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THE FATE OF CADMIUM IN THE MAMMALIAN ORGANISM

by

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A Thesis

Submitted in partial fulfilment of the requirements

for the Degree of Doctor of Philosophy.

Department of Biochemistry
Dalhousie University
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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "The Fate of Cadmium in the Mammalian Organism" submitted by Zahir Ahmad Shaikh in partial fulfillment of requirement for the degree of Doctor of Philosophy.

Dr. Henry A. Schroeder, Department of Physiology, Dartmouth Medical School acted as external examiner, but was unable to attend the examination. He sent a letter recommending the acceptance of this thesis.

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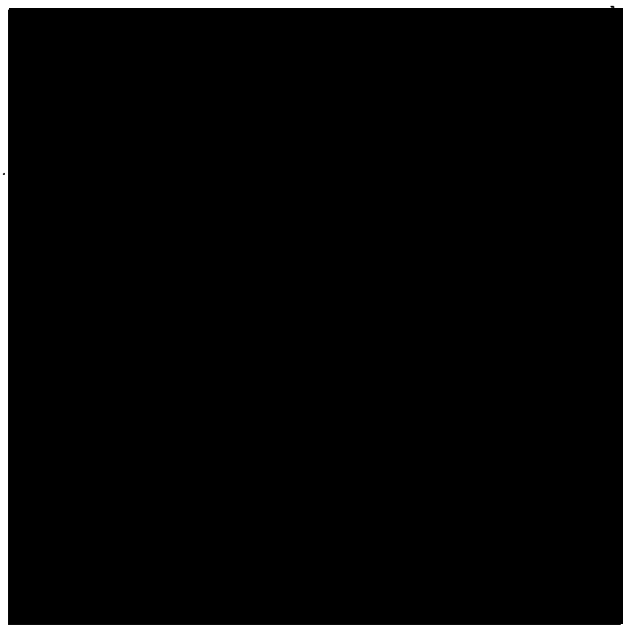

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ABSTRACT

The tissue distribution, intracellular binding, and turnover of cadmium was studied in rats and mice. Distinct differences were found between the metabolic fate of ^{109}Cd and that of ^{65}Zn or ^{203}Hg. Cadmium-109 was accumulated mainly by the liver and by the kidney tissues, where it was tightly bound to specific intracellular proteins. These proteins were also capable of binding ^{65}Zn and ^{203}Hg. A procedure for the isolation of cadmium-binding proteins (Cd-BP) was developed. The Cd-BP were also isolated from the splenic, pancreatic, and placental tissues of rats. Two electrophoretically distinct components were separated from rat liver Cd-BP. The molecular weights of the two proteins, Cd-BP 1 and Cd-BP 2, were 11,400 and 12,000 respectively. The cadmium content of Cd-BP 1 was 6.9 g-atoms/mol and that of Cd-BP 2 was 6.5 g-atoms/mol . Of the total amino acid residues half-cystine accounted for 30-33%. In addition, lysine and serine were also abundant. The synthesis of Cd-BP was increased *in vivo* after exposure of rats to drinking-water containing $CdCl_2$, or after injection of $CdCl_2$ solution. This effect was dose dependent. The increase in Cd-BP was not produced by Zn^{2+}, Hg^{2+}, Pb^{2+}, Co^{2+}, or Ni^{2+}. Incorporation of exogenous cystine-^{14}C, lysine-^{14}C, and threonine-^{14}C into rat liver Cd-BP was demonstrated *in vivo*. In rat liver the biosynthesis of Cd-BP became accelerated between 5 and 12 hours after injection of $CdCl_2$. Two components of Cd-BP were also isolated from

human kidney tissue, which showed chromatographic properties similar to those of the rat liver Cd-BP. It is suggested that in mammalian cells the Cd-BP represent a biological detoxifying mechanism and the biosynthesis of intracellular Cd-BP is regulated by the body burden of cadmium.

ABBREVIATIONS

The symbols and abbreviations used in this thesis are in accordance with those suggested in J. Biol. Chem., 246 : 1-8 (1971). In addition, the following abbreviations have been utilized.

Cd-BP	=	Cadmium-binding proteins
-SH	=	Sulfhydryl group
BAL	=	British anti lewisite (dimercaptopropanol)
dpm	=	Disintegrations per minute
% T	=	Percent transmission
w/v	=	Weight/Volume
MW	=	Molecular weight

The names of scientific journals are abbreviated as described in Index Medicus, 12 : 1-26 (1971).

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CHAPTER I. INTRODUCTION

This work was presented in parts at the following scientific conventions:

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54th Annual Meeting of the Federation of American Societies for Experimental Biology (F.A.S.E.B.), Atlantic City, New Jersey, April 13, 1970.

13th Annual Meeting of the C.F.B.S., Montreal, Quebec, June 12, 1970.

55th Annual Meeting of the F.A.S.E.B., Chicago, Illinois, April 13, 1971.

14th Annual Meeting of the C.F.B.S., Toronto, Ontario, June 16, 1971.

1. ELEMENTS AND THE ENVIRONMENT

In the geosphere the relative abundance of elements, with few exceptions, follows an inverse relationship to the atomic weight (Burbidge et al., 1957). This relationship extends from hydrogen (atomic weight, 1) to molybdenum (atomic weight, 96). All bulk and trace elements of the biosphere, except iodine (atomic weight, 127) fall within this range.

Life most probably took its origin in water, which covers nearly three-quarters of the surface of the earth. In the language of a chemist, the water reservoirs represent a dilute solution of almost all elements. This means that all primitive forms of life were exposed to various elements present in their natural environment. As the living organisms differentiated into more complex forms, they adapted themselves to environmental conditions. A selectivity towards elements according to physiologic need for sustaining life developed and some of these were incorporated into cellular processes. Within the organism specific control mechanisms evolved which served for conservation of essential elements to prevent a deficiency and for excretion of those elements absorbed in excess.

2. THE TRACE ELEMENTS

The term "trace element" in living organisms originated from the analytical work done at the turn of this

century. It was assigned to those elements that were detected in microgram and sub-microgram quantities. The definition of trace elements has since changed. Now these elements can be measured with accuracy by emission and atomic absorption spectrometry, by neutron activation analysis, and other physicochemical methods. For the sake of convenience, however, the term "trace element" is still accepted and widely used.

The description of a biologically essential trace element, as provided by Arnon (1950) is any element required for growth and the completion of the life cycle; it cannot be replaced completely by any other element; and it is directly involved in the metabolic processes of the organism.

Cotzias (1967) further elaborated on the concept of an essential trace element. According to him an essential element is present in healthy tissues of all living organisms. Its concentration from one animal to the other of the same species is nearly constant. The withdrawl of this element from the organism induces the same structural and physiological abnormalities accompanied by specific biochemical changes regardless of the species studied. Finally, its addition in the diet either prevents or reverses these abnormalities. Schroeder (1965a) adds that an essential trace element comprises less than 0.01% of the total body weight of an organism, and nutritionally it is more important than vitamins.

There are specific homeostatic control mechanisms operating in the organism which regulate the absorption, retention, and excretion of essential elements, and a constant balance is maintained within the tissues. Non-essential elements, on the other hand, do not find themselves under such controls.

The essential elements are necessary for the organism within a limited range of concentration. Their deficiency retards growth and development, while an excess is toxic. There are some elements that are poisonous even at low concentrations and are, therefore, recognized for their toxic properties only. The list of these elements is short and includes: arsenic, lead, mercury, and cadmium (Underwood, 1971a).

3. METALS AS POLLUTANTS

Our ancestors, some 7 to 8 thousand years ago learned to use copper. This was followed by bronze and later by brass and other metals and their alloys (Wertime, 1964). Metals have been in use ever since and contributed towards development of human civilization over the centuries.

In modern times, ever increasing growth of metallurgical and other forms of industries have increased the requirement for these metals and more and more of the metallic ores have been brought from deep down to the surface of the earth. These practices have disturbed the natural balance of elements in the environment. In fact,

advances in science and technology have created vast arrays of new problems in environmental health. The release of undesireable compounds, including metals, into water reservoirs, air, and the food chain of man and animals has continuously increased during the past century. Carelessness on the part of industry has contributed to the pollution of our environment. As a result of these disturbances in the ecosystems of the biosphere, the ecologists predict potential danger to life on this planet. There is no doubt that most of these metallic contaminants, i.e., titanium, tin, cadmium, mercury, and lead, are cumulative poisons and Schroeder (1965a) has linked these elements with the pathogenesis of some chronic diseases. The investigations of this thesis are concentrated mainly on cadmium. Along with cadmium two other chemically related elements, zinc and mercury, are also briefly reviewed.

4. ELEMENTS OF THE II-B SUBGROUP

In the periodic table of elements zinc, cadmium, and mercury are all grouped together. Their natural abundance in the lithosphere and hydrosphere is in decreasing order of atomic weights; zinc predominates, while cadmium is present in a relatively higher concentration than mercury. Of these three elements only zinc is recognized as an essential one in plant and animal nutrition; the other two may act as its antagonists.

5. ZINC

Zinc is a soft white metal with a bluish tinge. Its atomic weight is 65.38; specific gravity, 7.1; melting point, 419.5°C; and the boiling point is 907°C. The main ores are saphalerite (ZnS) and calamine ($ZnCO_3$) (Browning, 1969c).

The essentiality of zinc lies mainly in its role as a constituent of various metalloenzymes, i.e., carbonic anhydrase, carboxypeptidase A, carboxypeptidase B, neutral proteases, alcohol dehydrogenases, glutamic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase, malic dehydrogenase, cytochrome reductase, alkaline phosphatases, aldolase, phospholipase C, and dipeptidase (Vallee and Wacker, 1970a). It also serves as a cofactor for arginase, enolase, lecithinase, carnosinase, oxaloacetic decarboxylase, histidine deaminase, and various peptidases (Vallee, 1959; Orton, 1966).

Involvement of zinc in DNA, RNA, and protein synthesis and metabolism has also been suggested in plants (Schneider and Price, 1962), microorganisms (Nason et al., 1953) and animals (Fujioka and Lieberman, 1964; Sandstead and Rinaldi, 1969; Weser et al., 1969; Holt et al., 1970).

Nutritional deficiency of zinc in humans is rare, and has only been noticed in certain parts of Iran and Egypt. This was due to the diet high in phytate, which prevented zinc absorption. The principal symptoms were summarized by Prasad and Oberleas (1970) as: hypogonadism, dwarfness, roughened skin, hepatospleenomegaly, iron deficiency anemia,

and general lethargy. Halsted and Smith (1970) measured plasma zinc in a variety of clinical disorders. The plasma level in healthy adults was reported as 96 μg Zn/100 ml, and in healthy children, 89 μg Zn/100 ml. No significant variations due to sex, food consumption, or diurnal changes were noted. Low plasma values of zinc were observed in cirrhosis of the liver, active tuberculosis, indolent ulcers, uremia, cystic fibrosis with growth retardation, growth retarded Iranian villagers, pregnancy, and in women taking oral contraceptives (Halsted and Smith, 1970). Malabsorption and repeated infections also induced zinc deficiency resulting in stunted growth, lack of sexual development, and poor wound healing (Prasad and Oberleas, 1970).

In animals, zinc affects growth, development of nails, horns, hooves, feathers, hair, bones, and primary and secondary sex characteristics, as well as gonadal function (Underwood, 1971b).

Metabolic interrelationship of zinc and cadmium will be discussed on a comparative basis under the main heading of cadmium.

6. MERCURY

Mercury is a silvery white metal and is liquid at room temperature. Its atomic weight is 200.61; specific gravity, 13.55; melting point, -38.87°C; and the boiling point is 357.3°C. Cinnabar (HgS) is the chief natural ore. (Browning, 1969b).

Mercury is present in food, water, and air. The hazards of mercury and mercury compounds have long been known in certain industries and rare instances of accidental poisoning have been reported. Outbreaks of mercurial poisoning from eating fish and other sea food from water reservoirs polluted with industrial mercurial waste were reported in Minamata and Niigata, Japan, during 1953-1964. The use of organic mercurials Guanosan-M (ethyl mercury compound), Agrosan-GN (ethyl mercury chloride and phenyl mercury acetate), and Panogen (methyl mercury dicyandiamide) as fungicides for seed preservation also induced mercurial poisoning in Iraq, West Pakistan, Guatemala, and New Mexico (U.S.A.) during mid to late sixties (Eyl, 1971).

The maximum allowable concentration of mercury in food is internationally accepted as 0.5 μg/g; only Sweden accepts the limit as 1 μg/g. The new safe limit for mercury under consideration by the World Health Organization is 0.05 μg/g. Recently, U.S. Food and Drug Administration detected mercury levels of over 0.5 μg/g in samples of canned tuna (Eyl, 1971). Safe daily intake of mercury is estimated to be 0.1 mg (Archives of Environmental Health, 1969). The limit set by U.S. Public Health Service for drinking water is 5 μg/liter (Hammond, 1971).

Vapour of metallic mercury and soluble mercury salts are readily absorbed by the mammalian organism. In the blood the erythrocytes tend to contain more mercury than plasma.

Inorganic mercury is universally distributed in tissues and a high accumulation is found in the kidneys. Uptake of inorganic mercury by the brain is a slow process and may lead to tremor and erethism in the affected individuals. In addition, gingivitis and proteinuria are frequently observed. Excretion of mercury takes place both through the urine and feces, although the latter route is predominant (Nordberg, 1969). In rats, Friberg (1956a) noted that almost all subcutaneously injected mercuric chloride was excreted in the urine and feces within two weeks. Inorganic mercury also appeared in rat milk (Parizek et al., 1969a).

Among organic mercury compounds the phenyl and methoxyethyl derivatives are distributed in the body similar to the inorganic compounds. Methyl, ethyl, and other alkyl derivatives, however, have an entirely different distribution pattern. Mercury is firmly bonded to a carbon atom in these compounds, therefore, the molecule is not broken down and is retained for longer periods in the organism (Goldwater, 1971). The characteristic difference is accumulation of about 15% of the total ingested dose in the brain. In addition to crossing the blood-brain barrier, methyl mercury also crosses the placental barrier and high levels are found in fetal blood as compared with the maternal blood. Blood levels of more than 100 μg Hg/100 ml are accompanied by toxic symptoms (Archives of Environmental Health, 1969). In the fetus methyl mercury damages the central nervous system and also causes teratogenic effects during the early stages of

mercury secreted in the bile. The biotransformation takes place in the large intestine by bacteria and inorganic mercury which is so released, is excreted in the feces.

Mercury found in fish is mostly methyl mercury. According to Hammond (1971) the half-life of methyl mercury in an unspecified variety of fish is about 200 days, which is three times higher than in man. Industrial waste is a direct source of methyl mercury for fish. Inorganic mercury is largely released in industrial waste and fossil fuel smoke. From the atmosphere it is brought down with the rain and eventually ends up in water reservoirs. This inorganic mercury is converted to methyl mercury compounds under anaerobic conditions by a bacterium Methanobacterium omelianskii which lives in mud at the bottom of lakes and rivers. From this bacterium, methyl mercury is taken up by plankton and thus enters the food chain of fish, and subsequently that of man (Wood et al., 1968). This biological transformation of a relatively less toxic form to highly toxic compounds of mercury is the real problem presently facing pollution control authorities.

In contrast to mercury, cadmium has different biological and biochemical properties. These will be described in detail subsequently, and compared with those of zinc and mercury.

the development. This disease has been first reported from Minamata district in Japan and it is, therefore, named the "Minamata disease" (Nordberg, 1969). Paul et al. (1971) have observed that injected methyl mercury (^{203}Hg) chloride finds its way into guinea pig milk.

Methyl mercury poisoning in adults causes irreversible damage to the nervous system. In order of increasing severity, the following manifestations are observed: paresthesia of mouth, lips, tongue, hands, and feet; fatigue; difficulty in speech; concentric constriction of visual fields, with abnormal blind spots; hearing difficulty; emotional instability; difficulty in writing, lack of memory; uncoordinated movements. Paralysis, coma, and death may result in extreme cases (Eyl, 1971). A high frequency of lymphocyte chromosome breakage has recently been reported from Sweden in persons eating methyl mercury contaminated fish (Skerfving, 1970).

The biological half-life of methyl mercury in man is about 70 days. Daily excretion is only 1% and 80% of this is via the feces (Archives of Environmental Health, 1969). The mechanism of mercury excretion has been studied by Norseth and Clarkson (1971) in rats injected with methyl mercury chloride. It is reported that in the bile methyl mercury is excreted mainly as methyl mercury cysteinate. This, however, is completely reabsorbed. Mercury found in the feces is derived predominantly from methyl mercury in intestinal cell sheddings and the protein-bound methyl

7. CADMIUM

7A. Properties and Uses:

Cadmium is a white metal with a bluish tinge; atomic weight 112.41, specific gravity 8.65, melting point 321°C and boiling point 770°C. It was first isolated by Strohmeyer of Göttingen in 1817. In its natural form cadmium exists as greenockite (CdS), but the major sources are zinc and lead ores which contain cadmium as an impurity and thus it is obtained as a by-product during processing of these ores (Browning, 1969a).

Cadmium is used for electroplating iron, steel and copper as protection from corrosion; in making alloys with other metals such as copper, lead, bismuth, nickel, silver and aluminum for use as electrical conductors, solders, antifriction and bearing metals, alkaline storage batteries, and jewelry; in the glass, paint, and plastic industry as a pigment (CdS); and as an insecticide in the form of the oxide and hydroxide (Heyroth, 1949; Lancet, 1964; Browning, 1969). Furthermore, cadmium stearate is used as a stabilizer to increase the longivity of plastic products (Suzuki et al., 1965).

Total world production of cadmium during 1968 was 14.1×10^6 Kg which was an increase of 2.3×10^6 Kg over the last five year period (Report of the Study-Group for Critical Environmental Pollution, 1970). It is obvious that only a small proportion of mined cadmium will go back to earth for redeposition, the rest will be retained by the

biosphere.

7B. Distribution in Nature:

Cadmium is present in various mineral deposits, and cadmium concentration ranges from 35-300 ng/g, with Cd:Zn ratio 1:570 to 1:320. Sea water contains 0.1 μg/liter Cd, (Cd:Zn, 1:100) (Bowen, 1966). The impact of environmental contamination with cadmium is also seen in the fresh water reservoirs. A report by Durum et al. (1971) indicated high concentrations of cadmium in water near larger cities. Out of more than 720 samples of water obtained from lakes and rivers in the United States, 42% of the samples had cadmium concentration ranging from 1-10 μg/liter. About 4% of the water samples had more than 10 μg/liter Cd which is the U.S. Public Health upper limit. The highest concentration found was 130 μg/liter in a sample from Arizona. Hasegawa (1970) reported that in Japan the maximum limit for drinking water was equivalent to the U.S. limit. Transport of water by galvanized iron, copper, or plastic pipes increases cadmium content of water (Schroeder et al., 1967). The sources are apparently the impure zinc used for galvanizing iron pipes, the copper pipe and the solder used for pipe fittings, and the cadmium salts used in the manufacture of polyvinyl plastic (Schroeder et al., 1967). Soft water (low in Ca^{2+}, Mg^{2+}, and other ions) used for municipal water supplies corrodes metal pipes. This

water tends to be acidic and makes soluble salts with metals (Schroeder, 1966). The concentration of dissolved metals increases the longer the water stands in the pipes. Stagnant water analyzed by Schroeder et al. (1967) had cadmium content as high as 77 μg/liter. Hard water, on the other hand, forms insoluble residues which deposit on the inner surface of the pipes and eventually forms a coating, thus protecting metallic pipes from corrosion.

The only country where federal limits are imposed on the cadmium content of industrial waste is Japan. According to Hasegawa (1970) the industrial drainage should not contain more than 100 μg Cd/liter.

The highest concentration of cadmium in air is found near mines and smelteries. Soot and smoke from incinerators and from industrial plants using fossil fuel, also increases the level of cadmium in the atmosphere. Coal, for example, contains 250 ng Cd/g (Bowen, 1966). The ambient air around the factories in Japan should not contain more than 0.1 μg Cd/m^3 (Hasegawa, 1970). The maximum allowable limit of cadmium in the air of industrial workshops is internationally accepted as 0.1 mg/m^3 (Lancet, 1969). Air samples collected from various U.S. cities contained 0.002 - 0.37 μg Cd/m^3 (Schroeder, 1970). In addition to industry, the general population contributes to atmospheric pollution by burning petroleum products in automobiles and heating furnaces. The extent of pollution from these sources can be visualized by comparison of cadmium analyses in samples

of melted snow picked from two different locations in New Hampshire, U.S.A. The first sample taken 20 meters from a street in Hanover had 1.5 µg Cd/liter, and the second from a hill top, 100 meters from the road, which was comparatively less exposed to motor vehicle exhaust contained only 0.35 µg/liter (Schroeder et al., 1967). Cigarette smoke is another source of cadmium. A pack of 20 cigarettes according to Schroeder and Balassa (1961) contained 30 µg Cd. Lower values, with an average of 22.7 µg were reported by Nandi et al. (1969). These investigators calculated that after passing through the filter, the smoke still retained 69% of the total cadmium. It was speculated that about 10-20% of the inhaled smoke is absorbed by the lungs (Nandi et al., 1969). This would mean that a person smoking a pack of 20 cigarettes per day may absorb about 1-3 µg Cd, additional to that from dietary sources.

