics.

Of the metabolic inhibitors investigated, only NaCN caused a decrease in the uptake of hexavalent chromium. The cyanide treatment caused marked hemolysis which may be associated with an interruption of the metabolic energy pool supplying the energy requiring process necessary for maintenance of cell membrane and its permeability. In some way the cell becomes modified by cyanide treatment causing a change in the reactive groups on the membrane surface or by a more indirect approach by way of the effects on the energy metabolism of the cells.

Na fluoride, azide and arsenite result in inhibition of different enzyme systems involved with active transport of ions (Maizels, 1951), but none had any significant effect

on chromium accumulation by turtle erythrocytes.

The study of the distribution and valence state of chromium in the cell was approached from many different directions. Separation of soluble cytoplasmic proteins from the cell walls and nuclei proved to be a major problem. The turtle erythrocyte was found to be very resistant to hemolysis and once the cellular breakdown was accomplished the liberated proteins would immediately clot. Very dilute solutions of these cytoplasmic proteins were able to form thin gelatinous masses. Removal of Ca^{++} ions or treatment with heparin did not prevent this coagulation. Similar results (Maizels, 1954) have been reported for the turkey and chicken red cell. Because of this problem the isolation of clean cells ghosts, free from cytoplasmic proteins such as hemoglobin, was very difficult. There is also an indication that hemoglobin is an integral part of the stroma material. All workers have found that their stroma preparations contain this protein, and have found great difficulty in removing it (Prankerd, 1961). It appears possible that hemoglobin may be important in maintaining the structural stability of the cell membrane. The great affinity of chromium for hemoglobin thus gives a feasible explanation for the relatively high accumulation on the cell stroma.

The living cell can be looked upon as a metal binding agent which has both cytoplasmic and surface binding sites. In many cases the cell surface represents a temporary barrier to metallic ions. As the metal ion moves into the cell it must first come in contact with the so-called metal binding sites located in the cell wall. A process then exists whereby the binding sites can give up the metal ion to deeper agents that will bind the metal with more stability.

The rapid accumulation of chromium may be associated with saturation of the transient binding sites located in the cell wall. The saturated metal binding sites then may serve as "a conveyor belt" to carry additional ions into the cytoplasmic pool.

Many different metabolic substances can pass very rapidly through the many types of membrane pores. Other substances like metallic ions are transported much slower. Christensen (1961) stated that the transport of metal ions may well occur more as a result of sequential complexing on the mobile chelating agents and mobile metal complexes than by the free movement of the metal ions themselves. The metal ion may therefore enter the cell in combination with some carrier substance. The carrier may be removed

by a metabolic process in the cytoplasm or it may remain firmly attached. The metal then in the case of chromium, is transferred to a permanent binding site on the soluble proteins. The concentration of hemoglobin is greater than other proteins so a larger percentage of chromium is associated with this protein. Chromium was found only on the globin portion of the hemoglobin molecule.

Correlations between the exposed chelating sites (transient binding sites of the cell wall) and the sites functioning in the transport of other materials may exist. When Mn^{++} ions or Mg^{++} ions are fixed on yeast cells the process of glucose uptake is enhanced. This may indicate that the metal-binding site is part of the glucose transport mechanism. Addition of uranyl ions (Christensen, 1961) which fill the superficial sites inhibits the transport of glucose. Accordingly Stein (1958) showed that metallic ions bind to N-terminal histidine, which is part of the glycerol transport site and Mertz (1961) showed a relation between glucose transport and chromium ions.

If the above hypotheses were true then accumulation of metallic ions by an apparently non-energy requiring process, but still against a concentration gradient may occur. The rate of uptake would be dependent upon the

transport of the material across the cell wall, that is, its rate of interaction with the transient binding sites.

The large accumulation of trivalent chromium after 5 minutes incubation with hexavalent chromium means that there is an intracellular reduction of chromium VI to chromium III which occurs at a rapid rate. The disappearance of free intracellular chromium III could be by oxidation to chromium VI or removal by binding to cytoplasmic proteins.

At temperature extremes of 2°C. and 54°C. no significant chromium accumulation occurred with time. At other temperatures, between these extremes a linear uptake of chromium was observed over an 8 hour period. The uptake rate increased with temperature, reaching a maximum rate at about 40°C. The Q_{10} values calculated for several temperature ranges indicated that the process of chromium accumulation is probably similar to simple diffusion.

Brauer, Long and Halloway (1957) calculated Q_{10} values for the overall reaction rate for the uptake of $CrPO_4$ radiocolloid by isolated rat liver. They found that over the temperature range of 20°C. to 35°C. the Q_{10} was substantially constant and equal to 1.92. This agrees with data on the red cell of the turtle. The reaction of

radiocolloid with the liver is believed to be an irreversible reaction between the colloid and an effectively unlimited fixed active phase, presumably the surface of the Kupffer cells.

Under the conditions existent in the living body the possibility of conversion of soluble chromate to colloidal forms of chromium can not be ruled out. This type of surface attraction based on surface charges may then play an important role in chromium accumulation.

The temperature effect can be explained in one of two ways: (1) Because the rate of simple diffusion is directly related to the molecular motion, i.e. kinetic energy, of the diffusing molecules, one would predict an increased rate of diffusion with an increased temperature. (2) The temperature elevation may cause an increase in the available reaction sites (transient metal binding sites) resulting in an increased influx of the material.

The effects noted at the extreme temperature ranges were probably caused by alteration in the physical properties of the proteins. Boiling of liver and kidney tissue slices also resulted in a decrease in the chromium accumulation. This effect is probably due to the denaturation of the membrane proteins and corresponding changes in membrane permeability.