The highest content of cadmium is found in sea-foods and meat products. Among sea-foods analyzed by Schroeder et al. (1967) oysters contained 3.14 - 3.66 µg Cd/g. Canned anchovies had 5.39 µg/g, probably due to contamination during food processing. The average in other sea-foods excluding oysters was 0.79 µg Cd/g (Cd:Zn, 1:22). The data of Kropf and Mallinckrodt (1968) showed the highest cadmium concentration in beef kidneys ranging between 1-40 µg/g. According to Schroeder et al. (1967) meats had an average of 0.88 µg Cd/g (Cd:Zn, 1:35). Certain oils, like those of cod liver, castor beans, and

olives had more than 1 μg Cd/g. The mean value for oils and fats was 0.75 μg/g (Cd:Zn, 1:11). Molasses and honey contained more than 0.5 μg/g. Cereals, grains, and dairy products averaged 0.16 μg Cd/g. Vegetables had lower values ranging from 0.01-0.45 μg/g. Although superphosphate (CaH_2PO_4 and $CaSO_4$) used as fertilizer had 8.97 μg Cd/g, the vegetables grown in soil containing 20% superphosphate did not show a significantly higher accumulation of cadmium than those grown in unfertilized soil. Spices in general had 0.1-0.2 μg Cd/g. Refining wheat flour and polishing rice increased cadmium concentration. A considerable quantity of zinc present in the germ was lost during this process. This resulted in an increase in Cd:Zn ratio from 1:65 in whole wheat flour to 1:26 in the refined product (Schroeder et al., 1967). Japanese rice analyzed by Schroeder et al. (1967) had 0.077 μg/g and among U.S. varieties, the rice from Mississippi contained as high as 0.137 μg/g Cd. The maximum limit set by the Ministry of Health and Welfare in Japan for rice is 1 μg/g for the unpolished rice and 0.9 μg/g for the polished variety (Hasegawa, 1970). This is supposed to keep the daily intake of cadmium below the maximum safe limit of 0.3 mg.

7C. Metabolism of Cadmium:

By definition the term metabolism is applied to biosynthetic as well as degradative process within cells and tissues (McCann, 1966). An element within an organism is

neither degraded nor biosynthesized, apart from a change in valance state for certain elements. In spite of this, the scientific literature accepts the term "trace element metabolism" in biological systems. The reason probably is convenience, and this short term replaces the phrases: "absorption, transport, cellular deposition, molecular binding, turnover, and excretion of a trace element". Therefore, in this thesis metabolism represents all processes involved in the biological cycle of a trace element.

7D. Cadmium in Human Organism:

In liver and kidneys of newborn babies Stitch (1957) reported 40 and 20 µg Cd/g ash in one out of six and one out of five specimens respectively, using spectrographic methods. Schroeder et al. (1967) using atomic absorption could not detect cadmium in the livers and kidneys from stillborns, newborns, and infants up to the age of 5 months. In children over one year of age cadmium was always present in these organs. Widdowson (1969) reported that human milk contained cadmium and week-old infants fed on mothers' milk showed a positive balance of 0.73 µg/Kg/day. This may be related to intestinal accumulation of cadmium as shown recently by Lucis et al. (1971a) in newborn rats.

The daily intake of cadmium and zinc in adults varies according to the composition of the food and it ranges between 115-500 µg and 8-13 mg respectively (Schroeder et al., 1967; Kropf and Mallinckrodt, 1968). According to the

latest calculations by Schroeder (1970) a total of 160 μg Cd and 14.5 mg Zn are ingested daily by an adult American. The intake from the air depends on local pollution levels and it may be as high as 7.4 μg for Cd and twice this amount for Zn (Schroeder, 1970). It has not been shown exactly what percentage of the ingested and inhaled cadmium is absorbed. An indirect estimate from the maximum total body content of 50 mg (Schroeder, 1970), shows that about 3 μg of Cd is retained by the organism per day.

Imbus et al. (1963) measured cadmium in the blood of healthy subjects and reported a range of 0.3-0.5 μg/100 ml. Blood samples from 243 males were also analyzed by Kubota et al. (1968) who found less than 0.5 μg Cd/100 ml in more than 50% of the cases, and less than 1 μg in 83% of all samples. No cadmium transporting protein has been isolated from the blood, although a zinc-binding α_2-globulin has recently been separated by Parisi and Vallee (1970) from human serum.

From the blood, cadmium is taken up by the kidneys and liver and the accumulation in these organs increases with the age of the individual (Perry et al., 1961; Schroeder and Balassa, 1961). It was shown that accumulation of cadmium in the kidneys occurred until the fourth decade of life. Older individuals showed a lower cadmium content in these organs (Schroeder and Balassa, 1961). In these subjects a decrease in zinc concentration and Cd:Zn ratio was also

observed (Schroeder et al., 1967). Curry and Knott (1970) in England attempted to establish an age-linked trend for cadmium accumulation, but their results revealed no definite pattern.

Variations in cadmium intake are related to geographic area and dietary habits (Perry et al., 1961). Sea-foods provide more cadmium than a vegetable diet (Schroeder et al., 1967). Kidneys of primitive people from Rwanda and Burundi in Africa, with no exposure to industrialization, had a mean concentration of 710 µg Cd/g ash. In comparison, kidney specimens from the United States analyzed by Perry et al. (1961) showed a value of 2120 µg Cd/g ash, and the specimens from Japan had three times higher cadmium content. The concentration of cadmium in the liver tissue had a similar trend. In general, Mongoloids from East and Far Eastern countries contained higher concentrations of cadmium and zinc in their tissues than Americans, Europeans, and Asiatic Caucasoids, or African Negroids (Perry et al., 1961).

Factory workers exposed to cadmium fumes during casting of cadmium alloys, had increased cadmium concentrations in the lungs, liver, and kidneys as compared to workers in other industries. High tissue levels persisted even 18 years after the exposure had ceased (Smith et al., 1960). Friberg (1957a) reported the highest values in the livers of alkaline accumulator battery workers which ranged from 2-14 mg Cd/100 g wet weight. The kidneys of these workers contained 1-8 mg Cd/100 g wet weight.

Sex differences were observed for cadmium in human hair. The mean value reported in male hair was 2.76 μg/g and that for female hair was 1.77 μg/g (Schroeder and Nason, 1969). A similar difference for zinc distribution in human hair was also noted (Strain and Pories, 1966).

In human kidneys cadmium was bound to metallothionein (Pulido et al., 1966). This protein of 10,500 ± 1,050 molecular weight was rich in sulfhydryl groups of cysteine (26 residues/mole) which accounted for the binding of divalent metal ions. Human kidney metallothionein contained 4.2% Cd and 2.6% Zn , by weight. In addition to these elements, 0.5% Hg and 0.3% Cu were also present (Pulido et al., 1966). The ratio of metal ion to sulfhydryl groups in this protein was reported as 1:3. The physiological function of metallothionein has not been elucidated.

It has been pointed out earlier in this chapter that of the total cadmium ingested, about 3 μg is retained per day; the rest of the ingested cadmium is excreted in the feces. A recent estimate indicates that urinary excretion of cadmium is 5 μg/day (McCaull, 1971). Higher values, of the order of two - (Smith and Kench, 1957; Perry and Perry, 1959) to five-fold (Schroeder et al., 1967) have been reported by others. Excretion of cadmium in urine of copper-cadmium alloy and alkaline accumulator factory workers may reach a value as high as 420 μg/liter (Smith and Kench, 1957).

Potts et al. (1950) using ^{115}Cd, demonstrated that in dogs and mice, inhaled $^{115}CdCl_2$ was absorbed from the lungs within 30 minutes and was deposited in the liver. In dogs, the intravenously injected $^{115}CdCl_2$ was cleared rapidly from the plasma (Walsh and Burch, 1959) and concentrated by the liver as well as by the kidneys (Potts et al., 1950). Similar results were reported in rats by Decker et al. (1957). After initial depletion, the remaining blood radioactivity was localized in the blood cells (Friberg, 1952). Electrophoresis of the hemolysate revealed that ^{115}Cd was bound to hemoglobin (Carlson and Friberg, 1957). Friberg (1952), and Gunn and Gould (1957) showed that in the kidneys ^{115}Cd was retained mainly by the cortex.

The availability of ^{109}Cd facilitated whole body sagittal section autoradiography. Berlin and Ullberg (1963) using this technique found that one hour after intravenous injection of $^{109}CdCl_2$ in mice, the highest radioactivity appeared in the liver followed by the kidneys and pancreas. The distribution pattern of radioactivity remained unchanged for 16 days. Within the liver, the concentration was higher towards the periphery of the lobules than towards center of the organ. In the kidneys, the cortex contained more ^{109}Cd than the medulla. Radioactivity was also detected in the hair follicles, and in interstitial tissue of the testes. Lucis and Lucis (1969) injected an equimolar mixture of $^{109}CdCl_2$ and $^{65}ZnCl_2$ to four inbred strains of mice and

7E. Cadmium in Animal Organism:

Rats and mice maintained on cadmium-free diet from the time of weaning grew normally and no effect on their life span was observed. Schroeder et al. (1963a,b) concluded that cadmium was not an essential trace element for these species of animals. This is probably true for other animals and man as well. Deficiency of cadmium has never been reported to cause any metabolic disorder. On the other hand, exposure to this element produces a variety of acute and chronic toxic effects in both animals and humans which will be described later under toxicity of cadmium.

The earliest work on the metabolism of cadmium in animals was published in 1867 by Marmé and cadmium was detected in the blood, liver, kidneys, heart and brain. In most of the early work, the element in animal tissues was measured chemically after oral (Schwartze and Alsberg, 1923), subcutaneous (Friberg, 1952) and intravenous administration (Tepperman, 1947) of cadmium salts, or after inhalation of cadmium dust and fumes by the animals (Friberg, 1950; Princi and Geever, 1950). After oral administration, deposition of cadmium occurred chiefly in the kidneys and liver. Inhaled cadmium was absorbed from the lungs and concentrated by the kidneys and liver. Administration of cadmium salts by other routes resulted in a higher uptake of cadmium by the liver than by the kidneys.

found significant variations in the uptake of ^{109}Cd by the testes of different strains. In contrast, other tissues showed no strain differences in the uptake of ^{109}Cd. ^{65}Zn concentration in various tissues was similar in all strains studied. Studies by Lucis et al. (1969) in rats, after subcutaneous injections of $^{109}CdCl_2$ revealed that most tissues reached a maximum level of ^{109}Cd within 4 hours. The turnover of the isotope was slow.

It was shown by Cotzias et al. (1962) and Cotzias and Papavasiliou (1964) that the total body pool of zinc was controlled by a homeostatic mechanism which regulated the absorption and excretion of this element. Cadmium, acting as an antimetabolite of zinc, replaced some zinc from the tissues (Cotzias et al., 1961a). The distribution of cadmium in the tissues was, however, not influenced by high dietary or injected loads of cadmium. Absorption continued from the intestine in spite of high tissue levels, suggesting that no homeostatic mechanism existed to control the absorption of cadmium (Cotzias et al., 1961b). It is possible that in animal tissues this element is bound to proteins similar to horse kidney metallothionein (Margoshes and Vallee, 1957) which has greater affinity for cadmium than for zinc (Kägi and Vallee, 1961).

Excretion of cadmium takes place mainly via feces (Burch and Walsh, 1959). Lucis et al. (1969) observed that in one week 2% of subcutaneously injected ^{109}Cd appeared in rat feces and 1% in the urine. While a decrease

in ^{109}Cd content of stomach wall and small intestine wall occurred with time, large intestine wall showed a continuous increase (Lucis et al., (1969). It is possible that intestinal tract may have a dual role, one for the excretion of cadmium and the other for its reabsorption.

7F. Effects of Cadmium in Humans:

Cadmium metal is soluble in dilute acetic and other organic acids which are commonly present in food. Until the middle of the twentieth century, the use of cadmium in electroplating household utensils and food containers caused a number of acute cadmium poisonings (Griebel and Weiß, 1931; Frant and Kleeman, 1941; Taylor and Hamence, 1942; Schiftner and Mahler, 1943). As a result of these reports, the Sanitary Code of New York City was amended and the use of cadmium, in articles which came in contact with food and drinks, was prohibited (Fairhall, 1945). The symptoms of cadmium poisoning were described as: increased salivation, immediate vomitting, abdominal pain, and diarrhea, lasting for about 24 hours. Acute poisoning with cadmium was usually followed by a complete recovery (Bonnell, 1965).

Chronic accumulation of cadmium in the human organism was suspected by Schroeder (1964a) as one of the contributing factors in the pathogenesis of hypertensive cardiovascular disease. Carroll (1966) found a positive correlation between the concentration of cadmium in the air and the death rates from hypertension and arteriosclerotic

heart disease. Studies by Schroeder (1960a,b; 1966) revealed that in municipal drinking water there was a negative correlation between water hardness and cardiovascular disease. On the other hand, soft water showed a positive correlation. The corrosive property of soft water was suggested as a contributing factor towards increased death rates due to hypertension and arteriosclerotic heart diseases (Schroeder, 1966). It was later discovered (Schroeder et al., 1967) that soft water, during passage through the pipes could acquire levels of cadmium higher than the permissible limit of 10 μg/liter. Further evidence which actually connected cadmium with the hypertensive cardiovascular disease came from the studies of Tipton and her colleagues (Tipton and Cook, 1963; Tipton et al., 1965). Analysis of their data by Schroeder (1964a; 1965b) showed that kidney specimens from subjects who died from hypertensive complications had a higher molar Cd:Zn ratio than the tissue specimens from individuals where death was caused by accidents and other non-cardiovascular causes. Similar studies on hospitalized patients by Morgan (1969) failed to confirm Schroeder's observations. Bonnell et al. (1959) and Holden (1969) who conducted routine examination of cadmium-exposed factory workers, reported that they did not find any elevation in the blood pressure of their patients. It was also reported by Perry and Schroeder (1955) that hospitalized hypertensive patients excreted significantly higher quantities of cadmium in urine as compared to the normotensive controls. This

was not confirmed by Szadkowski et al. (1969) who performed similar analyses. Due to controversial reports on this subject by different investigators, it is not possible to reach a conclusion. Further work is needed to prove or disprove the role of cadmium in pathogenesis of cardiovascular diseases.

Another chronic disease where cadmium pollution is suspected to have affected the general population has been reported in Japan (Tsuchiya, 1969a). This disease which is called "itai-itai" in Japanese is characterized by severe pain in the joints which slowly leads to complete disability and crippling. Bones become thin and fragile. Deformation of stature due to bending of bones is also noted. Clinical laboratory tests showed proteinuria, aminoaciduria, glycosuria, hypercalcuria, hyperphosphaturia, and an increase in serum alkaline phosphatase activity (Ishizaki and Fukushima, 1968; Tsuchiya, 1969a). The incidence of itai-itai disease was limited to Fuchu-machi town in Toyama prefecture. River and well water in this area contained more than 10 μg Cd/liter. Rice and soybean grown in soil containing high concentrations of cadmium accumulated considerable quantities of cadmium along with lead and zinc, which were also abundant in the soil of this area. The source of these heavy metals was a mine located upstream at the Jintsu river, which released raw waste into river water. A positive relationship was found between the concentration of cadmium in the soil and the geographic distribution of the disease (Tsuchiya, 1969a,b). Once again, it remained to be clarified whether cadmium was

the only agent causing itai-itai disease or merely one of the factors in the etiology of this chronic ailment.

Cadmium was also evaluated for a possible involvement in inducing tumorigenesis. Morgan (1970) found that the plasma of patients with bronchogenic carcinoma had 2.2 µg Cd/100 ml as compared to the normal values of 1.2 µg/100 ml. Autopsy specimens of liver and kidneys of these subjects showed approximately 50% increase in cadmium concentration in comparison to the control group. Zinc, on the other hand, showed 20% decrease in plasma values with no change in liver concentration, while the kidney tissue contained 20% more zinc. Earlier, Kipling and Waterhouse (1967) examined case histories of 248 factory workers who had been exposed to cadmium for more than one year during their occupation. An increased incidence of prostatic carcinoma among these workers, as compared to the general population, was reported. The carcinomas of all other sites including the bronchus were found in a number close to the epidemiological figures. Regarding the prostatic carcinoma, Kipling and Waterhouse (1967) commented that "it would be manifestly unwise to infer from the present evidence alone the existence of an industrial hazard".

However, this statement should not give the impression that cadmium does not cause chronic industrial poisoning. In fact, diseases of the respiratory tract and kidney are common among workers casting cadmium alloys, manufacturing alkaline storage batteries, and handling

cadmium pigments. Friberg (1950) published a complete account of chronic cadmium toxicity in workers of alkaline accumulator industries. The predominant feature of respiratory tract damage was pulmonary emphysema. This was also reported by Baader(1952), Bonnell (1955), and Holden (1965). Other disorders included: fatigue (Friberg, 1950), anosmia (Friberg, 1950; Adams and Crabtree, 1961; Holden, 1965; Potts, 1965), chronic rhinitis (Bonnell et al., 1959; Bonnell, 1965), and ulceration of the nasal mucosa (Friberg, 1959). In addition, golden-yellow rings at the alveolar margin of the teeth were noticed (Princi, 1947; Friberg, 1950; Baader, 1952). Among the urinary tract disorders proteinuria was the most frequent observation. It was characterized by urinary excretion of proteins ranging in molecular weight from 20,000 to 30,000 (Friberg, 1950; Bonnell et al., 1959; Potts, 1965). Proteinuria was regarded as a diagnostic test and it could occur even in the absence of any signs of emphysema. Chronic cadmium poisoning was declared as an insidious disease, since in a number of cases, both proteinuria and emphysema developed many years after the last exposure to cadmium (Friberg,1957b; Bonnell, 1965). Piscator (1962) reported that the amount of protein excreted in the urine was proportional to the length of exposure to cadmium. The same investigator (Piscator, 1962; 1966a) noted that in comparison with normal urinary proteins, cadmium-exposed subjects excreted more proteins having electrophoretic mobility similar to serum globulins. The

molecular weight range of these proteins was measured by gel filtration to be between 10,000 to 30,000 (Piscator, 1966a). According to Kazantzis et al. (1963) and Piscator (1966c) renal damage in cadmium poisoning was tubular in origin. The low molecular weight proteins found in urine were also detected in serum by immunoelectrophoresis (Piscator, 1966b). Abnormal albumins of molecular weight between 5,000 - 20,000 were isolated by Kench and Sutherland (1965) from both urine and serum of men poisoned with $CdCl_2$. These proteins were termed "minialbumins" due to their antigenic similarity on Ouchterlony plates with albumin of normal molecular weight, and a very similar amino acid composition (Kench and Sutherland, 1965). Other manifestations which accompanied or followed cadmium-induced proteinuria in factory workers were: anemia (Friberg, 1950); renal calculi (Friberg, 1950; Ahlmark et al., 1960); aminoaciduria (Clarkson and Kench, 1956; Kazantzis et al., 1963); glycosuria (Bonnell et al., 1959; Kazantzis et al., 1963); hypercalcuria, impaired acid-base balance, and hyperchloremic acidosis (Kazantzis et al., 1963).

7G. Effects of Cadmium in Animals:

It was shown by Schwartze and Alsberg (1923) that ingested cadmium chloride could produce strong emesis in cats. This effect was similar to cadmium poisoning in humans.

Marmé in 1867 first described damage to the kidneys of pigeons, rabbits, cats, and dogs after intravenous,

subcutaneous, and oral administration of cadmium salts. Renal damage was also observed by Severi (1896) and later by Alsberg and Schwartze (1919) in rats, rabbits, cats, and dogs.

Cadmium fumes were used by Prodan in 1932 to produce emphysema and inflammatory changes in the liver and kidneys of cats. Feeding cadmium chloride to rats bleached the yellow incisor teeth (Wilson et al., 1941). Friberg (1950) produced severe anemia, emphysema, and proteinuria in rabbits after prolonged exposure to cadmium sulfate. The proteinuria developed experimentally in rabbits had similar characteristics to that observed in factory workers (Axelson and Piscator, 1966a). The increase in urinary proteins was accompanied by an increase in cadmium excretion (Friberg, 1952). Specific damage to the renal tubules and interstitial fibrosis in rats and rabbits was reported by Dalhamn and Friberg (1957) and Bonnell et al. (1960). Autoradiography of mouse kidney sections, 24 hours after intravenous injection of $^{109}CdCl_2$ revealed that ^{109}Cd was localized in the first part of the proximal convoluted tubules (Berlin et al., 1964). In rabbits exposed to 0.25 mg $CdCl_2$/Kg, 5 times a week for a maximum of 29 weeks, renal lesions were characterized by degenerative cytoplasmatic and nuclear changes in the proximal tubular epithelium (Axelson et al., 1968). Axelson and Piscator (1966a) reported that in rabbits the glomerular filtration rate was not affected; glucose absorption was impaired; and the alkaline phosphatase activity in the renal

cortex was lowered. Spontaneous repair of tubular damage occurred with time, although large deposits of cadmium which were accumulated during a 24 week exposure to 0.25 mg $CdCl_2$/Kg, 5 times a week, were still present in the tubular epithelium. Piscator and Axelson (1970) explained that this was due to increased synthesis of metallothionein which tightly bound metal ions and the toxic effects on renal tubules were thus neutralized. Treatment of cadmium poisoned animals with BAL (Tepperman, 1947; Dalhamn and Friberg, 1955), EDTA (Friberg, 1956b), and cysteine (Kennedy, 1968) increased urinary excretion of cadmium. At the same time the severity of renal lesions became more pronounced. This was due to the fact that more cadmium in the chelated form was diverted from other tissues to the kidneys. It was not shown, however, whether increased cadmium deposits found in the kidneys were in the form of chelated cadmium or in the form of cadmium ions. The mechanism of accumulation of cadmium by the kidneys is still poorly understood.

Other abnormalities which were commonly reported in animals after exposure to cadmium were: accelerated destruction of erythrocytes (Berlin and Friberg, 1960), increase in plasma volume (Berlin and Piscator, 1961), hemolytic anemia (Piscator, 1963), and ahaptoglobulinemia (Piscator, 1963; Axelson and Piscator, 1966b).

"Minialbumins" were isolated from the blood and urine of cadmium-poisoned monkeys by Kench and Sutherland

(1965). These proteins differed from normal serum albumin with respect to molecular weight and tryptophan content. Molecular weights of minialbumins ranged from 5,000 to 20,000 and tryptophan was completely absent. Apart from this, the comparison of amino acid composition revealed that minialbumins had less lysine and cysteine residues than the normal serum albumin. In addition, serum albumin of normal molecular weight obtained from cadmium-poisoned monkeys was low in tryphophan. These abnormal molecules were considered to have originated by aggregation of small molecular weight peptides. It was suggested by Kench and Sutherland(1967) that minialbumins were produced from the fission of normal albumin molecules and during this process tryptophan and some lysine and cysteine residues were cleaved off from the polypeptide chain. However, the possibility that cadmium may interfere at some stage during the biosynthesis of albumin, cannot be ruled out.

Experimentally, hypertension was induced in rats either by chronic feeding of 5 mg Cd/liter as $CdCl_2$ in drinking water (Schroeder and Vinton, 1962; Schroeder, 1964b) or by a single intraperitoneal injection of 2 mg cadmium acetate/Kg body weight (Schroeder et al., 1966). It was reported that the incidence of hypertension was greater in female than in male rats. In its severity, cadmium-induced hypertension was comparable to that produced by unilateral renal artery constriction (Schroeder et al., 1966). Schroeder (1964b) reported that once the animals were made hypertensive, they

remained so while maintained on a normal diet. In addition, normal rats developed hypertension if maintained on soft water which was known to contain cadmium (Schroeder, 1967). Elimination of cadmium from the diet and from the drinking water resulted in gradual lowering of blood pressure (Schroeder et al., 1968). Schroeder (1967) pointed out that the molar ratio of Cd:Zn in the kidneys of rats played an important role in experimental hypertension. The minimum Cd:Zn ratio required to induce hypertension was described as 0.37, while if it exceeded 0.46, the animals were always hypertensive. In rats with Cd-induced hypertension, injection of a chelate of zinc, 2-diaminocyclohexane-disodium-zinc-tetra-acetate, in a dose equivalent to 9.1 mg of Zn decreased renal Cd:Zn ratio and the hypertension regressed (Schroeder and Buckman, 1967). It was shown in vitro, that the chelating agent trans-cyclohexane-1 , 2-diamine-N,N,N′,N′-tetra-acetic acid had greater stability constant (log K) for cadmium as compared to zinc. Perry and Yunice (1965) gave intraarterial injections of 10-40 μg Cd^{2+} as $CdCl_2$ in anesthetized rats and observed a pressor effect. This effect was dose dependent. At higher dose levels cadmium showed depressor activity. Under these conditions mercury displayed pressor activity at all dose levels and zinc had no effect on the blood pressure. It can be deduced that in rats cadmium can elevate blood pressure and make the animal hypertensive. The significance of critical Cd:Zn ratio in the kidneys is still not known.

Alsberg and Schwartze in 1919 noted that cadmium salts caused changes in the appearance of rat testes and the testes looked blue. This observation remained overlooked for a long period. In 1956 Parizek and Zahor described hemorrhage followed later by necrosis of the testicular tissue, in mature male rats after a single subcutaneous injection of 0.03 mmole cadmium chloride or cadmium lactate. This damaging effect to testes was specific for cadmium since mercuric chloride was not effective. The observations of Parizek and Zahor were soon confirmed by other groups (Meek, 1959; Kar and Das, 1960; Gunn et al., 1961) using injections of 0.02-0.04 mmole $CdCl_2$/Kg body weight. It should be noted here that only a small fraction of this dose of cadmium reached the testes, and caused specific damage; other tissues, such as liver and kidneys which concentrated most of the injected cadmium, did not develop such degenerative changes at this dose level. Gunn et al. (1963a) stated that in addition to testis, the proximal end of the caput epididymis was also damaged by cadmium. Parizek (1960) demonstrated that newborn rats were not susceptible to testicular damage by cadmium for the first 9 days of life. It was shown histologically (Kar and Das, 1960; Mason et al., 1963) and by electron microscopic studies (Chiquoine, 1964) that the primary effect of cadmium was on the capillary endothelium and not on the seminiferous tubules as postulated by Parizek (1957). Clegg and Carr (1967) reported that the capillaries and venules began to

leak within 10 minutes after cadmium-induced damage, and the leakage was mainly between the endothelial cells. In rats the blood flow to testes started to decrease within 3 hours after injection of cadmium (Waites and Setchell, 1966) and it was completely blocked by 6 hours (Niemi and Kormano, 1965). The interlobular edema followed by anoxia caused degeneration of the testicular tissue (Mason et al., 1964a). The damage to seminiferous tubules was permanent and resulted in sterilization of the male rats (Parizek, 1957; 1960). However, the interstitial tissue (Leydig's cells) regenerated with time (Parizek, 1957, 1960; Meek, 1959; Kar and Das, 1960; Gunn et al., 1961) along with the establishment of new blood vessels (Niemi and Kormano, 1965). The proliferation of interstitial tissue continued and Gunn et al. (1963b) observed that 70-80% of the rats and mice developed interstitial cell tumors one year after a single subcutaneous injection of 0.03 mmole $CdCl_2$/Kg. The tumor cells were completely irresponsive to the interstitial cell-stimulating hormone (Gunn et al., 1963b). Morphologically these cells appeared normal, but their testosterone synthesizing ability in vitro was markedly reduced (Gunn et al., 1965c; Favino et al., 1966). Lucis et al. (1971b) reported that actually the conversion of androstenedione to testosterone was remarkably lowered. Apparently, in the tumor cells, the enzymatic activities involved in steroidogenesis were altered.

All animal species with scrotal testes, i.e., mouse, rat, rabbit, hamster, guinea pig, and gerbil, with the exception of opossum and ferret were found to be cadmium-sensitive, while those with abdominal testes, i.e., fowl and other birds, were resistant to testicular damage by subcutaneously injected cadmium salts (Chiquoine, 1964; Chiquoine and Suntzeff, 1965). In addition, strain specificities to testicular damage were observed among the sensitive species (Chiquoine and Suntzeff, 1965; Gunn et al., 1965a). After intratesticular injection of cadmium, however, the testes of all species were damaged (Cameron, 1965; Chiquoine and Suntzeff, 1965). Prevention of testicular injury was achieved by various agents. Simultaneous administration or pretreatment of rats with 100 fold molar excess of zinc salts prevented the injury to testes (Parizek, 1956, 1957; Gunn et al., 1961) and also protected the vascular epithelium of the testes (Gunn et al., 1963a). Development of interstitial tumors was also inhibited with this dose of zinc (Gunn et al., 1963b). Selenium as selenium dioxide, when administered simultaneously in twice molar excess to cadmium chloride, prevented damage to rat testes (Kar et al., 1960; Mason et al., 1964b; Mason and Young, 1967). Protection against cadmium damage to the testes was also offered by estrogens but not by androgens (Gunn et al., 1965b). Among sulfhydryl compounds, BAL, cysteine and glutathione were also effective in prevention of testicular necrosis (Gunn et al., 1966). A natural defense mechanism against cadmium

toxicity was suggested by Terharr (1965). Oral treatment of rats with 20mg $CdCl_2$/Kg at least 7 hours prior to a subsequent dose of 100 mg $CdCl_2$/Kg, prevented testicular atrophy as well as death. It is possible that cadmium exerts its harmful effects on the testes by reacting with sulfhydryl groups of the enzymes in the vascular epithelium which apparently require zinc for optimal activity. The mechanism of protection by pretreatment with small doses of cadmium will be discussed later in this thesis.

Parizek and Zahor (1956), in their earliest study reported no effect of cadmium on the ovaries of female rats. However, Kar et al. (1959) observed that in prepubertal rats, subcutaneous injection of 1 mg $CdCl_2$/100g induced profound cellular and vascular changes in the ovaries. The ovaries of nonovulating rats which where experimentally maintained in persistant estrus, showed extensive hemorrhage and necrosis after receiving 0.02-0.04 mmole cadmium chloride or cadmium acetate per Kg (Parizek et al., 1968b). The placenta was also damaged by cadmium. Parizek (1964) observed that in pregnant rats injected subcutaneously with 0.04 mmole $CdCl_2$/Kg on the 17th and 21st day of pregnancy, the fetal part of placenta was destroyed, causing death of the embryos. The abortive effect of cadmium was also documented in mice (Chiquoine, 1965). It was also reported by Chiquoine (1965) that fertility in mice was not permanently affected. Injection of 0.02 mmole cadmium acetate/Kg during the last 4 days of pregnancy caused severe toxicity

in the female rats. Symptoms of this "toxemia of pregnancy" as described by Parizek (1965) were the appearance of blood in the urine within 6 hours, followed by violent convulsions and death in 76% of the animals. The presence of a placenta was essential for the "toxemia of pregnancy". Removal of fetuses alone did not lower mortality of the mothers, unless the placenta was also excised (Parizek et al., 1969a). Female rats suffered necrosis of the acinar epithelium in lactating mammary glands, when exposed to cadmium chloride after delivery (Parizek et al., 1969a). The toxic effects of cadmium in female rats were not produced by equimolar mercuric chloride. Parizek et al. (1969a) found that the toxicity of cadmium was prevented by 100 times molar excess of zinc during all stages of pregnancy and lactation. They reported that simultaneous injection of equimolar sodium selenite along with cadmium chloride was also effective in protection against cadmium toxicity. The mechanism of protection by selenium seems to be different from that by zinc. Selenium apparently combines with cadmium in the organism, and alters its reactivity and tissue distribution. The circulating levels of cadmium bound to macromolecules were increased after treatment with selenium salts (Parizek et al., 1968a; 1969c). Zinc in contrast did not affect the concentration of cadmium in the blood (Parizek et al., 1969b). It probably competes with cadmium for the binding sites on essential biological molecules. The reason for the diversion of more cadmium to the liver after injection of zinc

(Parizek et al., 1969b) has not been explained.

Teratogenesis by cadmium was first observed by Ferm and Carpenter in 1967, in golden hamsters. A single intravenous injection of 2 mg $CdSO_4$/Kg to the animals on the 8th day of gestation produced specific facial malformations in 60% of the embryos. The most significant features were unilateral and bilateral cleft lips and palates. Using radioactive cadmium Ferm et al. (1969) demonstrated the presence of ^{109}Cd in the embryos, 24 hours after the injection of 2 mg $^{109}CdCl_2$/Kg intravenously into the mother. Simultaneous injection of equimolar $ZnSO_4$ did not stop the passage of ^{109}Cd into the embryos (Ferm et al., 1969), although teratogenesis by cadmium was completely prevented (Ferm and Carpenter, 1968). Mulvihill et al. (1970) also reported deleterious effects of cadmium on cartilage and bone formation especially in the areas where clefts were present. However, they failed to determine whether the effect of cadmium was local in the embryonic tissue or if it was secondary to metabolic changes in the mother.

In recent years the effect of sublethal doses of cadmium on the central nervous system of experimental animals has been investigated. In newborn rats injected subcutaneously with 1 mg $CdCl_2$/100 g (0.055 mmole/Kg) between day 1 and day 20 of life caused hemorrhagic lesions in the brain (Gabbiani et al., 1967b). In older animals, and during adult life, the lesions were produced in the sensory ganglia of rats, rabbits, guinea pigs, and hamsters (Gabbiani, 1966;

Gabbiani et al., 1967b). Electron microscopic examination of rat sensory ganglia 24 hours after injection with cadmium chloride revealed the destruction of endothelial cells of small vessels (Gabbiani and Baic, 1969). The ganglionic lesions healed spontaneously within 15 days. Pretreatment with 6 mg $CoCl_2$, 17 hours before injection of 2 mg $CdCl_2$ in adult rats, inhibited hemorrhage of the sensory ganglia (Gabbiani et al., 1967a). Animals receiving intravenously 200 μg $CdCl_2$ daily for 5 days acquired resistance to 800 μg $CdCl_2$ given on the 6th day. This dose injected alone was toxic to the nervous system and to the testes (Gabbiani et al., 1967a). These observations suggest once again that a natural defense mechanism exists in animals which can be triggered by injection of small prophylactic doses of cadmium salts. Effects of zinc have not been investigated.

Administration of cadmium by subcutaneous, intramuscular, and intratesticular routes causes a local inflammatory reaction in the tissues. This initial inflammatory response diminished with time. A variety of tumors developed at the site of injection. Guthrie (1964) produced teratomas and Sertoli cell adenomas in fowl testes as early as 53 days after intratesticular injection of cadmium chloride. Rhabdomyosarcomas and fibrosarcomas were induced in rats 13 weeks after subcutaneous and intramuscular injection of metallic cadmium suspension (Heath and Daniel, 1964). Sarcomas were also reported after subcutaneous injection of cadmium sulfate solution (Haddow et al., 1964). These tumors were

also observed in rats after subcutaneous and intramuscular injection of water insoluble cadmium sulfide; subcutaneous injection of water insoluble cadmium oxide (Kazantzis and Hanbury, 1966); and after subcutaneous injection of cadmium chloride (Gunn et al., 1964; Lucis et al., 1971b). Gunn et al. (1964) found that zinc was effective in preventing these sarcomas, if given in 100 times molar excess to the dose of cadmium.

7H. Effects of Cadmium on Cells and Subcellular Elements:

In the past, the biochemistry of heavy metals has not been adequately explored. However, with the increasing awareness for pollutants, the primary mechanisms by which these elements affect the biological systems are now being investigated in some detail.

The effect of the cadmium ions on pulmonary alveolar macrophage (PAM) cells of sheep has been reported by Cross et al. (1970). These cells are directly exposed to environmental inhalants. In the lungs the macrophages carry out chemotaxis, pinocytosis, phagocytosis, and bacterial destruction. Metabolically, PAM cells have a high oxygen consumption in vitro. Respiration of these cells is inhibited in vitro by 5 mM Cd^{2+} added to the medium. The mitochondrial respiration is much more sensitive and is inhibited by 0.5 mM Cd^{2+}.

All multivalent cations, with the exception of alkaline earths, agglutinate the red blood cells in vitro

(Passow et al., 1970). Generally, there is no hemolysis at concentrations of metal ions which can produce agglutination. Cd^{2+} and Hg^{2+} are exceptions, and produce hemolysis at much lower concentrations; 50 μ moles $CdCl_2$/ml and 100 μ moles $HgCl_2$/ml cause hemolysis of human erythrocytes suspended in isotonic saline. A concentration of 1 μ mole $ZnCl_2$/ml agglutinates erythrocytes without hemolytic action. The presence of plasma proteins in the medium, however, prevents agglutination and hemolysis of red blood cells by metal ions. This may reflect a natural defense mechanism existing in the blood.

It is known that Ca^{2+} is required for the functioning of neurones in vitro. According to Goldman (1970) Zn^{2+} and Cd^{2+} can replace Ca^{2+} on a molar basis and the effectiveness of these two ions on the lobster axons is in the order: $Zn^{2+} > Cd^{2+}$. It is questionable whether Cd^{2+} and Zn^{2+} actually take part in the transport of nerve impulses in the in vivo system.

A review on mitochondrial ion transport by Mela (1970) states that both Cd^{2+} and Zn^{2+} increase the permeability of beef heart and rat liver mitochondria to Mg^{2+} and K^{+}, in vitro. The increased accumulation of K^{+} and Mg^{2+} produced by Cd^{2+} and Zn^{2+} is effective only in the presence of an energy source and inorganic phosphate. Furthermore, it is sensitive to uncouplers of oxidative phosphorylation. In the presence of 100 μ M Cd^{2+} or Zn^{2+}, the accumulation of Mg^{2+} by the mitochondria is increased more than two-fold. Zn^{2+} binding

to mitochondria, in turn, is inhibited by K^+ and Mg^{2+}. The effect of Cd^{2+} and Zn^{2+} on the mitochondrial membrane seems to be of a specific nature, since permeability to all ions is not increased. The accumulation of Ca^{2+}, Sr^{2+}, and Mn^{2+} in the mitochondria is also energy dependent, but it is not affected by Cd^{2+} or Zn^{2+}. The energy dependent K^+ accumulation by mitochondria is also induced by Hg^{2+}, but to a lesser extent than by Cd^{2+} or Zn^{2+}. In the red blood cells Hg^{2+} inhibits the ATPase system and increases the passive transport of K^+ and Na^+, but not that of choline (Passow, 1970). The ATPase of pulmonary alveolar macrophage cells is also sensitive to Cd^{2+} and 50% of this enzyme system is inhibited by 2 mM Cd^{2+} (Cross et al., 1970). The effect on ion transport in these cells has not been studied.

7I. Effects of Cadmium on Macromolecules:

Metal ions, i.e., Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, and Cu^{2+} can bind to the nucleic acid molecules and to the synthetic polynucleotide chains at phosphate groups of the ribose phosphate moiety and also to the proton donor sites at the heterocyclic bases (Eichhorn et al., 1970). Binding of metals, i.e., Mg^{2+} to the phosphate group increases the stability of molecular structures like the DNA helix, by neutralizing the repelling negative charges of the phosphate groups. In contrast, the binding of Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} to the phosphate groups of RNA or polyribonucleotide structures causes degradation of the molecule by cleavage of

the phosphodiester bonds at the 5' position. This reaction requires heat and the presence of the 2'-hydroxyl group on the sugar moiety. Binding of metal ions with bases destabilizes the ordered structure of DNA by displacing the hydrogen bonds. In other words, metal binding with the phosphate group of DNA increases the stability of the molecule and hence increases the half-melting temperature (Tm), while metal-base interaction has an inverse effect. Reaction of DNA with II-B metal ions (Zn^{2+}, Cd^{2+}, Hg^{2+}) as well as Mg^{2+} reveals that all these ions can bind with both phosphate and the base, but their relative binding to the base as compared to the phosphate differs and is in the increasing order: $Hg^{2+} \gg Cd^{2+} > Zn^{2+} \gg Mg^{2+}$, as determined by Tm studies. When DNA is heated in the presence of these metal ions, randomly coiled single strands are produced, which are held together by cross-links through metal-ion bridges. The extent of cross-linking is dependent on the relative affinity of the metal ions for the bases. On cooling the heat-denatured DNA in the presence of Mg^{2+}, no rewinding is observed; Zn^{2+} on the other hand, completely rewinds the two strands. The rewinding of the heat-denatured DNA by Cd^{2+} is brought about only in the presence of an electrolyte. The effects of Zn^{2+} and Cd^{2+} on the unwinding and rewinding during replication and transcription of DNA molecules, or on the stability of transfer RNA molecules *in vivo* are still obscure.

It has been described earlier that zinc participates in a variety of catalytic reactions in the body. Replace-

ment of zinc by cadmium or mercury may be one way of exerting toxicity in the tissues. On an enzyme molecule the potential metal binding sites are: the sulfhydryl group of cysteine, the imidazole group of histidine, the phenolic group of tyrosine, the ϵ-amino group of lysine, the secondary carboxyl groups of glutamic and aspartic acids, and the α-amino and α-carboxyl groups of the polypeptide chain (Vallee and Wacker, 1970b). Among all these groups, Zn^{2+}, Cd^{2+}, and Hg^{2+} have the highest affinity for the sulfhydryl groups. Mercury binds most strongly with this group and its association constant ($\log K$) for the $R\text{-}S^-$ ligand is more than 20. Log K for Cd^{2+} is 8, and that for Zn^{2+} is 7 (Passow, 1970). The inhibition of enzymic activity by metal ions is, in many cases, proportional to the solubility products of metal sulfides (Vallee and Wacker, 1970c).