It was also noted that increased temperature caused a general increase in the "Y" intercept of the fitted lines. Therefore it can be concluded that the rapid accumulation of Cr^{51} on the cell membrane is accelerated by increasing temperatures. This may be due to a greater availability of surface reaction sites.

The cataphoretic velocity (i.e. electrophoretic mobility) is not influenced by either size or shape of the microscopic particle suspended in an electrolyte-containing medium. The differences in red cell velocities of many different species are given by Abramson (1929) and he believes these differences are almost certainly representative of surface constituents of the different cells. The influence of salts on membrane potentials and cataphoretic potentials is discussed by Loeb (1923).

The net charge of a cell is dependent only on the ionization of the chemical groups making up the cell membrane. Chromium tagging significantly decreases the negative charge. Trivalent cations such as Fe^{+++} and Al^{+++} are also very effective in reducing the negative zeta potential of proteins (Oliver and Barnard, 1924). In the present studies chromium VI may have been reduced at or on the surface of the cell, then bound to the cell wall proteins

An increase in the chromium concentration of the medium was shown to decrease the net negative charge on turtle erythrocytes.

In an attempt to compare the metabolism of chromium by the nucleated erythrocytes with liver and kidney slices, it was found that tissue slices present the difficult problem of distinguishing extracellular from cellular fixation, and identifying the barriers that retard metal entrance into cells. Indications of this work on turtle tissues are that the chromium enters liver and kidney cells probably in the same manner that it enters the red cell.

The assumption was made that 1.0 ml. of whole blood is equivalent to 1.0 gm. of wet tissue. Taking the blood activity as 1.0 it was found that only the kidney and the liver had activities above that of the blood. Liver activity undoubtedly is associated with the phagocytic activity of the Kupffer cells, and their removal of colloidal chromium. The ability of the kidney to accumulate chromium above blood levels has been reported many times previously, where chromium, in some way is said to interfere with the transport mechanism of the first portion of the proximal convoluted tubule.

Historadioautographs indicated that the radioactive chromium is located in the hepatic cells as well as in the extracellular spaces. There is a possible indication that chromium-51 may be concentrated in the lumen of the tubular network, however, because of the relatively poor resolution (with AR50) no definite statement as to the exact intracellular localization of chromium can be made.

The only other tissue showing an appreciable accumulation of chromium was the lungs. Histologically the lungs of the turtle appear to have a normal alveolar epithelium. This is infiltrated with fibers and sheets of smooth muscle. The muscle tissue appears to be connected to the alveoli by areas of loose connective tissue. Radioautographs revealed many scattered areas of chromium accumulation, but no positive localization of the activity in any specific tissue was possible. The many areas of chromium activity may be related to lymphatic activity.

Circulating leukocytes having the power to engulf colloidal particles by phagocytosis are known to exist. These cells could take up and concentrate large amounts of chromium. Labeled leukocytes could travel to the lungs or they might originally be located in small areas throughout the thoracic area, resulting in the patchy areas of chromium

accumulation as observed in the lungs of the turtle.

Bierman, Kelly and Cordes (1956) studied the leukocyte concentration entering and leaving the lungs of man and found a very great capacity of the lungs to hold leukocytes, causing an arterio-venous difference. The term "leukocyte withdrawal" was used to describe this phenomenon. A similar phenomenon may occur in the turtle. Inhalation of particles of chromium in man results in focal concentrations in the lungs.

Measurement of extracellular space by use of Na^{22} gave values which compare favorably with those reported for the Na space of mammals. Calculation of the Na^{22} space of the liver and the assumption that the chromium in this space was in equilibrium with the plasma chromium permitted a calculation of the total cellular and extracellular chromium of the liver. On a per gram basis the hepatic cell did not seem to be able to accumulate chromium to a greater extent than the nucleated erythrocyte.

The metabolism of the nucleated erythrocytes is low when compared with other tissue of the body, but it is considerably higher than that normally associated with anucleated cells. The metabolic rate of the turtle erythrocyte was found to be extremely constant over a 2.5 hour period at

37°C. This temperature was used in order to get a good rate of oxygen utilization. All indications point to the fact that this temperature was not harmful to the metabolic processes of the cells. There is a general consensus of opinion that the body temperature of turtles reaches values above 37°C. when they are sunning themselves. It was mentioned earlier that chromium accumulation attained its optimum rate at a temperature of around 40°C. Addition of hexavalent chromium to a concentration of 200 p.p.m. (200 mg./l.) in the incubation medium resulted in a decreased Q_{O2}. This is explained on the basis of the ability of chromium to precipitate soluble proteins, or instead of a true precipitation, denaturation may have occurred. Alteration of the cell membrane could allow for the build-up of waste products, leading to changes in cellular pH and/or inhibition of metabolic enzymes. The availability of metabolic substances may also be affected by changes in cell permeability.

SUMMARY AND CONCLUSIONS

The investigation of the *in vitro* accumulation of hexavalent chromium (available as chromate) by nucleated erythrocytes of the common painted turtle *Chrysemys picta* has shown that:

1. The accumulation of chromium was substantially less than that reported for the nucleated human erythrocytes measured under similar conditions.
2. Within a physiological range, rate of uptake varied directly with temperature, reaching a maximum value at about 40°C. Higher temperatures caused inhibition of chromium uptake.
3. Accumulation of chromium is a non-energy requiring process, taking place in two phases. The initial, rapid phase has been associated with binding at or on the cell surface. A subsequent slower phase has been associated with movement of chromium through the cell wall and its ultimate binding with cytoplasmic elements (probably proteins).
4. Measurements of cataphoretic velocities confirms the binding of chromium to surface sites on the cells.
5. By means of a physical and chemical separation, checked by electrophoresis, all Cr^{51} bound to hemoglobin was on the

globin portion of the molecule. Hydrolysis of Cr^{51} labeled globin resulted in the release of free Cr^{51}, preventing a precise localization of binding sites on the globin molecule.