The effects of Cd^{2+} on enzyme activities *in vivo* and *in vitro* have been studied by several investigators. Weber and Reid (1969) found that after feeding cadmium acetate (412 µg Cd/g of synthetic diet) to chicks, the activities of malic dehydrogenase and glucose-6-phosphate dehydrogenase were increased in the liver and heart tissues, but not in the kidneys. The succinic dehydrogenase of these tissues was not affected. Earlier, Simon *et al.* (1947) reported inhibition of succinic dehydrogenase activity by 0.1 mM $CdCl_2$ added to the lung, liver, and kidney tissue preparations, *in vitro*. The histochemical observations of Singh and Mathur (1968) revealed an increase in the activity

of this enzyme in testicular tissue of the desert gerbil 12-24 hours after the subcutaneous injection of 0.5 mg $CdCl_2$/ 100 g. At this dose level two other enzymes ATPase and 5'-nucleotidase of the testis were inhibited. A recent report published by Kench and Gubb (1970) indicated that Cd^{2+} affected the activities of several enzymes of chick liver, both in vivo and in vitro. Chick embryos were injected with 0.11-0.13 μ mole $CdCl_2$ into a superficial blood vessel 7 days before hatching. The liver tissue of the newly hatched chicks was analyzed for cadmium content and the activities of various enzymes were assayed. Purified enzymes were also studied in vitro using concentrations of cadmium comparable to those detected in the tissue fractions. It was reported that the tryptophan oxygenase was stimulated by less than 0.2 mM Cd^{2+} in vitro and by 70 μM Cd^{2+} in vivo. This enzyme was, however, inhibited in vivo at Cd^{2+} concentrations exceeding 0.5 mM. The activities of δ-aminolevulinate dehydratase, erythrocytic ATPase, and malate dehydrogenase in the liver cytosol, were enhanced by Cd^{2+} at certain unspecified concentrations both in vivo and in vitro. In contrast, lipoamide dehydrogenase, δ-aminolevulinate synthetase and xanthine dehydrogenase were inhibited both in vitro and in vivo. Cytochrome oxidase and succinic dehydrogenase were inhibited by Cd^{2+} in vitro but not in vivo. Some other mammalian enzymes like, histidine ammonia-lyase, arginase (Vallee, 1959), and phosphopyruvate hydratase (Dixon and Webb, 1964) were activated by Cd^{2+}; and carnosinase (Lehninger, 1950), flavine mononucleotide

phosphatase, homoserine dehydratase, 3-hydroxybutyrate dehydrogenase, and leucine aminopeptidase (Webb, 1966) were inhibited by Cd^{2+} *in vitro*. Carboxypeptidase A has been studied in its purified form. This enzyme has 1 Zn atom/molecule in the native state and exhibits both esterase and peptidase activities. Vallee *et al.* (1963) found that Cd^{2+} and Hg^{2+} enhanced the esterase activity of this enzyme and completely abolished the peptidase activity. The effect of cadmium on enzyme systems *in vivo* still needs further studies.

8. OBJECTIVES OF THIS INVESTIGATION

As reviewed in the previous pages, the metabolism of cadmium has not been fully investigated. The accumulation of this element in the kidneys and liver of animals as well as humans has been well documented. From these tissues cadmium- and zinc-binding proteins have been separated, however, the biological function of these proteins is still not clear. It is generally believed that cadmium competes with zinc for binding sites at biological macromolecules and thereby interferes with their normal function, resulting in growth retardation and other functional abnormalities. In view of this background, the present investigation was planned and some aspects of cadmium metabolism were explored.

First of all the intestinal absorption of cadmium was compared with zinc, using radioactive isotopes, ^{109}Cd and ^{65}Zn. To find the transport mechanism, plasma was analyzed for ^{109}Cd and ^{65}Zn binding. The uptake of injected

^{109}Cd and ^{65}Zn by various tissues and the turnover of these isotopes from the tissues was followed in rats and mice, to study the biological differences between these two elements. These comparative distribution studies were further expanded from the tissues to the subcellular and molecular levels. This led to the finding of ^{109}Cd-binding proteins in rats and mice. Basing on the assumption that cadmium-binding proteins (Cd-BP) may represent a biological detoxication mechanism against heavy metals, Cd^{2+}, Zn^{2+}, Hg^{2+}, as well as Pb^{2+}, Ni^{2+}, and Co^{2+} were tested for their effect on Cd-BP synthesis. The cadmium-induced Cd-BP were isolated and purified, and their molecular weights and amino acid compositions were determined. The question arose as to whether cadmium was bound to the degradation products of normal cellular proteins, or to specific protein molecules synthesized de novo. Therefore, the biosynthesis and degradation of Cd-BP were investigated utilizing exogenous ^{14}C-labeled amino acids, under a variety of experimental conditions. In addition to liver and kidneys, the pancreas, spleen, and placenta were also analyzed for the presence of Cd-BP. To verify the presence of these proteins in primates, tissue specimens from Rhesus monkey and humans were also subjected to similar analyses. The isolated human kidney proteins were labeled in vitro with ^{109}Cd and purified. Since mercury is chemically related to cadmium, the distribution and cytoplasmic localization of ^{109}Cd in rat tissues was compared with that of ^{203}Hg.

CHAPTER II. MATERIALS & METHODS

1. MATERIALS

1A. Animals and Tissues:

Male and female Wistar rats weighing 250 ± 50 g were purchased from Woodlyn Farms Ltd., Guelph, Ontario, Canada. Male mice of DBA/1J strain (average weight 23 ± 2 g) were obtained from Jackson Laboratories, Bar Harbor, Maine, U.S.A. Rhesus monkey liver specimen was removed surgically from a 2 year old female Macaca mulatta. Human liver and kidney tissues were autopsy specimens from a 64 year old male who died of myocardial infarction. These tissues were removed within 24 hours after death.

1B. Radioactive Isotopes:

Radioactive cadmium and zinc, and labeled amino acids were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. The radiochemical purity of $^{109}CdCl_2$ (carrier free) and $^{65}ZnCl_2$ (specific activity 3.78 mCi/mg) was greater than 99%. Radioactive amino acids used for experiments were: uniformly labeled L-cystine-^{14}C (specific activity 265 mCi/mmole), uniformly labeled L-threonine-^{14}C (specific activity 164 mCi/mmole), and DL-lysine-2-^{14}C monohydrochloride (specific activity 3.25 mCi/mmole). The analyses of the supplier indicated that the radiochemical purity of these amino acids was greater than 98%. $^{203}HgCl_2$ (specific activity 3.52 mCi/mg) was prepared by the Atomic Energy of Canada, Ltd.

1C. Chemicals:

All chemicals, except those specified otherwise, were purchased from the Fisher Scientific Company, Canada, and were of certified A.C.S. grade. Sephadex gels, DEAE-Sephadex, and molecular weight marker kit, were all purchased from Pharmacia, Canada. A separate protein molecular weight marker kit and additional proteins used for standardization of gel filtration columns were obtained from Mann Research Laboratories, Inc., U.S.A. Gel electrophoresis reagent kit was purchased from Canalco, Inc., U.S.A.

2. METHODS

2A. Experiments with Animals:

Rats and mice were maintained on Purina Rat Chow and distilled water. In one experiment distilled water was substituted by 0.5 mM $CdCl_2$ solution containing 56.2 mg Cd/liter. Usually the animals were kept in plastic cages, except in a few experiments where the rats were housed in stainless steel cages to collect the urine and the feces separately.

All radioactive and non-radioactive solutions used for injections were prepared in sterile isotonic saline (0.9% NaCl solution). Animals were injected under light ether anesthesia. Subcutaneous injections were given in the interscapular region. In case of multiple or repeated

injections, different sites were selected on the back of the rats for each injection. Intravenous injections were administered either in the tail vein or in the abdominal vena cava. In some experiments ^{109}Cd and ^{65}Zn containing solutions were injected intragastrically. For these experiments the rats were fasted for 24 hours and anesthetized with 10 mg/Kg sodium pentobarbital (Nembutal, Abbott Laboratories Ltd., Canada) intraperitonially. A polyethylene capillary tube was passed through the esophagus into the stomach and the radioactive solution was ejected.

Blood was collected from the abdominal aorta of rats, while the animals were under ether anesthesia. To draw the blood a hypodermic syringe containing approximately 0.05 ml of sodium heparin (10,000 I.U./ml, Connaught Medical Research Laboratories, Toronto, Canada) was used. After collection, the blood was mixed with heparin by gently inverting the syringe 3 or 4 times.

The animals were killed either by drawing the remaining blood, or by an overdose of chloroform inhalation. Tissues were dissected out, cleaned of fat and connective tissue, washed in isotonic saline, blotted, and weighed individually. Whole organs and weighed portions of liver, kidney, and skeletal muscle were placed in separate γ-counting tubes and were covered with 10% formaldehyde solution to make a total volume of 3 ml. Formaldehyde solution was used as a preservative for the tissues. The remaining protions of the liver and kidneys were processed within one hour for the

isolation of cadmium-binding proteins (Cd-BP), or frozen at -10 to -20°C for future experiments.

2B. Fractionation of Blood:

Five ml of heparinized blood was centrifuged within 10-15 minutes after collection, at 3,000 rpm for 15 minutes, in an International refrigerated centrifuge (model PR-6, rotor 269) at 4°C. After centrifugation the plasma was aspirated and placed in a γ-counting tube. The cells were re-suspended in 8 ml of isotonic saline solution and centrifuged again at 3,000 rpm for 15 minutes to obtain the washed blood cells. To prepare the hemolysate, 8 ml of distilled water was added to the washed cells and mixed thoroughly. After 5 minutes the hemolyzed suspension was centrifuged at 3,000 rpm for 15 minutes. The supernatant and the cell debris were transferred separately to γ-counting tubes.

2C. Fractionation of Tissues:

The tissue homogenization was carried out in an ice-cold solution (pH 8.6) which was 0.25 M with respect to sucrose and 0.001 M with respect to Tris. A 20% w/v homogenate was prepared using an all-glass homogenizer (Pyrex, model 7727) or glass homogenizer fitted with a Teflon pestle (Thomas, model C-14685). Human liver and kidney tissues were ground in a porcelain grinder before the final processing in glass homogenizer.

The procedure of Schneider (1964) was used for separation of subcellular fractions. The cell debris and nuclei were sedimented at 1,000 x g for 10 minutes in the International refrigerated centrifuge (model PR-6, rotor 169) at 4°C. After centrifugation the supernatant (S1) was removed by aspiration and the sediment was resuspended in 2 volumes of 0.25 M sucrose solution and recentrifuged. The mitochondrial fraction was obtained by centrifuging S1 at 7,500 x g for 10 minutes in an International preparative ultracentrifuge (model B-35, rotor A-147) at 4°C. The supernatant (S2) was transferred to another container and the mitochondrial pellet was washed with 2 volumes of 0.25 M sucrose solution. To sediment the microsomal fraction, the S2 was centrifuged at 105,000 x g for one hour in the model B-35 centrifuge, using swinging bucket rotor SB-283. The final supernatant was labeled as the soluble cytoplasmic fraction. Microsomes were suspended in 0.25 M sucrose solution and recentrifuged for one hour as described for S2, to obtain a washed microsomal preparation. Each subcellular fraction was suspended in distilled water and transferred quantitatively into γ-counting tubes. The washings of the subcellular particles were also transferred into separate γ-counting tubes. In most experiments, tissue homogenates were not subjected to differential centrifugation and only the last step, i.e., centrifugation at 105,000 x g was employed to obtain the soluble fraction directly.

2D. Gel Chromatography:

Sephadex G-75 and G-25 Fine were prepared for column chromatography as directed by the manufacturer (Pharmacia). Before use, the dry gel was added to at least 500 times (w/v) distilled water and swelled at room temperature for 24 hours or longer. After swelling, one volume of the gel was suspended in 5 volumes of distilled water and allowed to stand until gel particles, except the very fine ones, were settled. The supernatant liquid and the fine particles were decanted and this procedure was repeated 4-5 times. The gels were stored at 4°C in distilled water containing 0.02% sodium azide.

Glass columns were packed at room temperature. Each column was first filled up to 3/4 of its volume with distilled water and then the rest of the space was filled by adding gel slurry. The top of the column was fitted with a glass funnel which was filled with the gel slurry. The gel was allowed to settle by gravity. When 2-3 cm of the gel had settled at the bottom of the column, the outlet of the column was opened and a flow rate of 10-15 ml/hour was established. The funnel was disconnected after the gel bed was packed to 6-10 cm from the top of the column. Packed columns were stored in the cold room at 4°C. Before use, the newly packed columns were equilibrated with 3 void volumes of the eluting solvent.

A 2.5 x 90 cm column (Pharmacia) packed with Sephadex G-75 (total bed volume 460 ml) was used for gel filtration of the soluble fraction. Between 5-14 ml of soluble fraction was applied on the column under a layer of the eluting buffer (0.001 M Tris-HCl, pH 8.6) as described by Determan (1968). The column was eluted by downward flow and the flow rate was controlled by overhead pressure of the buffer reservoir at 18-20 ml/hour, or by a peristaltic pump (Technicon, Autoanalyzer pump II) at 27 $\pm$ 0.5 ml/hour. The effluent from the column was monitored at 2537 Å by an LKB Uvicord I (model 4700) and %T was recorded by LKB recorder (model 6520A). Fractions were collected directly into the γ- counting tubes every 10 minutes, using an LKB RadiRac fraction collector (model 3400).

Protein molecular weights were determined by a 1.5 x 85 cm Sephadex G-75 column (bed volume 120 ml). About 5 mg of the protein and 1 mg of sucrose was dissolved in 1 ml of the buffer, applied on the column, and eluted with 0.05 M Tris-HCl, pH 7.5 buffer containing 0.5 M KCl (Andrews, 1964). The flow rate of the column was controlled by the peristaltic pump at 12 $\pm$ 0.2 ml/hour. Standardization of the column was performed by using pepsin dimer (MW 71,000), bovine albumin (MW 67,000), ovalbumin (MW 45,000), chymotrypsinogen A (MW 25,000), ribonuclease A (MW 13,700), and cytochrome C (MW 12,400). The elution volume of each standard was plotted on a semi-logrithmic graph paper, against the respective molecular weight (Andrews, 1964). Unknown molecular weight

of a protein was determined from the standard curve.

For desalting operations, 2.5 x 56 cm column (bed volume 200 ml) was packed with Sephadex G-25 Fine. Tris and sodium azide were removed from the cadmium-binding proteins by gel filtration. A 0.1-1 % solution of freeze-dried protein containing salts was applied to the column as described earlier, and eluted with distilled water. The flow rate was controlled by the peristaltic pump at 25 $\pm$ 0.5 ml/hour. The effluent was monitored at 2537 Å and collected in separate γ-counting tubes.

2E. Ion-Exchange Chromatography:

DEAE-Sephadex A-25 was prepared and packed in the columns as described for the other Sephadex gels. Glass columns (Pharmacia) measuring 1.5 x 27 cm and 2.5 x 34 cm were used for analytical and preparative purposes. The total bed volume of 1.5 x 27 cm column was 40 ml and that of 2.5 x 34 cm column was 175 ml. Their void volumes were 20 and 68.5 ml, respectively. Before application of the sample, the columns were eluted with 2 void volumes of 0.001 M Tris-HCl, pH 8.6 buffer containing 0.02% sodium azide. About 5-10 mg of protein and 1 mg of sucrose were dissolved in 1 ml of 0.001 M Tris-HCl, pH 8.6 buffer and applied under a layer of this buffer, to the 1.5 x 27 cm column. It was then eluted with a Tris gradient which was formed by mixing 0.25 M Tris-HCl, pH 8.6 buffer with 100 ml of 0.001 M Tris-HCl, pH 8.6 buffer. The higher concentration buffer was added to the

mixing flask at 12 ml/hour using a peristaltic pump. The mixture was drawn out of the flask at the same rate and fed to the DEAE column. On the preparative column (2.5 x 34 cm) 50 mg of freeze-dried protein dissolved in 2 ml of the starting buffer was applied. For this column the gradient was prepared by mixing 0.5 M Tris-HCl, pH 8.6 buffer with 500 ml of 0.001 M buffer at 23 $\pm$ 0.5 ml/hour using a peristaltic pump. The flow rate of the column was 23 $\pm$ 0.5 ml/hour. U.V. monitoring, recording, and the fraction collection was performed as described before. The ionic strength of the gradient was determined by conductivity measurements using a conductivity meter (Radiometer, Denmark, model CDM2e).

2F. Freeze-drying and Storage of Protein:

The eluted fractions which contained proteins, were shell frozen in VirTis flasks and lyophilized using a VirTis freeze-drier (model FD3-U7). Lyophilized samples were stored at $4^{o}C$ in a desiccator containing anhydrous $CaCl_2$.

2G. Amino Acid Analysis:

The protein samples ranging in weight between 0.5 to 1.0 mg were hydrolyzed with 6N HCl in a nitrogen atmosphere and under reduced pressure, at $110^{o}C$ for 24 hours. To estimate total cysteine and cystine residues (as cysteic

acid), the protein sample was oxidized with performic acid, before hydrolysis with HCl. The performic acid was prepared by adding 1 volume of 30% H_2O_2 to 9 volumes of HCOOH. This mixture was heated in a water-bath at 50°C for one hour and then cooled in an ice-bath. To about 1 mg of protein 1 ml of the ice-cold performic acid was added and stirred overnight at 4°C. The oxidized protein solution was diluted with distilled water and freeze-dried. It was then hydrolyzed with HCl as described above. The amino acid composition of the hydrolysate was determined on a Spinco (model 120C) amino acid analyzer, using the chromatographic system of Moore and Stein (1963).

2H. Electrophoresis of Plasma Proteins

Rat plasma was separated as described earlier. Paper electrophoresis was used for the separation of plasma proteins. The electrophoresis buffer 0.08 M barbital-sodium barbital, pH 8.6 was prepared by dissolving premixed buffer B-2 components (Beckman, U.S.A.) in distilled water. A Whatman 3 MM filter paper sheet measuring 17.5 x 41 cm was moistened with the buffer and placed in the electrophoresis chamber (LKB, model 3276B). With a capillary pipette 0.2 ml of the plasma was applied on the paper sheet and the electrophoresis was carried out at 200 volts for 12 hours, at room temperature. After electrophoresis, the filter paper sheet was taken out of the electrophoresis chamber and dried in an oven at 105°C.

A longitudinal strip measuring 1 cm in width was cut from the middle of the electrophorogram and stained for protein with Amido-black 10B as described in the instruction manual of LKB. The rest of the sheet was divided and cut horizontally into 0.5 cm wide strips. For determination of radioactivity each paper strip was folded and placed in separate γ-counting tubes containing 3 ml of distilled water.

2I. Electrophoresis of Cadmium-binding Proteins:

The homogeneity of the isolated cadmium-binding proteins was determined by electrophoresis on cellulose acetate strips and on polyacrylamide gel. The buffer used for the cellulose acetate electrophoresis was 0.028 M Tris-sodium barbital, pH 8.8, obtained in premixed form from Gelman Instrument Company. Before use, 2.54 x 17 cm cellulose acetate strips (Gelman, Sepraphore III) were soaked in the buffer for 15 minutes. The procedure described in the Gelman electrophoresis manual (No. 70176-A) was followed throughout. On the line of origin one-half of the strip was used for the application of purified Cd-BP and the other half for the crude protein. About 1-2 μl of each protein solution equivalent to 4-8 μg protein was applied on the cellulose acetate membrane. Electrophoresis was performed in a Gelman (model 51170) electrophoresis unit at 200 volts (1.5 mA/strip) for 90 minutes, at 4^{o}C. The protein bands were stained with Ponceau S. The strips were dehydrated with

methanol and made transparent with 10% acetic acid in methanol.

For disc electrophoresis, the reagent kit and equipment of Canalco Inc., U.S.A. (model 1200) were used. The method described in the instruction manual was based principally on the technique devised by Ornstein (1964), and Davis (1964). Three layers of gel were polymerized individually, one on top of another in a successive order. The lower most layer was the separating gel, the middle layer was the stacking gel, and the top layer consisted of the loading gel. Details of the constituents of each layer were as follows:

(a) Separating gel: (7.18% polyacrylamide, pH 8.9)

(1) HCl, 0.06 M

(2) 2-Amino-2-hydroxymethyl-1 ,3-Propandiol, 0.373 M

(3) N,N,N',N'-Tetramethylethylene-diamine, 2.58 mM

(4) Ammonium persulfate, 3.06 mM

(5) Acrylamide monomer, 7.0%

(6) N,N'-Methylenebisacrylamide, 0.184% (2.625% of total acrylamide)

(b) Stacking and Loading gels: (3.025% polyacrylamide, pH 6.7)

(1) HCl, 0.68 M

(2) 2-Amino-2-hydroxymethyl-1 , 3-propandiol, 0.0617 M

(3) N,N,N',N'-Tetramethylethylene-diamine, 4.95 mM

(4) Riboflavin, 0.011 mM

(5) Sucrose, 0.584 M

(6) Acrylamide monomer, 2.5%

(7) N,N'-Methylenebisacrylamide, 0.525% (21% of total acrylamide)

The component of the gels were mixed in appropriate proportions, just before use. For polymerization of gels, 0.5 x 7.5 cm glass tubes were used. First of all, 1 ml of the separating gel solution was delivered to each tube and polymerized at room temperature. After 30 minutes, 0.2 ml of the stacking gel solution was layered on top of the polymerized separating gel and left under fluorescent light at room temperature for another 30 minutes. Protein solution (10-15 μl) containing 20-25 μg cadmium-binding protein was then delivered to each tube and mixed with 0.2 ml of the loading gel. This layer of gel was also polymerized under fluorescent light for 30 minutes. Electrophoresis of the protein was carried out at room temperature, in 24.76 mM tris (2-amino-2-hydroxymethyl-1, 3-propandiol)— 191.82 mM glycine buffer, pH 9.5. A constant current of 5 mA/tube was supplied for a period of 45 minutes. After electrophoresis, one of the duplicate gels was stained and the other was assayed for radioactivity. Before staining with Coomassie Blue, the gels were fixed with 12% trichloroacetic acid. The gels were destained and stored in 7% trichloracetic acid. The unstained gels were sliced with a lateral gel slicer (Canalco, model 1802) into 1.5 mm thick discs. Each disc was placed in a separate γ-counting vial containing 3 ml of distilled water.