On the basis of the above a concept of chromium transport across the cell wall, resulting in the establishment of a concentration gradient without the requirement of metabolic energy, is described.

In addition to the above, in vitro studies indicated that liver and kidney tissues alone are capable of concentrating chromium above blood levels. Radioautographic localization of Cr^{51} in lung tissue showed isolated focal areas of concentration; however, these areas could not be associated with specific cellular structures.

Some of the more unusual aspects (pigmentation of the liver) of the histology of the painted turtle are reported and the problems attendant with the use of this animal in experimental work have been discussed.

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APPENDICES

APPENDIX A

PHOTOMICROGRAPHS AND AUTORADIOGRAMS OF VARIOUS TISSUES FROM *CHRYSEMYS PICTA*

Figure A

Normal Liver and Gall Bladder Section from the Turtle (H & E 100X)

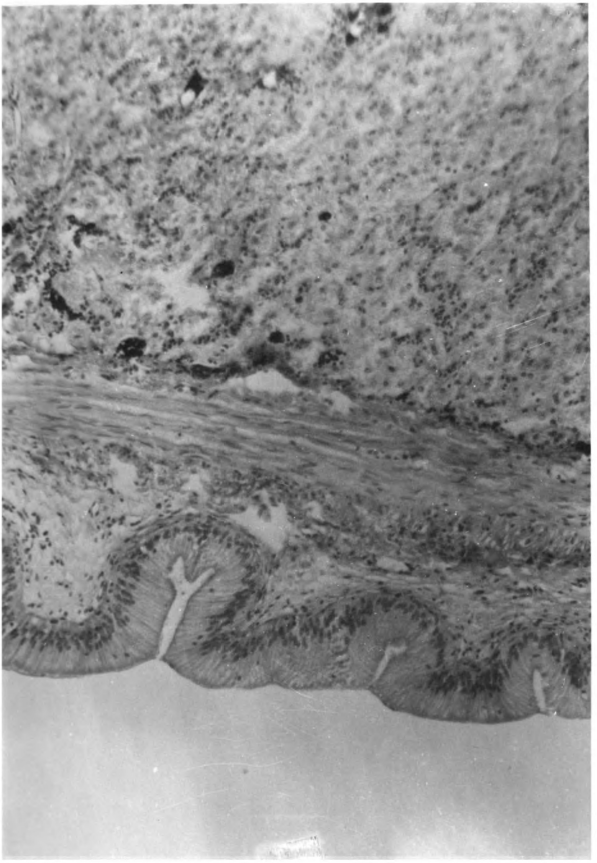


Figure B

Normal Kidney Tissue from the Turtle

(H & E 100X)

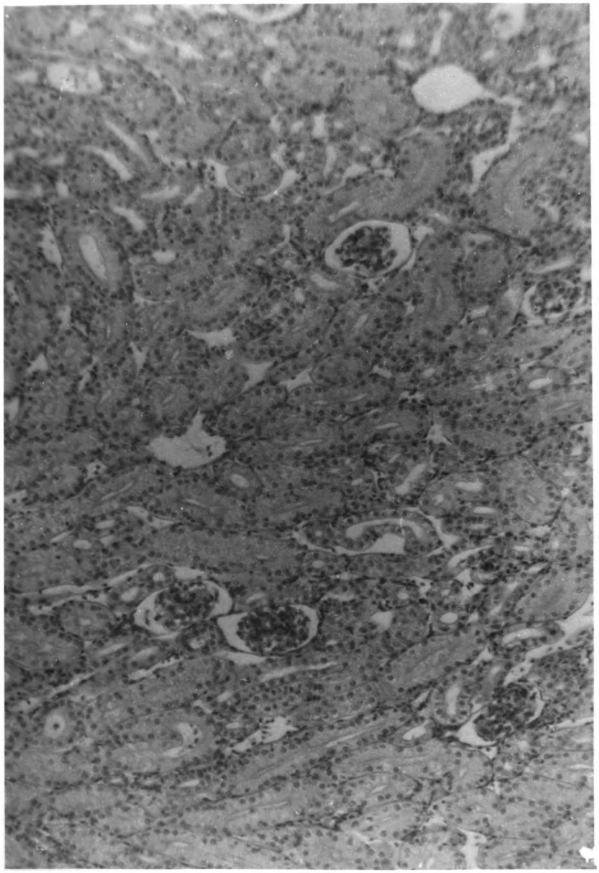


Figure C

Negative Test for Hemosiderin. Note Presence of Melanin Pigmentation.

(Turnbull's Blue Method 100X)

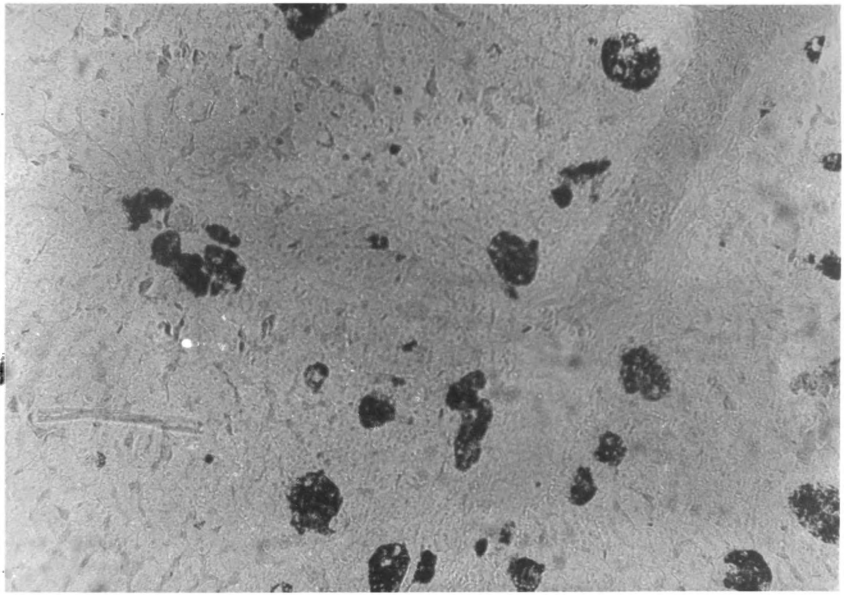


Figure D

Liver Section Showing a Positive Bleaching Reaction for Melanin (H & E 100X)

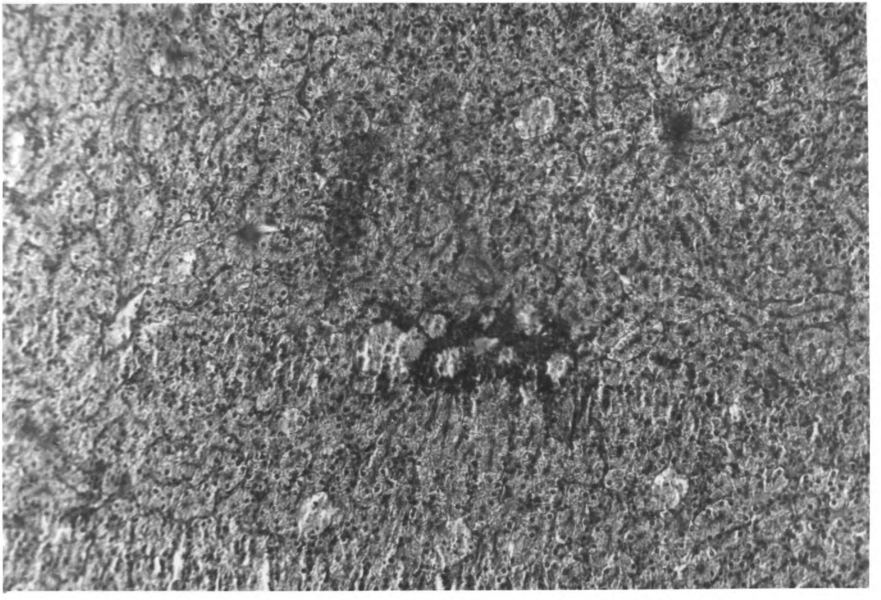


Figure E

Chronic Multiple Abscesses of the Turtle Neck.
The Contents Consist of Leukocytes and
Necrotic Tissue (H & E 100X)

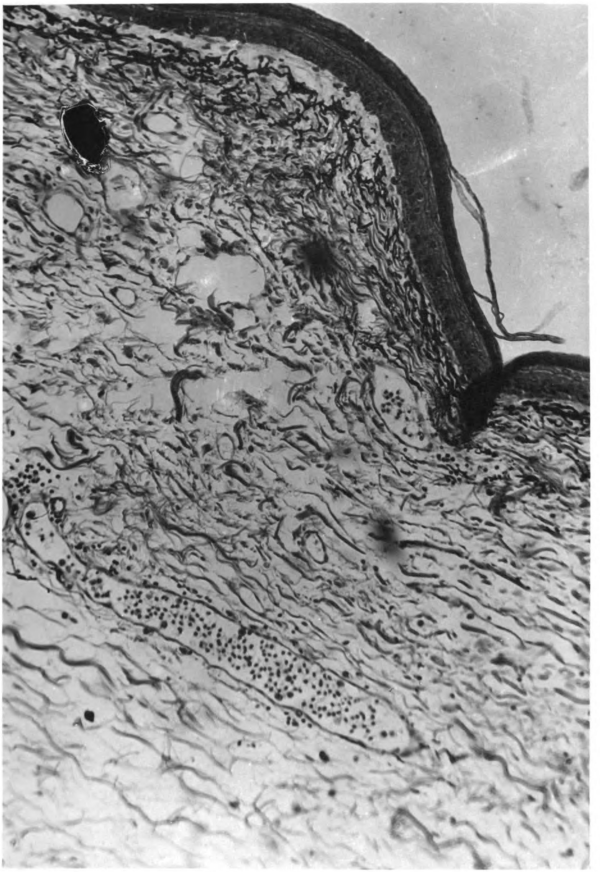


Figure F

Liver Section Showing Area of Lymphatic Infiltration (H & E 440X)

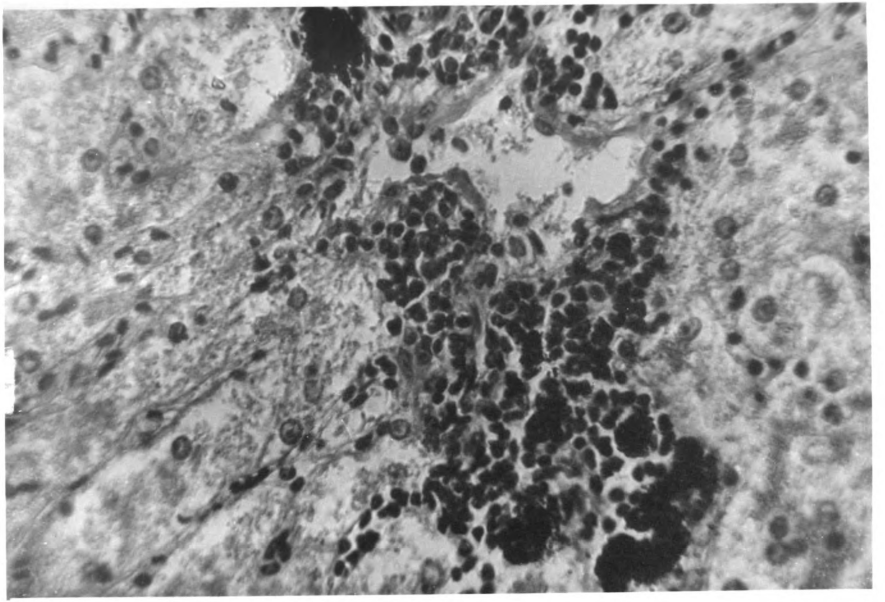


Figure G

Section of Aorta Taken Near the Heart. Note the Inflammation of the Tunica Interna. This Condition Appears Commonly in the Turtle. (H & E 100X)