2J. Determination of Radioactivity:

^{109}Cd and ^{65}Zn were counted in a Nuclear Chicago dual channel γ-spectrometer (model 1084), with one channel set for each isotope. While ^{109}Cd radioactivity was completely excluded from the ^{65}Zn-channel, 10-12% of ^{65}Zn radioactivity was detected by the ^{109}Cd-channel. The efficiency of the γ-spectrometer for counting NEN standard rods was 51% for ^{109}Cd and 8% for ^{65}Zn. A mixture of ^{109}Cd and ^{65}Zn when counted together, gave a value close to the expected number of counts up to 10^4 cpm for ^{109}Cd and 5×10^4 cpm for ^{65}Zn.

^{203}Hg was counted separately in another Nuclear Chicago γ-spectrometer (model 1084) set for this isotope. No ^{109}Cd was counted by the ^{203}Hg-channel, while 40% of ^{203}Hg contributed to ^{109}Cd-channel. Only two isotopes, ^{109}Cd and ^{203}Hg were counted simultaneously. Corrections were made to obtain the actual counts for each isotope.

A Packard β-spectrometer (model 3365) was used to quantitate the radioactivity of ^{14}C. The scintillation fluid was prepared by dissolving 14g of BBOT (2,5-bis-[2-(5-tert-butyl-benzenoxazolyl)] -thiophene) in 2 liters toluene and 1 liter of Triton X-100. Using an automatic pipette fitted with disposable plastic tip (Oxford, U.S.A.) 1 ml of the aqueous radioactive solution was transferred to the β-counting vial. To each sample, 10 ml of the scintillation fluid was added and mixed thoroughly. The clear solution was counted in the spectrometer, which was calibrated by using

benzoic acid-7-^{14}C standard (New England Nuclear Corp.). The β-counting efficiency was determined by an external standard and experimental color quench curve. The efficiency of the spectrometer for counting unquenched benzoic acid in the pressence of 1 ml of distilled water averaged 45%. An external standard was used to determine the extent of quenching for each sample.

2K. Determination of Protein:

Proteins were quantitated as described by Lowry et al. (1951). The following reagents were used:

Reagent A was prepared by dissolving 2% $(Na)_2CO_3$ in 0.1N NaOH.

Reagent B contained 0.5% $CuSO_4.5H_2O$ in 1% Na-tartarate. This reagent was prepared fresh daily.

Reagent C was a mixture of 50 ml of reagent A and 1 ml of reagent B.

Reagent D was commercially available Folin-Ciocalteu reagent (British Drug Houses), diluted with 2 volumes of distilled water.

Crystalline bovine serum albumin fraction V (Calbiochem, U.S.A.) was dissolved in distilled water to a concentration of 125 μg/ml. Duplicate sets were used for each standard and unknown sample. Reagent blanks consisted of 0.8 ml of distilled water. Standard protein samples ranged from 12.5 to 100 μg of bovine albumin. The unknown

protein solution containing approximately 20-60 μg of protein was used. The total volume in each tube was adjusted to 0.8 ml with distilled water. To all tubes, 2 ml of reagent C was added and mixed thoroughly. After 10 minutes at room temperature, 0.2 ml of reagent D was delivered to each tube and mixed with the contents at once. The reaction mixture was left undisturbed at room temperature for one hour. The optical density of the blue color was measured at 720 nm by a Baush and Lomb (model, Spectronic 20) spectrophotometer; or Karl Zeiss (model, PMQ II) spectrophotometer. The protein concentration in the unknown sample was calculated from the standard curve.

2L. Determination of Nitrogen:

The method used for nitrogen estimation was described by Lanni et al. (1950). All samples were prepared in duplicate in 1.3 x 10 cm Pyrex tubes. Standard $(NH_4)_2SO_4$ (100 μg nitrogen/ml) was pipetted into separate tubes using 0.05 -0.30 ml of solution equivalent to 5-30 μg nitrogen. The volume of the unknown protein solution varied between 0.05 to 0.3 ml, containing approximately 10-25 μg nitrogen. As reagent blank, 0.2 ml of distilled water was used. To all tubes 0.14 ml of diluted H_2SO_4 (1 volume of concentrated H_2SO_4 + 1.2 volume of distilled water) was added and the tubes were placed in a metal digestion rack. To avoid bumping during digestion, the water was driven off from the

samples by heating the metal rack over a low flame, in a fume hood. The flame was increased gradually to the maximum. After 30 minutes the flame was removed and the tubes were cooled. Two drops of 30% H_2O_2 were added at this stage to all tubes. The rack was reheated, starting from low flame and gradually increasing the flame. After heating for 30 minutes, the tubes were cooled again. If the samples were not completely decolorized, an additional drop of H_2O_2 was added to all tubes and the tubes were reheated as described above. To the completely digested samples 5 ml of distilled water was added and mixed thoroughly. The color was developed with 2 ml of Nessler's reagent, which was added to each tube and mixed with the contents by a footed glass stirring rod. After keeping the tubes at room temperature for one hour, the optical densities of the samples were read in a Karl Zeiss Spectrophotometer (model PMQ II) at 440 nm. A standard curve was constructed and the quantity of nitrogen in the unknown sample was determined from the graph.

CHAPTER III. RESULTS

1. THE FATE OF ORALLY ADMINISTERED ^{109}Cd AND ^{65}Zn

The intestinal absorption and fecal excretion of ^{109}Cd and ^{65}Zn was studied in male Wistar rats. One ml of aqueous solution containing 20 nmoles of $^{109}CdCl_2$ and 20 nmoles of $^{65}ZnCl_2$ was injected intragastrically in each of 2 rats and the animals were maintained in metabolic cages. One of the animals was killed after 24 hours and the other after 14 days. The fecal and urinary excretion of ^{109}Cd and ^{65}Zn was followed in the latter animal. Figure 1 shows the cumulative excretion of both isotopes in the feces. Within 2 days after the intragastric dose, 79.8% of the ^{109}Cd was eliminated by the feces. During the subsequent 12 days the excretion of ^{109}Cd was small and accounted for 1.75% of the original dose. The excretion of ^{65}Zn was also rapid during the first 2 days of the study, and 45.45% of the administered dose was recovered in the feces. After this period, the excretion of ^{65}Zn continued at a slower rate. No ^{109}Cd was detected in the urine, while 0.3% of the total dose of ^{65}Zn was excreted via this route during 14 days.

Analyses of tissues from the experimental animals, shown in Table 1, indicated that 24 hours after the intragastric dose, peripheral blood contained ^{65}Zn but no detectable ^{109}Cd. In the liver, kidneys, stomach, and the small and large intestines, ^{109}Cd was found together with ^{65}Zn. The concentration of ^{109}Cd in other tissues was less than 0.1 pmole/g. One day after the gastric dose, the highest concentration of ^{109}Cd was detected in the small

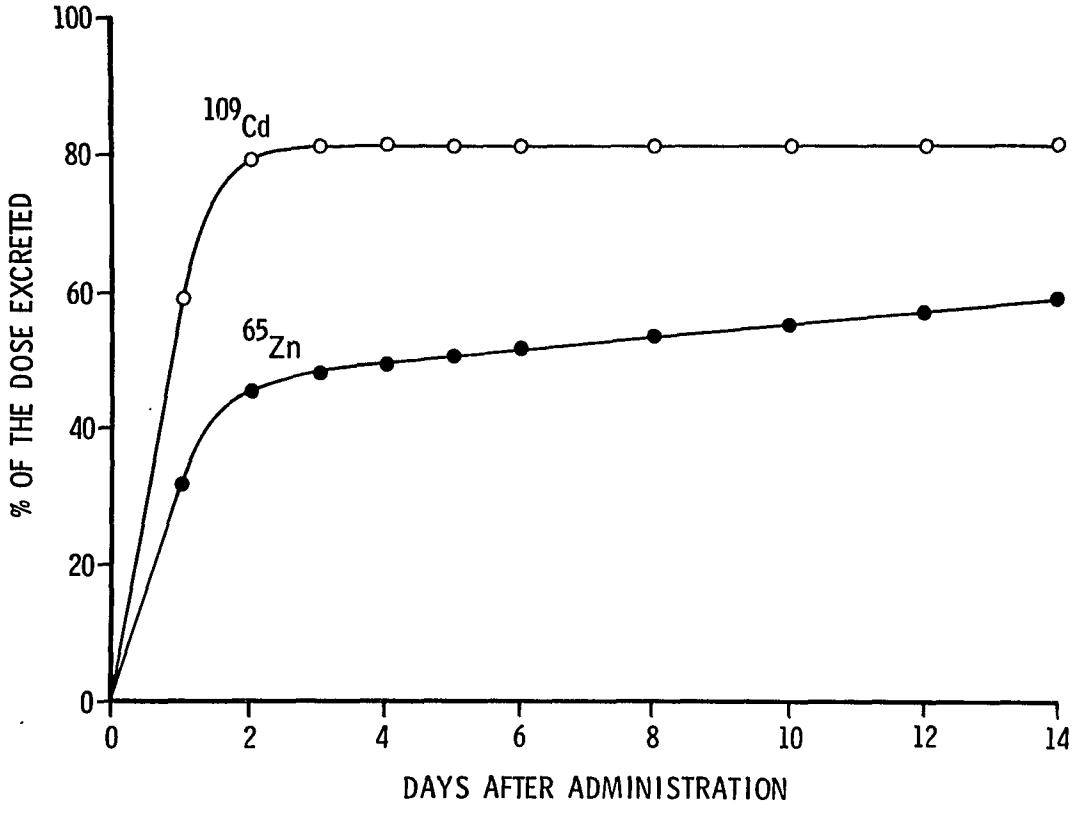


FIGURE 1. Elimination of ^{109}Cd and ^{65}Zn in rat feces after intragastric administration.

TABLE I. Distribution of ^{109}Cd and ^{65}Zn Injected Intragastrically

Time after Injection	Isotope	pmoles per gram of tissue							
		Blood	Liver	Kidneys (Total)	Kidney Cortex	Kidney Medulla	Stomach*	Small Intestine*	Large Intestine*
24 hr.	^{109}Cd	N.D.**	6.6	9.0	10.6	2.4	14.8	52.2	26.8
24 hr.	^{65}Zn	11.4	128.8	99.6	111.4	50.2	47.8	80.4	50.4
14 days	^{109}Cd	N.D.**	5.4	8.6	10.2	2.2	11.6	1.8	0.2
14 days	^{65}Zn	8.2	34.4	28.8	30.2	15.8	23.4	11.8	16.6

* Without contents.
**Not Detected.

intestine, followed by the large intestine and the stomach. Zinc-65 was absorbed to a greater extent from the alimentary canal than ^{109}Cd. Unlike ^{109}Cd, the concentration of ^{65}Zn in the liver and kidney tissues exceeded that of the small intestine. A marked depletion of ^{65}Zn was observed in all tissues after 14 days and of the total radioactivity detected after 24 hours, only one third was left in the liver and kidneys. In contrast, there was little change in ^{109}Cd content of these tissues. During the 14-day period, a loss of ^{109}Cd was observed in the stomach and the intestines. The uptake and retention of ^{109}Cd, per unit weight of tissue, was greater in the kidneys than in the liver, while ^{65}Zn was taken up more by the liver than by any other tissue studied. In the kidneys, both isotopes were localized predominantly in the cortical tissue.

2. DISTRIBUTION OF ^{109}Cd AND ^{65}Zn IN THE PLASMA

Since the disappearance of orally ingested ^{109}Cd in the blood was faster as compared to ^{65}Zn, it was suspected that these elements were transported by separate biological systems. An investigation of this hypothesis was performed. The intravenous injection was chosen to obtain the rapidly-labeled components of blood with high specific activity. One ml of isotonic NaCl solution containing 20 nmoles of $^{109}CdCl_2$ and $^{65}ZnCl_2$ each, was injected into the abdominal vena cava of an adult male rat. For the analyses, the blood

was collected into a heparinized syringe 10 minutes later, and centrifuged to separate the plasma and the cells. Of the total radioactivity in the blood, 94.5% of ^{109}Cd and 93.6% of ^{65}Zn was present in the plasma. Fractionation of the plasma by a Sephadex G-75 column revealed that 55% of the ^{109}Cd and 79% of the ^{65}Zn present in the plasma was bound to proteins eluted in the void volume. The plasma was also separated by paper electrophoresis to determine the nature of these proteins. Figure 2 shows that ^{109}Cd was associated mainly with the α-globulins, while ^{65}Zn migrated with both α- and β-globulin fractions. In addition, 17.9% of the ^{109}Cd and 18.2% of the ^{65}Zn was also bound to albumin.

3. DISTRIBUTION AND TURNOVER OF ^{109}Cd AND ^{65}Zn INJECTED SUBCUTANEOUSLY

The distribution and turnover of ^{109}Cd and ^{65}Zn was studied in adult male Wistar rats and male mice of the DBA/1J strain. The rats were injected subcutaneously with 1 ml of a solution containing 20 nmoles of each isotope. The mice received one-half of this dose. After injection, these animals were killed at regular intervals reaching up to 600 hours (25 days).

The fate of ^{109}Cd and ^{65}Zn in the blood of rats was followed for 48 hours. Analyses of blood, 30 minutes after injection, revealed that the plasma contained more ^{109}Cd and ^{65}Zn than the cells (Table II). In the plasma, ^{109}Cd

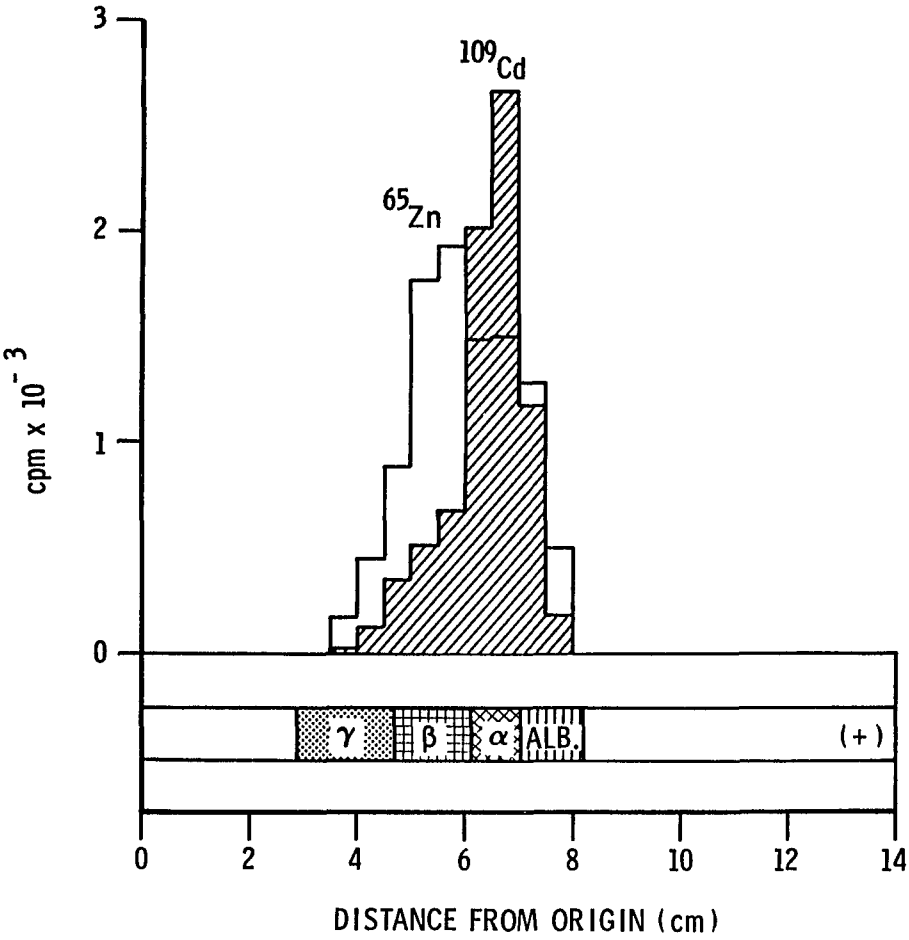


FIGURE 2. Association of ^{109}Cd and ^{65}Zn with rat plasma proteins.

TABLE II. Turnover of ^{109}Cd and ^{65}Zn in Rat Blood

Hours after Injection	(% of dose per ml)x100			
	Plasma*		Cells*	
	^{109}Cd	^{65}Zn	^{109}Cd	^{65}Zn
0.5	13.76	15.28	1.16	2.52
2	8.34	19.13	1.32	5.71
4	4.18	16.80	1.23	9.92
6	2.33	13.29	1.12	10.94
8	1.68	9.83	1.29	12.41
10	1.50	12.82	1.22	14.09
12	0.83	8.47	1.49	14.36
24	0.57	7.55	1.74	17.63
36	0.00	4.73	4.06	24.80
48	0.26	4.20	5.63	20.80

*Each value is average of two animals.

declined rapidly during the first 6 hours and then at a slower rate (Figure 3). By 48 hours, the radioactivity of ^{109}Cd in the plasma was negligible and close to the background (12 cpm). The $^{109}Cd/^{65}Zn$ ratio in the plasma decreased from 0.9 at the beginning of the experiment to 0.06 at 48 hours. In comparison with ^{109}Cd, the depletion of ^{65}Zn from the plasma was considerably slower. The concentration of ^{109}Cd in the blood cells remained constant for approximately 12 hours. Its uptake by the cells then accelerated and the $^{109}Cd/^{65}Zn$ ratio increased from 0.01 to 0.27. On the other hand, the appearance of ^{65}Zn in the blood cells was demonstrable from 30 minutes after injection, and the uptake continued for 48 hours.

Notable differences were also found in the uptake of ^{109}Cd and ^{65}Zn by other tissues. The greatest affinity towards ^{109}Cd was exhibited by the liver, in both rats and mice. In decreasing order of ^{109}Cd uptake, per gram of tissue, the liver was followed by kidneys, pancreas, adrenals, salivary glands, spleen, heart, lungs, thymus, testes, prostate, urinary bladder, epididymides, seminal vesicles, epididymal fat, skeletal (thigh) muscle, and the brain (Tables III - VI). In contrast to ^{109}Cd, the uptake of ^{65}Zn in both rats and mice was the highest in pancreas, followed by the kidneys and liver. The distribution of radioactivity in other tissues of rats and mice showed species specificity (Tables VII - X). Rat tissues in order of decreasing concentration of ^{65}Zn were: spleen, adrenals, salivary glands,

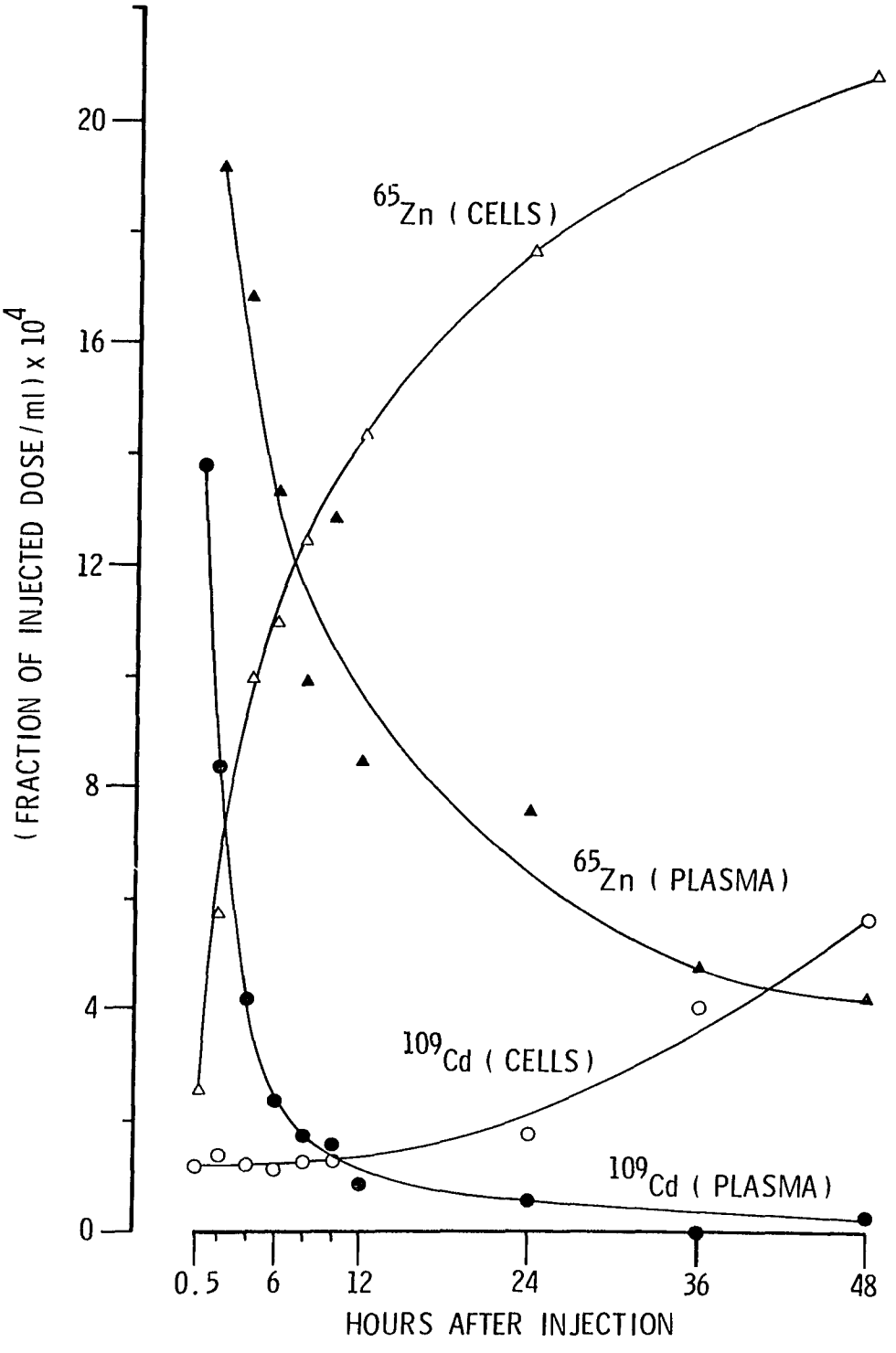


FIGURE 3. Turnover of ^{109}Cd and ^{65}Zn in rat blood after subcutaneous injection.