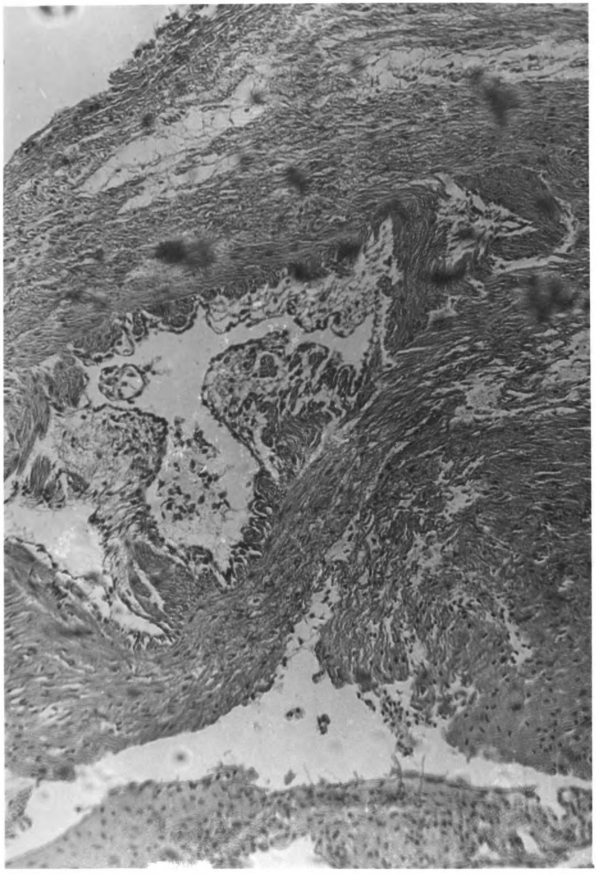


Figure H

Intravascular Parasite Located in a Coronary Artery (H & E 100X)

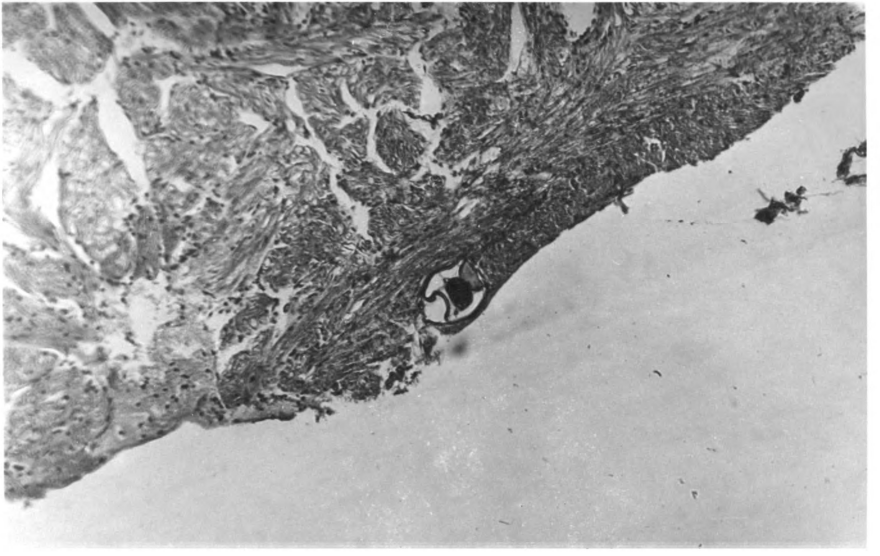


Figure I

Gross Specimen of Turtle Heart Following 6 Days of Daily Heart Punctures. Note Adhesion Which Connected the Ventricle to the Plastron.

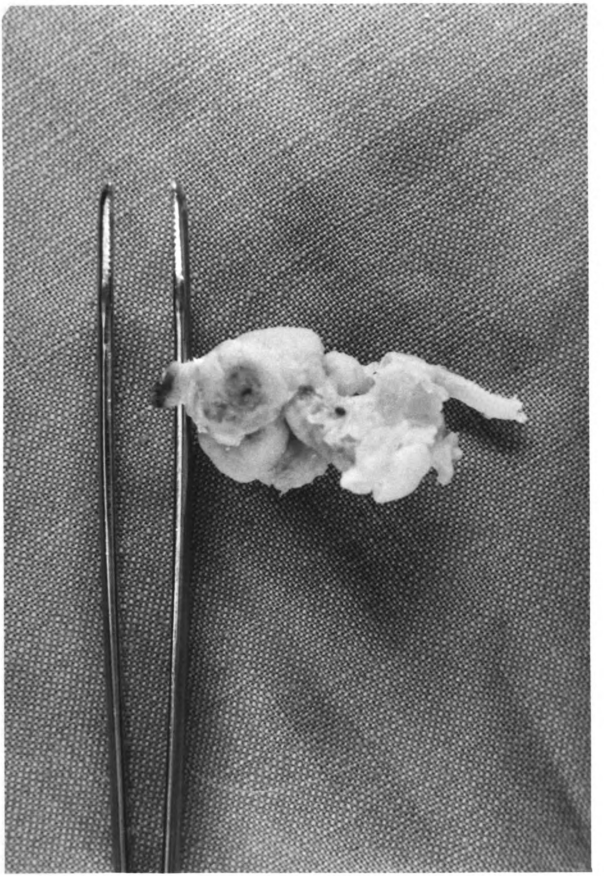


Figure J

Lung Section Showing Smooth Muscle

(H & E 100X)