TABLE III. Turnover of ^{109}Cd in Rats, 0.5 to 336 Hours after Injection

Hours after Injection	^{109}Cd *(% of injected dose per gram of tissue)x10							
	Skeletal Muscle	Testes	Epididymis	Epididymal Fat	Seminal Vesicles	Lungs	Thymus	Salivary Glands
0.5	0.07	0.25	0.19	0.07	0.09	0.53	0.39	0.80
2	0.06	0.50	0.36	0.10	0.28	0.67	0.54	1.16
4	0.09	0.77	0.53	0.12	0.28	0.91	0.67	2.03
6	0.07	0.77	0.54	0.16	0.33	0.88	0.69	2.02
8	0.11	0.87	0.54	0.16	0.41	0.98	0.56	1.99
10	0.07	0.80	0.53	0.15	0.43	0.99	0.44	2.22
12	0.34	0.89	0.60	0.17	0.44	1.09	0.86	3.07
24	0.08	0.93	0.61	0.18	0.47	0.82	0.62	2.28
48	0.14	0.81	0.56	0.18	0.43	0.96	0.69	2.17
168	0.15	0.88	0.72	0.16	0.61	1.20	0.89	3.01
336	0.15	0.86	0.65	0.16	0.47	0.93	0.83	2.93

*Each value is a mean of 2 animals.

TABLE IV. Turnover of ^{109}Cd in Rats, 0.5 to 336 Hours after Injection

Hours after Injection	^{109}Cd *(% of injected dose per gram tissue)x10								
	Pancreas	Heart	Spleen	Kidneys	Liver	Brain	Urinary Bladder	Prostate	Adrenals
0.5	2.26	0.70	0.72	2.63	8.06	0.05	0.25	0.17	0.81
2	4.42	1.01	1.18	5.06	18.63	0.07	0.33	0.26	1.84
4	6.25	1.57	1.93	8.96	28.93	0.06	0.52	0.47	2.03
6	7.26	1.61	1.93	11.19	36.99	0.07	0.38	0.51	2.74
8	5.38	1.88	3.17	19.27	41.96	0.06	0.55	0.71	2.53
10	7.57	1.76	3.60	25.94	36.70	0.06	0.61	0.61	2.38
12	9.34	1.62	2.83	22.98	47.88	0.06	0.56	0.78	3.12
24	7.32	1.57	2.38	18.95	40.63	0.06	0.52	0.64	2.58
48	7.82	1.78	2.28	22.90	39.42	0.06	0.62	0.55	2.54
168	10.34	1.64	2.36	16.37	42.30	----	0.82	0.90	3.50
336	9.50	1.24	2.22	16.27	37.44	----	0.83	0.90	3.27

*Each value is a mean of 2 animals.

TABLE V Turnover of ^{109}Cd in Mice, 0.5 to 600 Hours after Injection

Hours after Injection	^{109}Cd *(% of injected dose per gram of tissue)							
	Skeletal Muscle	Testes	Epididymis	Epididymal Fat	Seminal Vesicles	Lungs	Thymus	Salivary Glands
0.5	0.46±0.03	0.67±0.06	0.82±0.08	0.18±0.01	0.27±0.02	2.41±0.41	1.02±0.23	3.11±0.13
2	0.56±0.25	1.08±0.11	0.90±0.18	0.13±0.02	0.56±0.27	1.43±0.35	0.93±0.18	4.07±0.84
4	0.48±0.20	1.31±0.07	1.18±0.06	0.21±0.04	0.72±0.17	2.01±0.26	1.88±0.33	4.87±0.33
6	0.34±0.01	1.33±0.16	1.25±0.18	0.15±0.00	0.45±0.06	1.69±0.19	1.38±0.09	5.46±0.37
18	0.31±0.04	1.22±0.15	0.97±0.07	0.15±0.02	0.43±0.06	1.28±0.01	1.23±0.10	4.58±0.22
24	0.27±0.01	1.36±0.05	1.02±0.08	0.16±0.02	0.51±0.12	0.99±0.31	1.14±0.13	5.17±0.48
48	0.35±0.14	1.26±0.11	1.12±0.08	0.19±0.03	0.54±0.16	1.63±0.15	1.36±0.29	5.27±0.68
168	0.29±0.00	1.27±0.20	1.04±0.12	0.12±0.03	0.51±0.11	1.27±0.20	0.95±0.10	4.65±0.71
360	0.28±0.06	1.31±0.20	1.13±0.06	0.10±0.01	0.52±0.12	1.80±0.50	1.11±0.34	4.23±0.54
600	0.30±0.05	1.10±0.11	1.18±0.29	0.12±0.02	0.43±0.10	1.16±0.02	1.07±0.01	4.01±0.02

*Each value is mean ± S.D. in 3 animals.

TABLE VI. Turnover of ^{109}Cd in Mice, 0.5 to 600 Hours after Injection

Hours after Injection	^{109}Cd *(% of injected dose per gram of tissue)							
	Pancreas	Heart	Spleen	Kidneys	Liver	≠Stomach	≠Small Intestine	≠Large Intestine
0.5	4.96±0.60	2.30±0.20	1.69±0.27	8.11±0.69	18.87±3.56	1.16±0.48	3.69±0.17	1.21±0.29
2	9.41±1.98	2.46±0.30	2.26±0.08	13.98±1.83	33.90±3.49	1.65±0.19	5.63±0.78	1.95±0.43
4	13.06±1.09	2.64±0.27	3.01±0.96	18.76±3.23	40.48±1.49	0.78±0.25	5.95±0.81	1.87±0.13
6	12.00±2.30	2.73±0.48	2.74±0.25	14.37±1.61	35.25±3.15	1.01±0.40	6.62±0.84	1.81±0.22
18	10.28±0.83	2.56±0.34	2.54±0.49	14.04±1.50	37.97±1.57	1.46±0.05	5.83±0.49	3.37±0.25
24	10.48±0.88	2.76±0.30	2.21±0.04	16.19±0.71	44.95±2.86	1.81±0.13	5.09±0.79	2.80±0.66
48	11.42±1.51	2.78±0.11	2.93±1.09	17.60±1.68	41.67±6.85	1.70±0.12	2.79±0.26	2.78±0.31
168	10.69±0.99	2.54±0.43	2.45±0.59	19.97±4.30	39.65±4.77	1.44±0.62	0.92±0.15	0.76±0.01
360	11.71±0.77	2.56±0.32	2.47±0.11	24.56±2.60	47.56±7.42	0.89±0.11	0.80±0.15	0.65±0.10
600	10.89±0.94	1.99±0.14	2.54±0.50	27.53±2.53	41.30±3.00	0.53±0.30	0.52±0.12	0.36±0.07

*Each value is mean ± S.D. in 3 animals.

≠Including contents.

TABLE VII. Turnover of ^{65}Zn in Rats, 0.5 to 360 Hours after Injection

Hours after Injection	^{65}Zn *(% of injected dose per gram of tissue)x 10							
	Skeletal Muscle	Testes	Epididymis	Epididymal Fat	Seminal Vesicles	Lungs	Thymus	Salivary Glands
0.5	0.10	0.12	0.13	0.09	0.13	0.84	0.61	1.56
2	0.25	0.41	0.44	0.22	0.85	1.94	1.63	2.27
4	0.49	0.89	0.79	0.35	1.21	3.78	3.51	6.31
6	0.50	1.11	1.05	0.48	1.53	4.16	4.58	6.49
8	0.85	1.37	1.19	0.47	1.91	4.90	4.03	7.13
10	0.76	1.50	1.21	1.94	2.06	5.19	4.37	8.38
12	0.95	1.70	1.48	0.54	2.14	6.30	7.16	10.34
24	0.92	2.08	1.71	0.68	3.34	6.24	6.88	7.48
48	1.45	3.02	2.03	0.55	2.61	6.27	7.00	5.00
168	1.09	3.06	2.00	0.20	2.10	2.10	2.30	1.64
336	0.76	2.17	4.43	0.11	0.74	0.99	1.07	0.89

*Each value is a mean of 2 animals.

TABLE VIII. Turnover of ^{65}Zn in Rats, 0.5 to 336 Hours after Injection

Hours after Injection	^{65}Zn *(% of injected dose per gram tissue)x10								
	Pancreas	Heart	Spleen	Kidneys	Liver	Brain	Urinary Bladder	Prostate	Adrenals
0.5	3.05	0.79	2.05	3.94	3.10	0.13	0.40	0.25	2.45
2	10.96	1.62	4.59	9.26	7.55	0.22	1.10	0.81	6.22
4	18.43	3.00	8.19	13.03	11.80	0.37	2.15	1.44	7.97
6	25.33	3.40	8.50	15.91	13.38	0.46	2.45	1.82	9.61
8	21.05	4.17	10.74	20.10	17.37	0.60	4.07	3.00	10.47
10	19.39	4.53	10.23	17.46	17.94	0.64	3.04	2.97	10.56
12	16.88	5.04	11.57	19.06	19.10	0.92	3.49	3.86	13.71
24	10.46	4.88	8.88	12.96	12.76	0.90	3.49	4.29	7.92
48	6.93	5.18	7.20	8.42	10.55	1.37	4.64	3.18	6.93
168	2.38	2.06	2.44	2.90	2.75	----	3.23	2.37	1.87
336	1.39	1.01	1.06	1.24	1.52	----	2.24	0.96	0.77

*Each value is a mean of 2 animals.

TABLE IX. Turnover of ^{65}Zn in Mice, 0.5 to 600 Hours after Injection

Hours after Injection	^{65}Zn *(% of injected dose per gram of tissue)							
	Skeletal Muscle	Testes	Epididymis	Epididymal Fat	Seminal Vesicles	Lungs	Thymus	Salivary Glands
0.5	0.59±0.05	0.36±0.00	1.99±0.18	0.24±0.02	0.60±0.08	5.33±0.85	2.13±0.46	6.06±0.77
2	0.97±0.18	0.79±0.11	2.26±0.39	0.33±0.02	1.41±0.52	5.68±1.03	2.71±0.68	11.71±3.20
4	1.00±0.09	0.85±0.07	2.62±0.28	0.49±0.11	1.84±0.59	6.07±0.54	4.12±0.69	13.29±1.46
6	0.96±0.10	1.09±0.14	2.80±0.51	0.38±0.03	1.35±0.18	5.46±0.61	3.83±0.48	11.85±1.45
18	1.26±0.23	1.53±0.13	2.99±0.24	0.46±0.08	1.66±0.16	4.82±0.09	4.54±0.62	8.53±0.96
24	1.31±0.15	1.80±0.06	3.01±0.28	0.45±0.06	1.78±0.39	4.08±1.12	4.62±0.09	9.96±1.57
48	1.62±0.27	2.11±0.24	3.07±0.29	0.50±0.05	2.04±0.61	4.66±0.67	4.87±0.63	6.24±0.88
168	1.53±0.09	2.82±0.31	3.40±0.55	0.21±0.05	1.57±0.26	2.49±0.23	3.53±0.55	3.49±0.13
360	1.08±0.08	2.25±0.22	3.09±0.28	0.11±0.00	1.02±0.17	1.52±0.12	1.63±0.32	1.99±0.19
600	0.66±0.05	1.51±0.02	1.98±0.12	0.05±0.00	0.51±0.04	0.65±0.04	0.75±0.02	1.14±0.14

*Each value is mean ± S.D. in 3 animals.

TABLE X. Turnover of ^{65}Zn in Mice, 0.5 to 600 Hours after Injection

Hours after Injection	^{65}Zn *(% of injected dose per gram of tissue)							
	Pancreas	Heart	Spleen	Kidneys	Liver	≠Stomach	≠Small Intestine	≠Large Intestine
0.5	14.16±1.36	2.72±0.28	7.21±0.52	15.72±1.96	11.36±1.55	2.81±1.03	5.50±0.11	2.00±0.44
2	22.09±2.63	3.65±0.30	9.99±1.33	16.70±2.87	15.95±0.67	3.95±0.73	9.25±0.60	3.63±0.64
4	20.92±2.15	3.95±0.29	11.06±1.95	16.73±1.24	14.95±0.87	2.30±0.93	8.58±0.13	3.86±1.11
6	20.04±0.59	4.02±0.54	10.89±1.01	15.06±1.54	12.41±1.81	2.95±1.16	9.60±1.12	4.67±0.87
18	12.17±1.62	4.79±0.58	9.71±1.04	9.58±0.63	11.20±0.78	4.14±0.19	8.81±0.73	6.28±0.39
24	9.91±0.14	4.79±0.34	8.87±0.46	9.01±0.50	12.52±1.22	4.22±0.32	8.40±1.14	5.58±0.89
48	7.47±1.31	4.61±0.44	6.82±1.03	6.16±0.83	9.21±0.93	4.09±0.23	6.10±0.85	4.69±0.89
168	3.63±0.26	2.79±0.33	2.93±0.16	2.66±0.19	4.73±0.37	2.26±0.19	2.46±0.23	2.14±0.15
360	2.13±0.23	1.57±0.07	1.44±0.09	1.38±0.14	2.55±0.39	1.13±0.48	1.17±0.22	1.29±0.31
600	1.18±0.04	0.78±0.02	0.71±0.03	0.70±0.04	1.14±0.06	0.44±0.23	0.49±0.07	0.51±0.03

*Each value is mean ± S.D. in 3 animals.

≠Including contents.

thymus, lungs, heart, prostate, urinary bladder, seminal vesicles, testes, and epididymides (Tables VII,VIII). In mice, the salivary glands had higher radioactivity than the spleen, lungs, heart, thymus, epididymides, testes, or seminal vesicles (Tables IX,X). The lowest radioactivity of ^{65}Zn was detected in skeletal muscles, brain, and in the epididymal fat. As the time after injection increased, a loss of ^{109}Cd was observed in the stomach, small intestine and the large intestine of mice (Table VI). In other tissues, after the initial deposition, ^{109}Cd did not show a demonstrable depletion as observed for ^{65}Zn in all tissues (Tables VII - X).

The turnover of ^{109}Cd and ^{65}Zn in the liver of rats and mice is shown in Figure 4. In both species the liver tissue attained a higher concentration of cadmium than of zinc. The maximum uptake of ^{109}Cd was reached between 4 and 8 hours after injection. Cadmium-109 remained at this level throughout the experiment. The uptake of ^{65}Zn by the liver of rats was slower than that of the mice. After accumulating to a maximum concentration, ^{65}Zn was depleted from all tissues. A difference in the uptake of ^{109}Cd was observed in the kidneys of rats and mice (Figure 5). The kidneys of rats accumulated ^{109}Cd up to 10 hours after subcutaneous injection, while the uptake of ^{109}Cd in the kidneys of mice continued beyond 10 hours. The concentration of ^{65}Zn in rat kidneys increased to a maximum after 8 hours and as the time after injection increased, there was a continuous loss of this

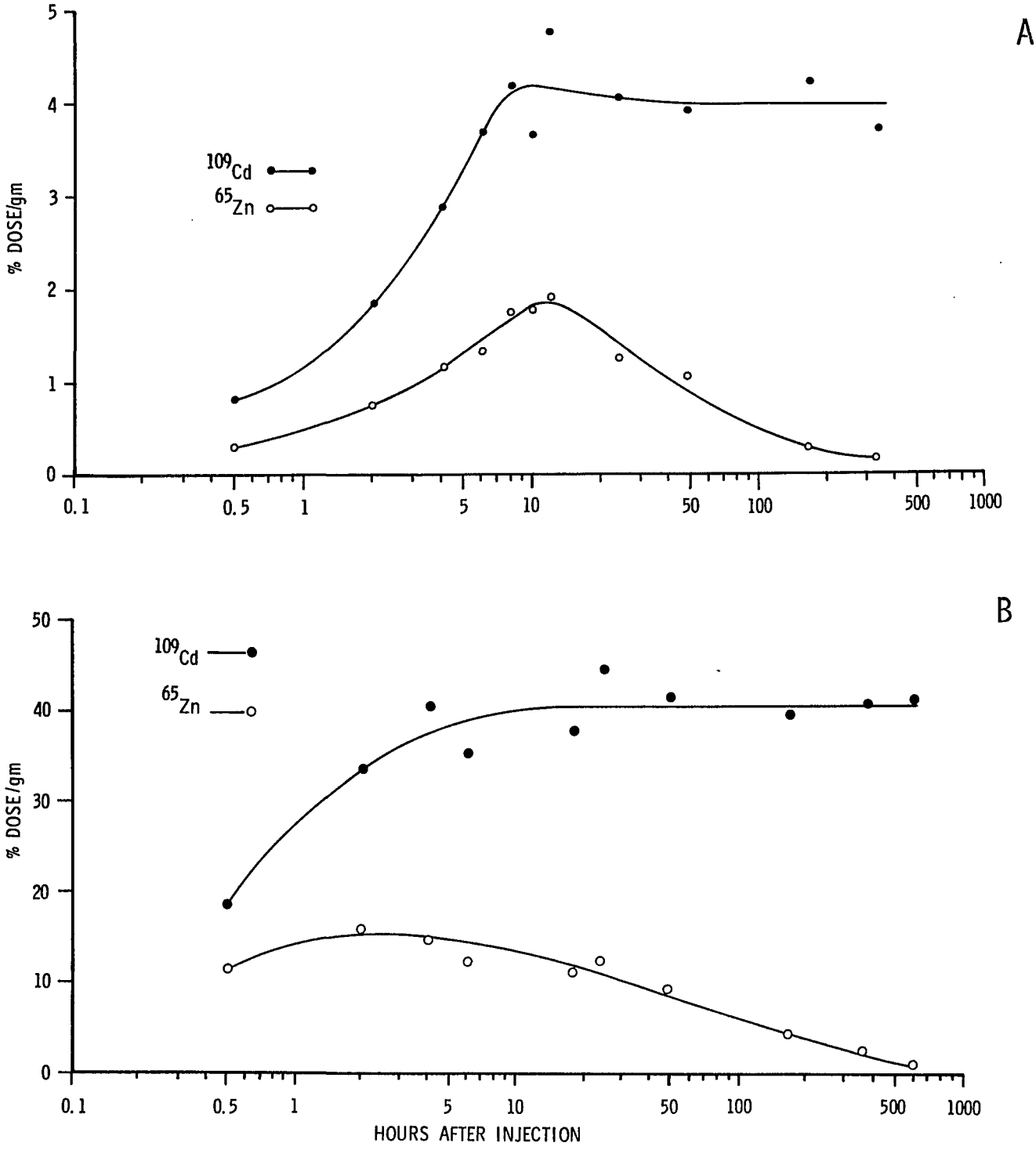


FIGURE 4. Turnover of ^{109}Cd and ^{65}Zn in the liver of rats (A) and mice (B).

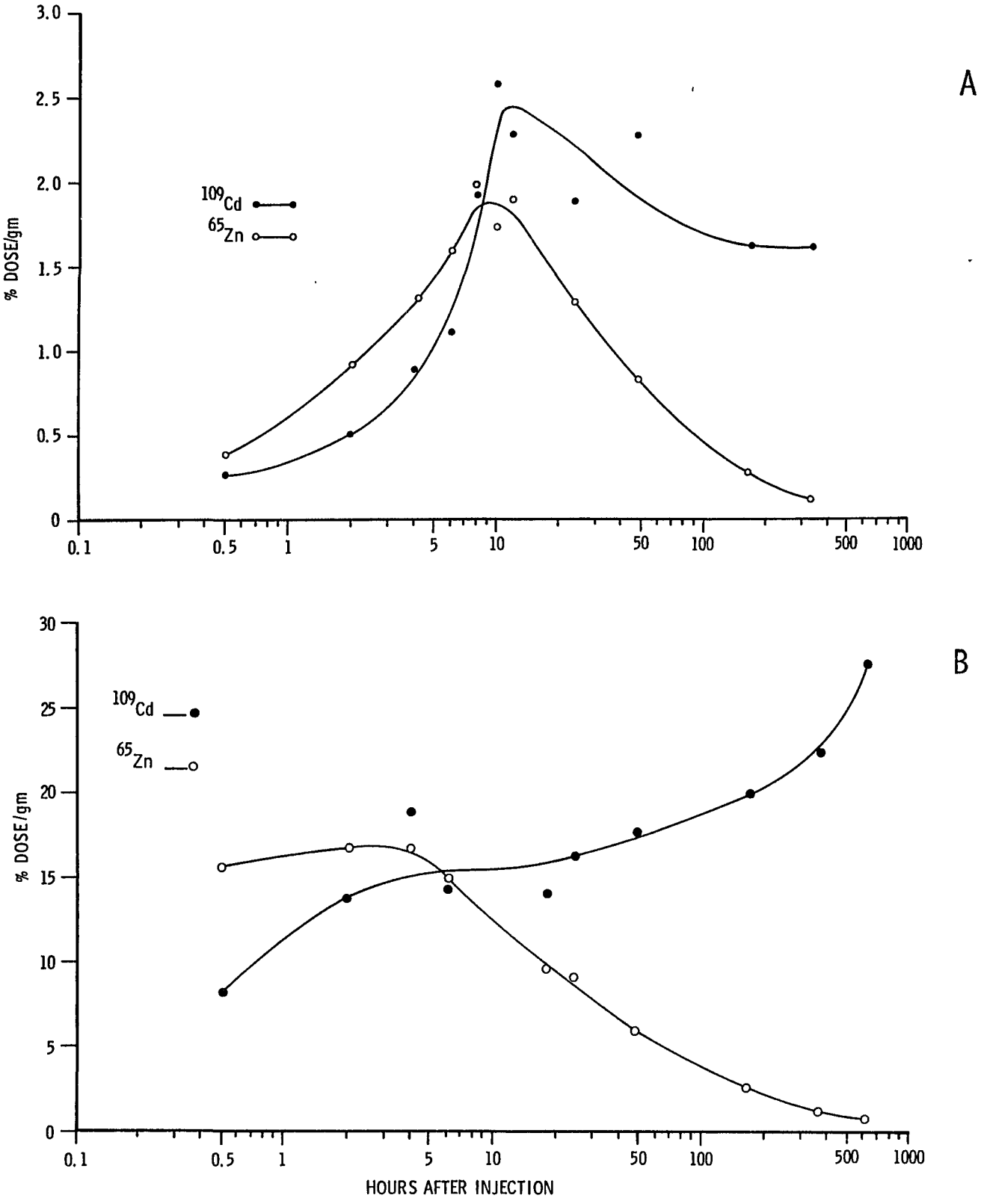


FIGURE 5. Turnover of ^{109}Cd and ^{65}Zn in the kidneys of rats (A) and mice (B).

isotope. In the kidneys of mice ^{65}Zn attained its maximal concentration between 2 and 4 hours; the isotope was depleted thereafter. Rat kidneys were further dissected into cortex and medulla. As indicated in Figure 6A, the cortex concentrated more ^{109}Cd than the medulla. A similar pattern for ^{65}Zn distribution was also obtained (Figure 6B). However, differences in the relative concentrations of ^{109}Cd and ^{65}Zn, per unit weight of tissue, were noted. In the kidney cortex, the concentration of ^{109}Cd was higher than that of ^{65}Zn, while in the kidney medulla the concentration of ^{65}Zn exceeded that of ^{109}Cd.

Figures 7A and B show the turnover of ^{109}Cd and ^{65}Zn in the pancreas. Cadmium-109 in this organ accumulated to the highest level within 12 hours. This isotope remained at an elevated level in the pancreas throughout the experiment. In contrast to ^{109}Cd, ^{65}Zn accumulated at a faster rate in the pancreas of both rats and mice. Of all tissues analyzed, pancreas showed the greatest affinity for ^{65}Zn. However, the concentration of ^{65}Zn in this tissue declined as the time after injection increased.

In contrast to the liver, kidneys, and pancreas, the testes and epididymal tissue of the experimental animals exhibited a striking difference in the turnover of ^{65}Zn and ^{109}Cd. Figures 8A and B illustrate the changes in the concentration of these isotopes in the testes of rats and mice. During the first 30 minutes after injection, the testes concentrated more cadmium than zinc. The maximal uptake of

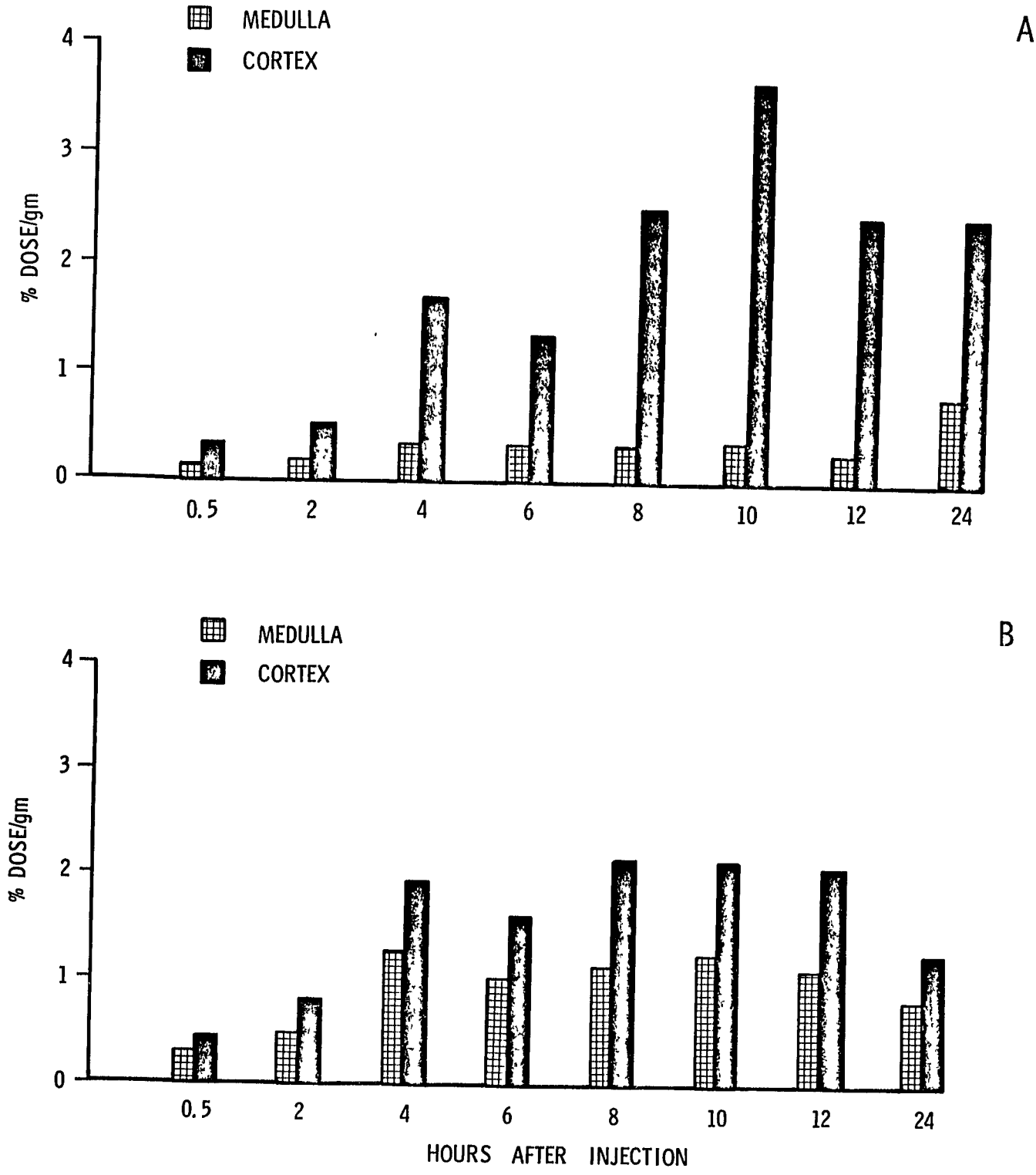


FIGURE 6. Distribution and turnover of ^{109}Cd (A) and ^{65}Zn (B) in rat kidney medulla and cortex.

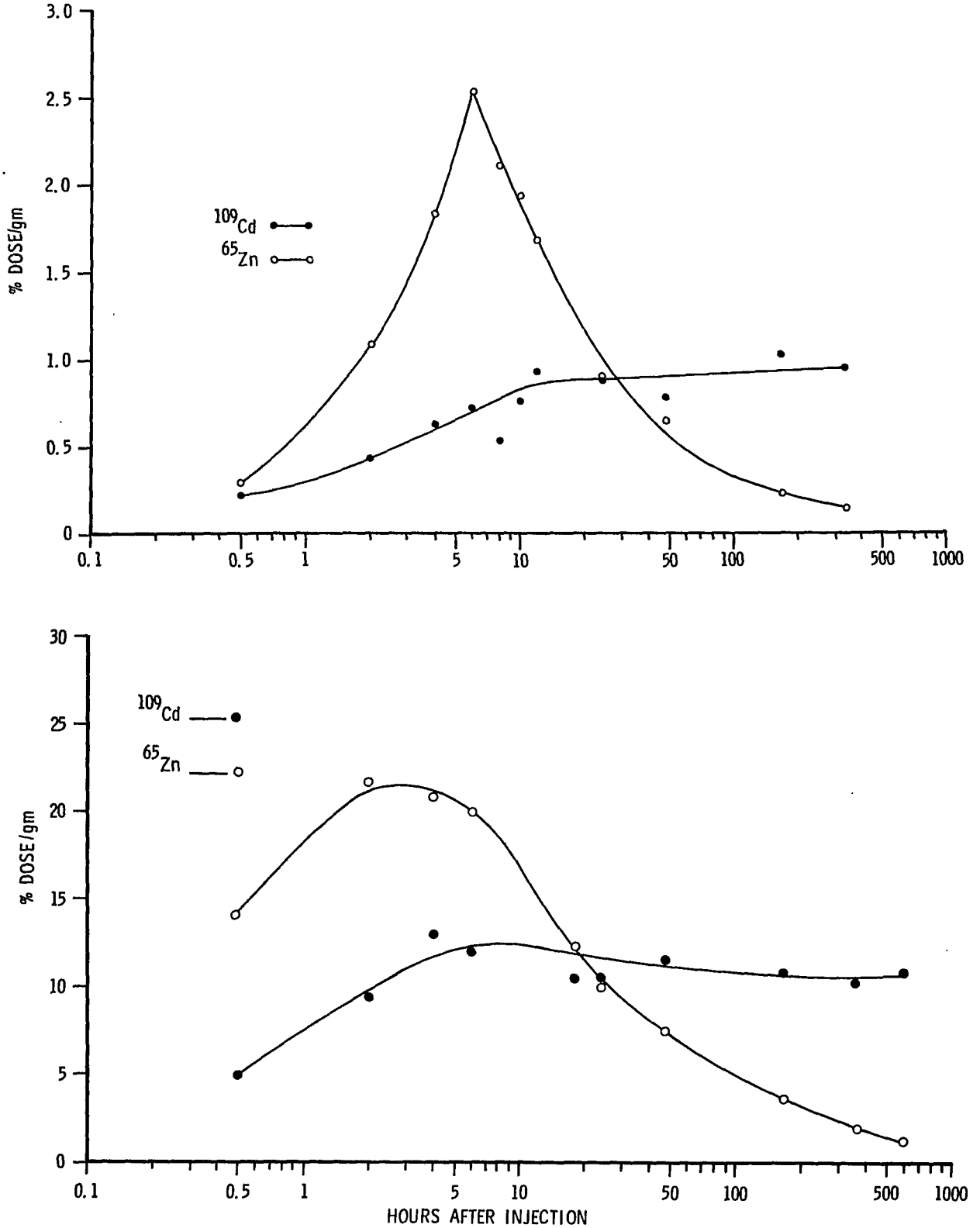


FIGURE 7. Turnover of ^{109}Cd and ^{65}Zn in the pancreas of rats (A) and mice (B).

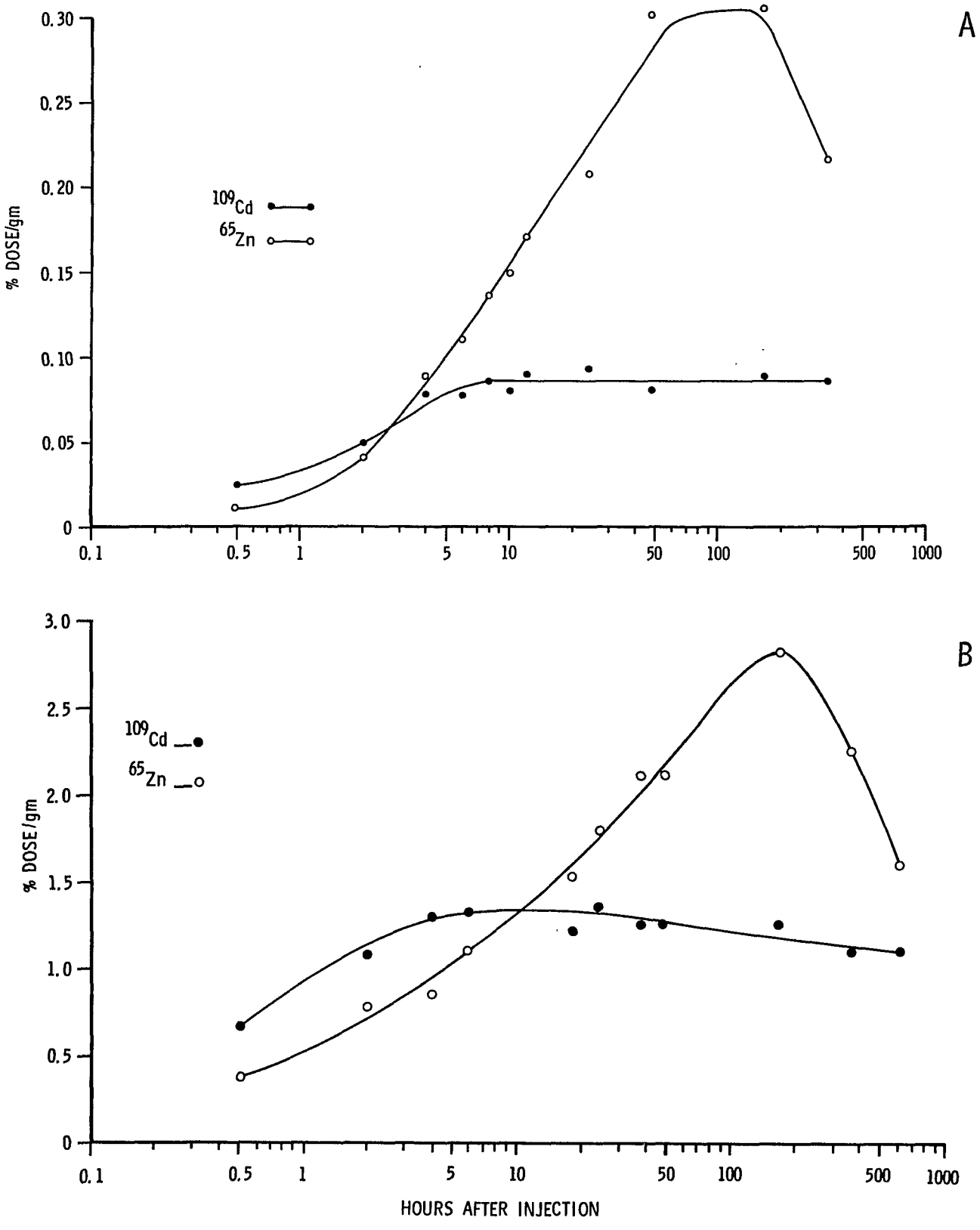


FIGURE 8. Turnover of ^{109}Cd and ^{65}Zn in the testes of rats (A) and mice (B).

^{109}Cd was achieved by these organs within 6-8 hours, and the isotope remained at a plateau thereafter. By contrast, the influx of ^{65}Zn in the testes continued for 168 hours, and then it was depleted. A prolonged accumulation of ^{65}Zn was also observed in the epididymal tissue of rats and mice (Tables VII,IX). In rats, the duration of accumulation was 336 hours, while in mice the highest concentration was observed at 168 hours following the subcutaneous dose.

Another tissue which showed a continuous increase in ^{65}Zn concentration was the brain (Table VIII). The value for zinc in rat brain, per gram of tissue, increased from 0.013% of the injected dose at 30 minutes, to 0.137% of the dose at 48 hours. Cadmium-109 was taken up by rat brain during the first 2 hours after injection (Table IV). Its level was low and persisted for the remaining 46 hours. The $^{65}Zn/^{109}Cd$ ratio in the brain at 30 minutes was 2.6, and by 48 hours this ratio had increased to 23.

The excretion of subcutaneously injected cadmium and zinc was studied by maintaining the rats in metabolic cages. It was observed that during the 5 day experimental period only 0.02% of ^{109}Cd and 0.34% of ^{65}Zn was excreted in the urine. In contrast, 5.3% of ^{109}Cd and 17.1% of ^{65}Zn was eliminated in the feces. Although the appearance of radioactivity in the feces started within the first 24 hours, it was on the 2nd and 3rd day that the highest excretion of both isotopes took place. During these two days, 3.54% of the dose of ^{109}Cd and 9.93% of the dose of ^{65}Zn was recovered from the feces.

4. DISTRIBUTION OF ^{109}Cd AND ^{65}Zn IN SUBCELLULAR FRACTIONS

As described in the preceding section, ^{109}Cd was retained in various tissues and its turnover, in contrast to that of ^{65}Zn, was rather slow. Amongst the tissues, the liver and kidneys concentrated between 50 and 70% of the total injected dose of ^{109}Cd. These observations suggested that ^{109}Cd was strongly bound to some intracellular components. To investigate the binding of ^{109}Cd and ^{65}Zn to subcellular fractions, male rats were injected subcutaneously with 18 nmoles of $^{109}CdCl_2$ and 18 nmoles of $^{65}ZnCl_2$. The animals were killed 24 hours later. The liver tissue was excised and homogenized in 0.25 M sucreose. Subcellular particles and the soluble cytoplasmic components were separated by differential centrifugation. The distribution of ^{109}Cd and ^{65}Zn in the liver homogenate and its fractions is presented in Table XI. It indicates that the homogenate contained 3.97 times more ^{109}Cd than ^{65}Zn. The content of cadmium in all subcellular fractions was higher than that of zinc. Of the total radioactivity present in the homogenate, the soluble cytoplasmic fraction accounted for 81% of ^{109}Cd and 71% of ^{65}Zn. Due to the difference in cadmium and zinc content, the $^{109}Cd/^{65}Zn$ ratio of this fraction was higher than that found in the homogenate.

TABLE XI. Distribution of ^{109}Cd and ^{65}Zn in Rat Liver Subcellular Fractions

Fraction	^{109}Cd	^{65}Zn	$^{109}Cd/^{65}Zn$
	pmoles/g liver	pmoles/g liver	
Homogenate	995	251	3.97
Nuclei and Cell Debris	6	3	2.00
Mitochondria	89	29	3.02
Microsomes	91	42	2.17
Soluble Cytoplasm	807	176	4.59

5. THE CADMIUM-BINDING PROTEINS

5A. Development of Isolation Procedure:

It has been known for some time that a cadmium- and zinc-binding protein, "metallothionein", exists in horse and human kidney cortex (Kägi and Vallee, 1960; Pulido et al., 1966). From the results presented in the previous sections, a similar protein was suspected to be present in rat tissues as well.

The procedure for the isolation of metallothionein as described by Pulido et al. (1966) was first followed to obtain the ^{109}Cd- and ^{65}Zn-binding protein from rat liver and kidneys. A rat was injected subcutaneously with 18 nmoles of $^{109}CdCl_2$ and 18 nmoles of $^{65}ZnCl_2$ and killed after 24 hours. The liver and kidneys were removed and processed for fractionation. Homogenization of each tissue was carried out in 0.05 M sodium phosphate, pH 7.0 buffer. The homogenate was centrifuged at 1,000 x g in an International model PR-6 centrifuge, for 1 hour. To the supernatant, 1.2 parts of 95% ethanol and 0.095 parts of chloroform, both prechilled at -20 C, were added and mixed. The mixture was centrifuged at 1,000 x g for 1 hour to sediment the precipitate. The clear supernatant was dialyzed overnight against two changes of distilled water. The fine precipitate which formed was removed by centrifugation at 33,000 x g for 1 hour in an International model B-35 centrifuge (rotor SB-283).

The distribution of ^{109}Cd and ^{65}Zn in various fractions

is summarized in Table XII. A considerable quantity of ^{109}Cd and ^{65}Zn was lost during precipitation of the buffer extract with organic solvents. As a result, the final yield was less than 50% for ^{109}Cd and less than 10% for ^{65}Zn. In one experiment, the freeze-dried final supernatant from rat liver preparation (Table XII), labeled with ^{109}Cd, was chromatographed on a Sephadex G-75, 1.5 x 85 cm column. The sample was eluted with 0.001 M Tris-HCl, pH8.6 buffer. The recovery of ^{109}Cd from the column was 98%. Of this, 92% was eluted as a single peak in a region where the ratio of elution volume (Ve) to the void volume (Vo) was between 1.66 and 2.14. In addition to this component, some lower as well as higher molecular weight UV-absorbing components were also separated. This indicated that the preparation was not homogenous. UV-absorption was recorded as per cent transmission, at the standard wavelength of 253.7 nm. Later investigations showed that the proteins described below had no absorption peak at 275-280 nm, and contained no tyrosine. The same is true of metallothionein (Kägi and Vallee, 1960).