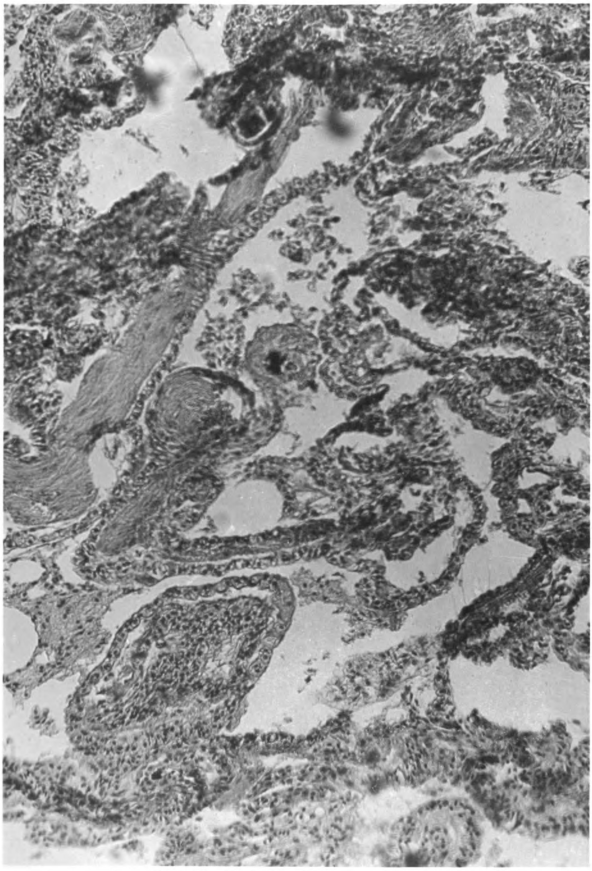


Figure K

Radioautograph of Lung Tissue

(H & E 100X)

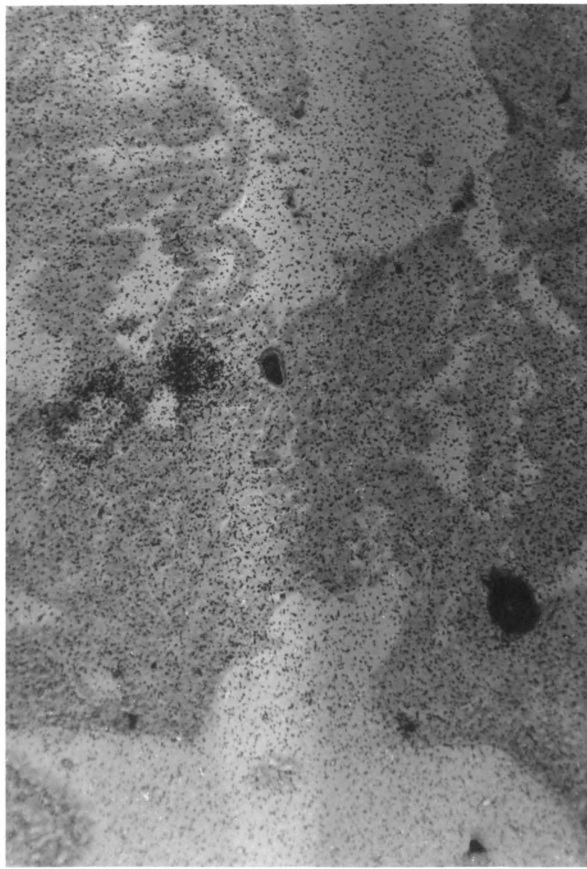


Figure L

Mesentery from Area of Stomach. Note Connective Tissue Between the Large Vessels. (H & E 100X)

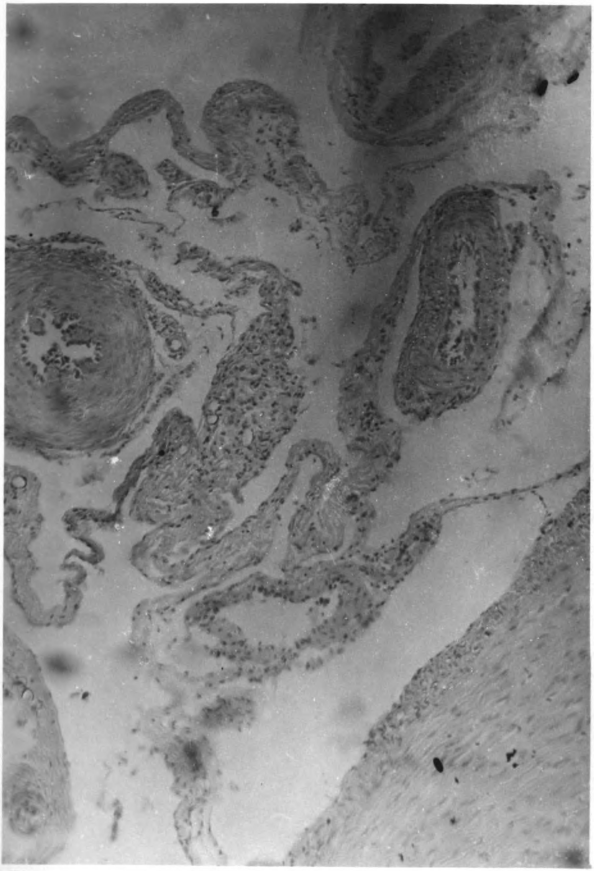


Figure M

Radioautograph of Mesentery Section Shown in Figure L.

(H & E 100X)

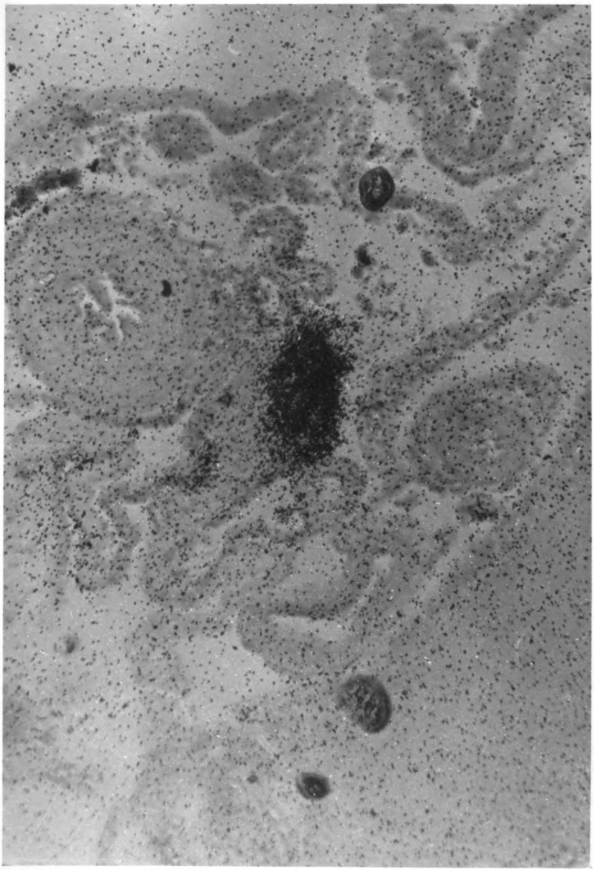


Figure N

Radioautograph of Nucleated Erythrocyte Tagged with Cr^{51} (H & E 440X)

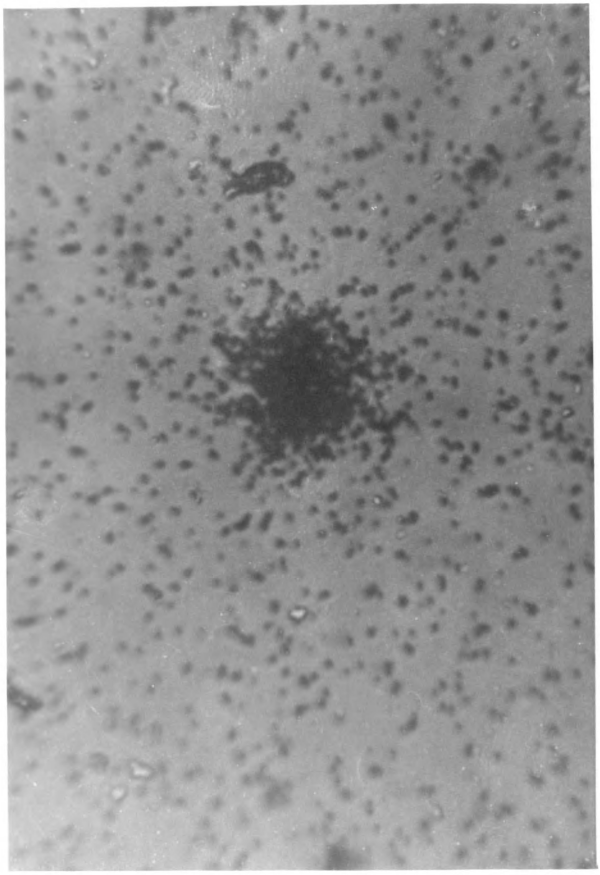
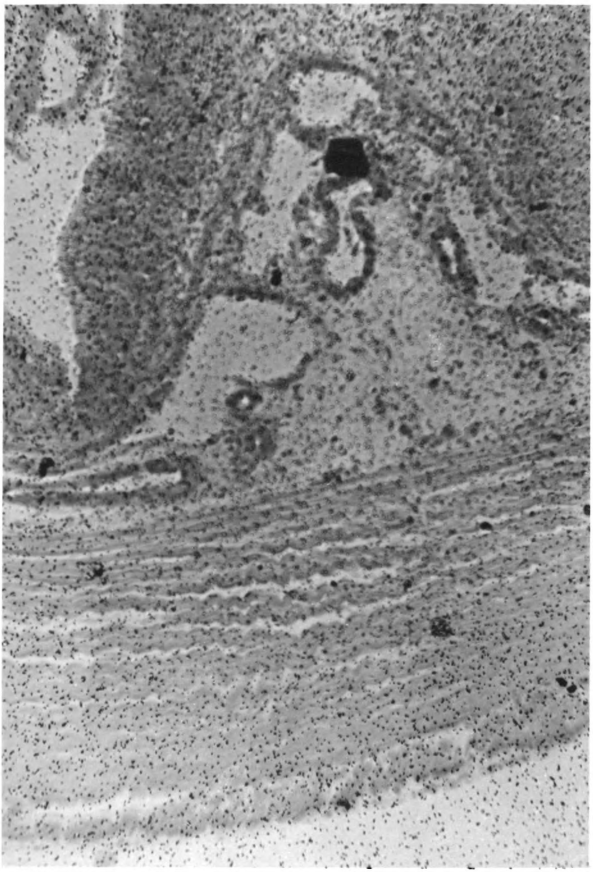


Figure O

Radioautograph of Small Intestine Showing No Localization of the Radioactivity from Cr^{51} (H & E 100X)



APPENDIX B

EFFECT OF METABOLIC INHIBITORS ON THE IN VITRO UPTAKE OF $Na_2Cr^{51}O_4$ BY TURTLE TISSUE

EFFECT OF METABOLIC INHIBITORS ON THE IN-VITRO UPTAKE OF $Na_2Cr^{51}O_4$ BY TURTLE TISSUE

Tissue	Treatment	N	a	b	r_{xy}
RBC	$NaCl_2$ (37°C.)	5	8785	1568	0.92+
RBC	NaAzide (37°C.)	5	6168	1858	0.91+
RBC	NaCN (37°C.)	5	244	975	0.27+
RBC	Saponin (37°C.)	5	1548	1562	0.99+
RBC	Normal (37°C.)	5	3588	3211	0.94+
RBC	Normal (56°C.)	5	1123	1135	0.85+
Liver	Normal (37°C.)	5	575	383	0.94+
Liver	Boiled	5	1134	160	0.46+
Liver	Glucose (37°C.)	5	541	346	0.97+
Liver	Normal (37°C.)	5	739	457	0.98+
Liver	Boiled	5	942	198	0.84+
Liver	Glucose (37°C.)	5	803	866	0.93+
Liver	NaCN (37° C.)	5	492	536	0.99+
Liver	NaAzide (37° C.)	5	500	687	0.96+
Liver	NaF (37°C.	5	21	732	0.99+
Liver	$NaASO_4$ (37°C.)	5	429	589	0.99+
Kidney	Normal (37°C.)	5	1253	466	0.90+
Kidney	Boiled	5	496	308	0.98+
Kidney	Glucose (37°C.)	5	1324	777	0.90+
Kidney	NaCN (37°C.)	5	740	989	0.89+
Kidney	NaF (37°C.)	5	1689	772	0.91+
Kidney	NaAzide (37°C.)	5	1038	512	0.94+
Kidney	$NaASO_4$ (37°C.)	5	1366	514	0.95+

+ significantly different from zero at the 5% level

* precipitated and not significantly different from zero

$y = a + bx$

where y = c.p.m./mg. dry weight
x = time
b = slope
a = y intercept

APPENDIX C

PREPARATION OF TISSUES FOR RADIOAUTOGRAPHS

PREPARATION OF TISSUES FOR RADIOAUTOGRAPHS (PELC, 1961)

1. Tissue cut in 5-6 mm. slices.
2. Fixed in Dietrich's Fixative.
3. Dehydration and paraffin embedding preparation as usual.
4. Sections cut at 6 microns.
5. Sections placed on microscope slides that have been previously prepared with a thin coat of Mayer's Egg Albumin. Albumin smeared on all parts of slide and allowed to dry completely.
6. Paraffin embedded sections dried overnight in 37°C. oven.
7. Hydration process (temperature not critical), i.e. xylene to distilled water.
8. Hydrolysis with trichloracetic acid to get rid of TON in cytoplasm. 5% TCA agitate slightly for 5 minutes at 0-3°C. Wash in distilled water while going to dark-room.
9. Placing film (AR-10 or AR-50) on tissue: safe-light (slide and tissue are both wet).
   a. About 1/4 inch of emulsion trimmed from all edges of film plate.
   b. Film plate divided into 12 equal parts by cutting emulsion with scapel.

c. Each film section stripped very slowly from plate with forceps and floated on distilled water at 21°C. with emulsion down. The film curls toward the emulsion. Blowing on film will uncurl it.

d. Film allowed to expand by water inbibition for approximately 3 minutes or until it has reached its maximum size.

e. The film is placed on the slide by lifting the film from underneath with the slide in such a manner that the film will overlap the slide.

f. Slides are dried approximately 1 hour in a horizontal position.

g. Slides placed in airtight, light-tight box with some calcium sulfate (Drierite). The slides are stored in the refrigerator at 3°C.

10. Development: Solutions:

I. Kodak D-19 Developer (full-strength)

II. Stop bath of approximately 1000 cc. distilled HOH with 1 or 2 drops of glacial acetic acid.

III. Fixer--Kodak Acid Fixer with Hardener. Dilute to 1 part fixer to 5 parts distilled water. This dilution is very important.

IV. Wash--distilled HOH.

All solutions are maintained at 17°C.

Treatment:

a. Developer -5 min. gently agitated every few minutes.

b. Stop bath approximately 45 seconds.

c. Fixer twice to three times the clearing time. It is important not to clear too fast.

d. Wash with 3 changes of water, 20 minutes each.

e. Slides are then placed on a test tube rack in a horizontal position and allowed to air dry.

11. Staining

Solutions: I. Harris Hematoxylin (stock)

Hematoxylin	5 gm.
Absolute alcohol	50 gm.
Ammonium or K alum	100 gm.
HOH	1000 cc.
Mercuric oxide	2.5 gm.

II. Eosin (stock)--0.25% Eosin Y made up in 25% alcohol.

III. Decolorizer--0.05% HCl in distilled HOH.

All staining to be done at 17°C.

Treatment:

a. Hematoxylin diluted 1:8 for 50 minutes.

b. Wash in distilled HOH.

c. Decolorizer (to pink color).

d. Blueing with distilled HOH and a few crystals of sodium bicarbonate.

e. Eosin Y, diluted 1:8, for about 1.5 minutes.

f. Distilled HOH wash for differentiation of Eosin.

12. Mounting process: Let sides air dry.

[illegible] LEE DILY

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FEB [illegible]