An alternate procedure was developed and compared with the previously described method. The soluble cytoplasmic fractions from rat liver and kidney tissues were chromatographed separately on Sephadex G-75. The tissues were obtained from a rat killed 24 hours after a subcutaneous injection of 18 nmoles each of $^{109}CdCl_2$ and $^{65}ZnCl_2$. Figures 9A and B show elution profiles obtained after gel filtration of 14 ml of the liver soluble fraction and 5.5 ml of the kidney preparation.

TABLE XII. Yield of ^{109}Cd and ^{65}Zn During Fractionation of Tissue Homogenate*

Fraction	Liver		Kidneys	
	^{109}Cd (%)	^{65}Zn (%)	^{109}Cd (%)	^{65}Zn (%)
Tissue Homogenate	100.0	100.0	100.0	100.0
Buffer Extract	90.9	90.7	90.0	89.5
Supernatant after Ethanol and Chloroform Precipitation	48.5	11.1	48.2	11.5
Supernatant after Dialysis	43.5	6.4	33.0	8.3

*By the method of Pulido et al. (1966).

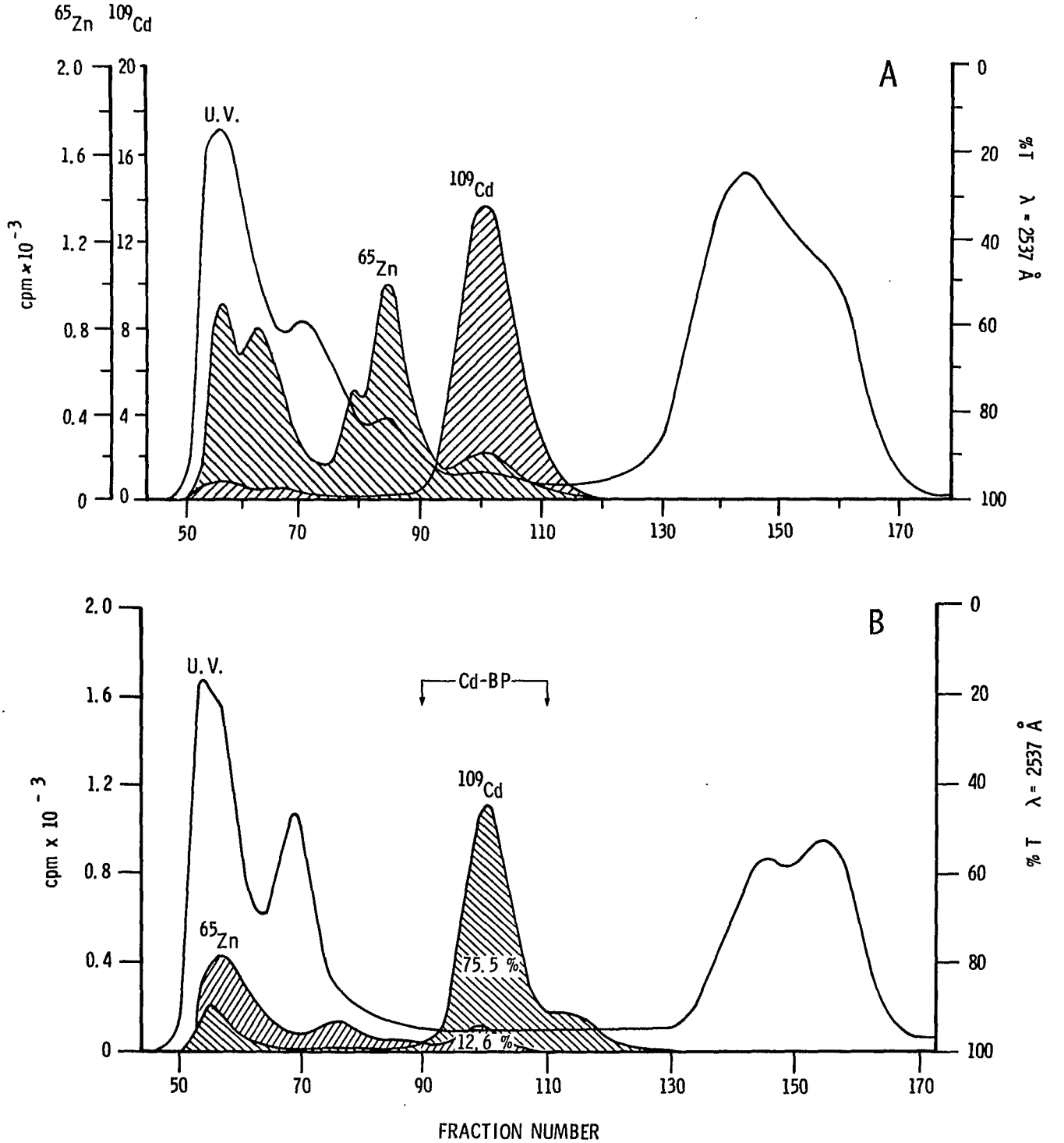


FIGURE 9. Distribution of ^{109}Cd and ^{65}Zn in rat liver (A) and kidney (B) soluble fractions, 24 hours after injection. Sephadex G-75, 2.5 x 90 cm column; flow rate, 19.2 ml/hour (A) and 19.14 ml/hour (B).

The soluble fraction of rat liver was resolved into five ^{65}Zn-containing components and one major and one minor ^{109}Cd-binding fraction (Figure 9A). Most of the ^{109}Cd was eluted in a region where UV-absorption was low. The Ve/Vo of ^{109}Cd peak was between 1.65 and 2.05. The recoveries of ^{109}Cd and ^{65}Zn from the column were 95 and 99%, respectively. Of the total ^{65}Zn recovered, 12.1% was associated with the ^{109}Cd-binding protein. Rat kidney soluble fraction from the same animal was also chromatographed on Sephadex G-75. As shown in Figure 9B, ^{65}Zn separated into 3 components, while 75.5% of the ^{109}Cd was again eluted in a region with a Ve/Vo from 1.65 to 2.05. Only 12.6% of ^{65}Zn was present in the ^{109}Cd-binding region. The recoveries of ^{109}Cd and ^{65}Zn were 98 and 94%, respectively.

From the observations mentioned above, the total yields of ^{109}Cd and ^{65}Zn associated with the cadmium-binding protein (Cd-BP) region were calculated. The results are presented in Table XIII. The recovery of ^{109}Cd by this procedure was higher than that obtained after employing the method of Pulido et al. (1966) (Table XII); although there was no difference in total yield of ^{65}Zn. An advantage of using the soluble fraction directly for the isolation of Cd-BP was that the procedure was shorter and less destructive for proteins. For all subsequent analyses the newly devised procedure described in Table XIII was used.

In order to establish the gel filtration characteristics of $^{109}CdCl$ and $^{65}ZnCl_2$ a solution containing carrier-free

TABLE XIII. Yield of ^{109}Cd and ^{65}Zn During Fractionation of Soluble Fraction by Gel Filtration

Fraction	Liver		Kidneys	
	^{109}Cd (%)	^{65}Zn (%)	^{109}Cd (%)	^{65}Zn (%)
Tissue Homogenate	100.0	100.0	100.0	100.0
Soluble Fraction	81.9	66.1	74.0	64.0
G-75 Column (Ve/Vo 1.65 - 2.05)	72.7	7.9	54.7	7.6

$^{109}CdCl_2$ and carrier-free ^{65}Zn was chromatographed on a Sephadex G-75 column. The isotopes were not eluted in 3 void volumes of 0.001M Tris-HCl, pH 8.6 buffer. Five ml of 0.4% disodium-ethylene-diamine-tetraacetate was then applied to the column and eluted with the Tris buffer. The chelated complex of the isotopes emerged at Ve/Vo 2.4. This corresponded to fraction number 131 at a flow rate of 19.2 ml/hour.

5B. Changes in the Distribution of ^{109}Cd and ^{65}Zn:

Although no change in ^{109}Cd content of the liver was noticed within 25 days, it was possible that with time some intermolecular redistribution of ^{109}Cd-binding within the cells might have taken place. The decrease of ^{65}Zn in the tissue could also have been accompanied by similar changes. To investigate this further, 2 male rats were injected subcutaneously with solution containing 18 nmoles of ^{109}Cd and 18 nmoles of ^{65}Zn. One of the animals was killed after 7 days and the other after 14 days. Determination of radioactivity in the liver of the first animal revealed that 4.23% of the dose of ^{109}Cd and 0.27% of the dose of ^{65}Zn was present in a gram of tissue. In the animal killed after 14 days, there was no change in ^{109}Cd content, while considerable decrease in the concentration of ^{65}Zn was observed. The concentration of ^{109}Cd per gram of the tissue was 4.20% of the dose, and that of ^{65}Zn was 0.15%.

Liver tissue from each animal was homogenized and

centrifuged at 105,000 x g to sediment the subcellular particles. The soluble fraction from the rat killed after 7 days contained 74.4% of the total ^{109}Cd and 61.4% of the total ^{65}Zn present in the homogenate. After 14 days the distribution of the isotopes in the soluble fraction did not change. The soluble fractions were separated on sephadex G-75 and analyzed for the distribution of ^{109}Cd and ^{65}Zn. Figure 10A indicates that in the sample obtained after 7 days, 88% of ^{109}Cd was eluted in the Cd-BP region. Zinc-65 was detected in 4 components. The concentration of ^{65}Zn was markedly diminished in all fractions and it was no longer found in the Cd-BP component. As shown in Figure 10B, the liver soluble fraction obtained 14 days after administration of the radioactive dose, separated into one principal ^{109}Cd-binding component and four ^{65}Zn-binding fractions. A further decrease in ^{65}Zn was demonstrable in all fractions. In contrast, no change in ^{109}Cd content or in its association with the Cd-BP was observed.

5C. Effect of Heavy Metals in vivo:

To study the effect of cadmium on the liver and kidney Cd-BP in vivo, one rat was injected subcutaneously with 0.03 nmole of $CdCl_2$/Kg body weight. A dose of 2 μCi $^{109}CdCl_2$ (carrier-free) was also injected and this served as a tracer. The injected dose of $CdCl_2$ induced hemorrhage in the testes of the animal within 24 hours. Figure 11 shows that UV-absorption in Cd-BP region of the gel-filtration

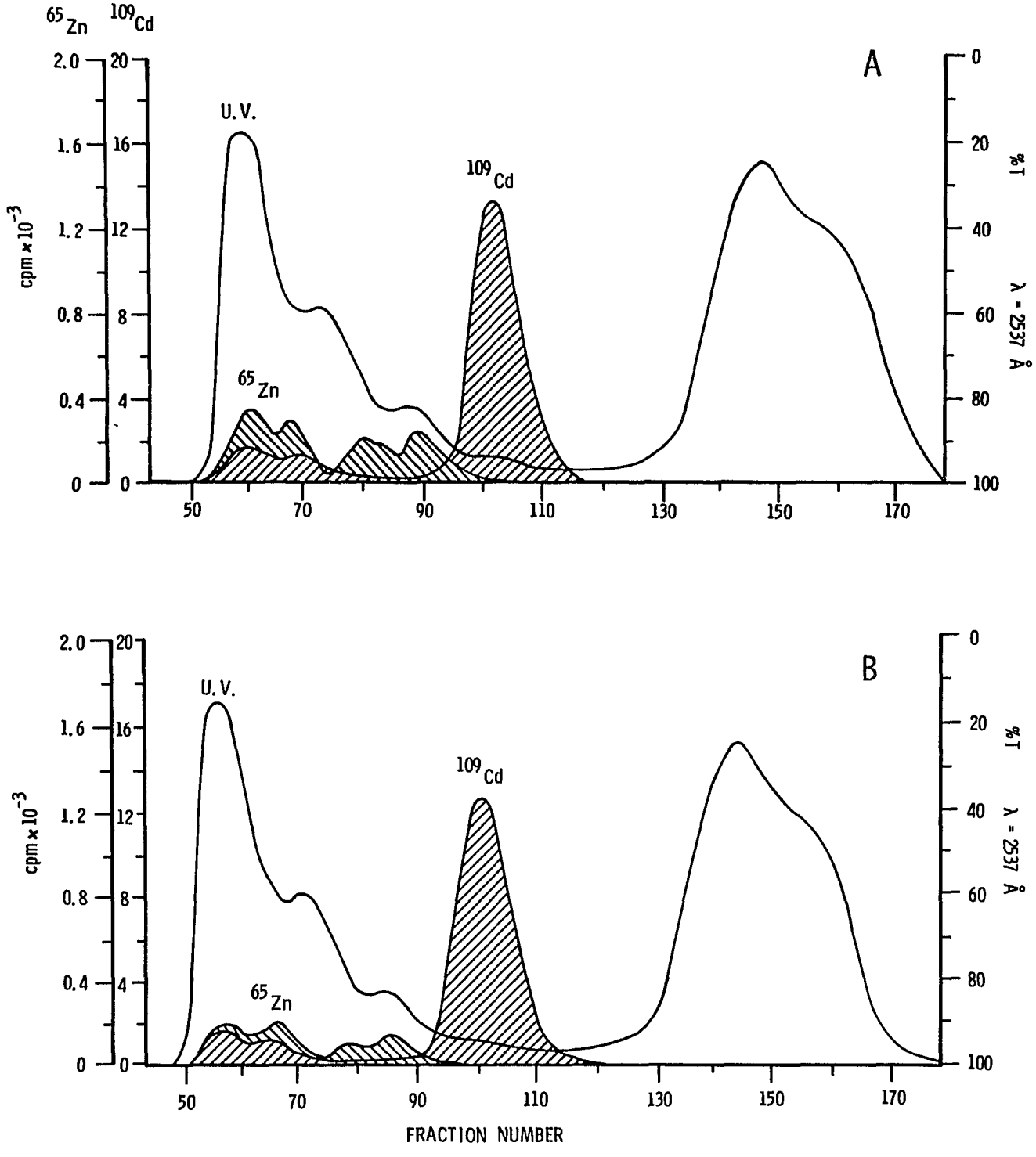


FIGURE 10. Distribution of ^{109}Cd and ^{65}Zn in rat liver soluble fraction, 7 (A) and 14 (B) days after injection. Sephadex G-75, 2.5 x 90 cm column; flow rate, 18.3 ml/hour (A) and 19.2 ml/hour (B).

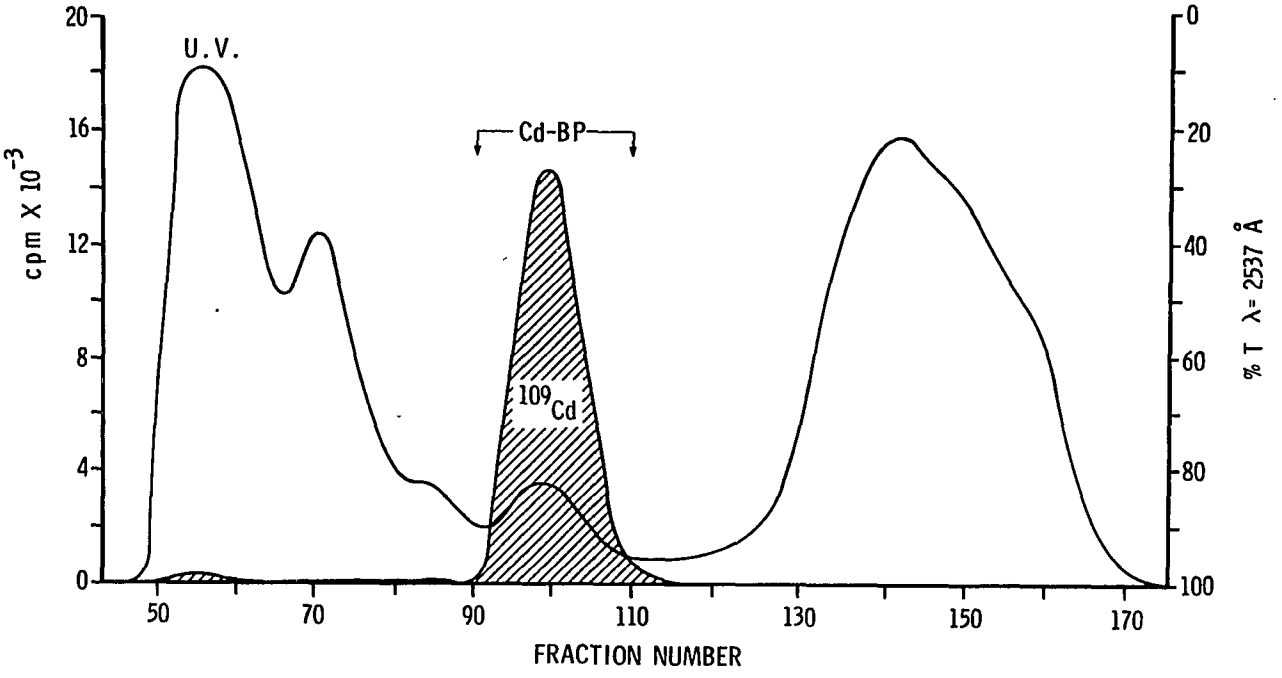


FIGURE 11. Rat liver soluble fraction, 24 hours after subcutaneous injection of 0.03 mmole $CdCl_2$/Kg. Sephadex G-75, 2.5 x 90 cm column; flow rate, 19.75 ml/hour.

profile became prominent and this was accompanied by an increase in the quantity of Folin-Ciocalteu-reactive protein. This observation suggested that the injected Cd^{2+} apparently accelerated the biosynthesis of Cd-BP.

Since the effects of other divalent metal ions on the biosynthesis of Cd-BP were not known, this problem was also explored. Separate rats were injected with a subcutaneous dose of 0.03 mmole/Kg using 30mM aqueous solutions of zinc chloride ($ZnCl_2$), mercuric chloride ($HgCl_2$), nickel chloride ($NiCl_2.6H_2O$), cobalt chloride ($CoCl_2.6H_2O$), or lead acetate ($Pb.(CH_3COO)_2.3H_2O$). These animals were killed 24 hours later. The morphological appearance of the testes showed no difference from the uninjected control. Liver tissue from each rat was homogenized and the soluble fraction was analyzed by gel filtration. Sephadex G-75 chromatography revealed no increase in UV-absorption in Cd-BP region as compared to the uninjected control. This indicated that not all heavy metals were capable of increasing the content of rat liver Cd-BP. Among the II-B subgroup metal ions, only Cd^{2+} produced a positive response, while Zn^{2+} or Hg^{2+} were ineffective.

5D. Acceleration of Biosynthesis:

The information obtained from the preceding experiments suggested that Cd-BP was specifically produced in response to a cadmium challenge. To further explore this phenomenon, the effects of different dose levels of cadmium

on the Cd-BP were studied *in vivo*.

Male and female rats were injected subcutaneously with varying doses of $CdCl_2$. Along with the last injection, a dose of carrier-free $^{109}CdCl_2$ was also given and it served as a tracer to locate the Cd-BP region. Table XIV shows that Cd-BP increased in quantity with increasing dose of cadmium. This effect was more pronounced when the total dose of cadmium was given in multiple divided injections, at daily intervals.

The gel filtration profile of the liver soluble fraction from a male rat which was injected with a single dose of 0.09 mmole $CdCl_2$/Kg and killed 24 hours later, is shown in Figure 12A. The increase in UV-absorption (recorded as %T) of the Cd-BP region was of a similar order of magnitude as that observed with 0.03 mmole $CdCl_2$/Kg (Figure 11). Total protein in the Cd-BP region as estimated by the method of Lowry *et al.* (1951) was 12.5 mg/3.5 g of wet liver tissue (14 ml of soluble fraction). Daily injections of 0.01 mmole $CdCl_2$/Kg for 9 days stimulated the biosynthesis of the Cd-BP to a greater degree (Figure 12B). The UV-absorption of the Cd-BP region was markedly increased. Estimation of protein revealed that a total of 20.3 mg of protein was present in fraction number 89 to 108 of the Sephadex column. This effect of $CdCl_2$ on the Cd-BP was also demonstrable in female rats as well as in castrated male rats.

Kidneys from rats injected with varying doses of $CdCl_2$ were also analyzed by gel filtration. An increase in

TABLE XIV. Effect of Subcutaneously Injected $CdCl_2$ on Rat Liver Cd-BP

Daily Dose	Total Injections	Total Cd Injected	Cd-BP* (G-75 Column)
mmole/Kg	No.	mmole/Kg	mg/g liver
0.00	(control)	0.00	3.1
0.00**	(control)	0.00	3.0
0.09	1	0.09	3.6
0.03	3	0.09	5.0
0.01	9	0.09	5.8
0.01**	9	0.09	6.7
0.01	12	0.12	7.5
0.01	15	0.15	8.8

*Determined by Lowry's method in one animal
**Female Rats.

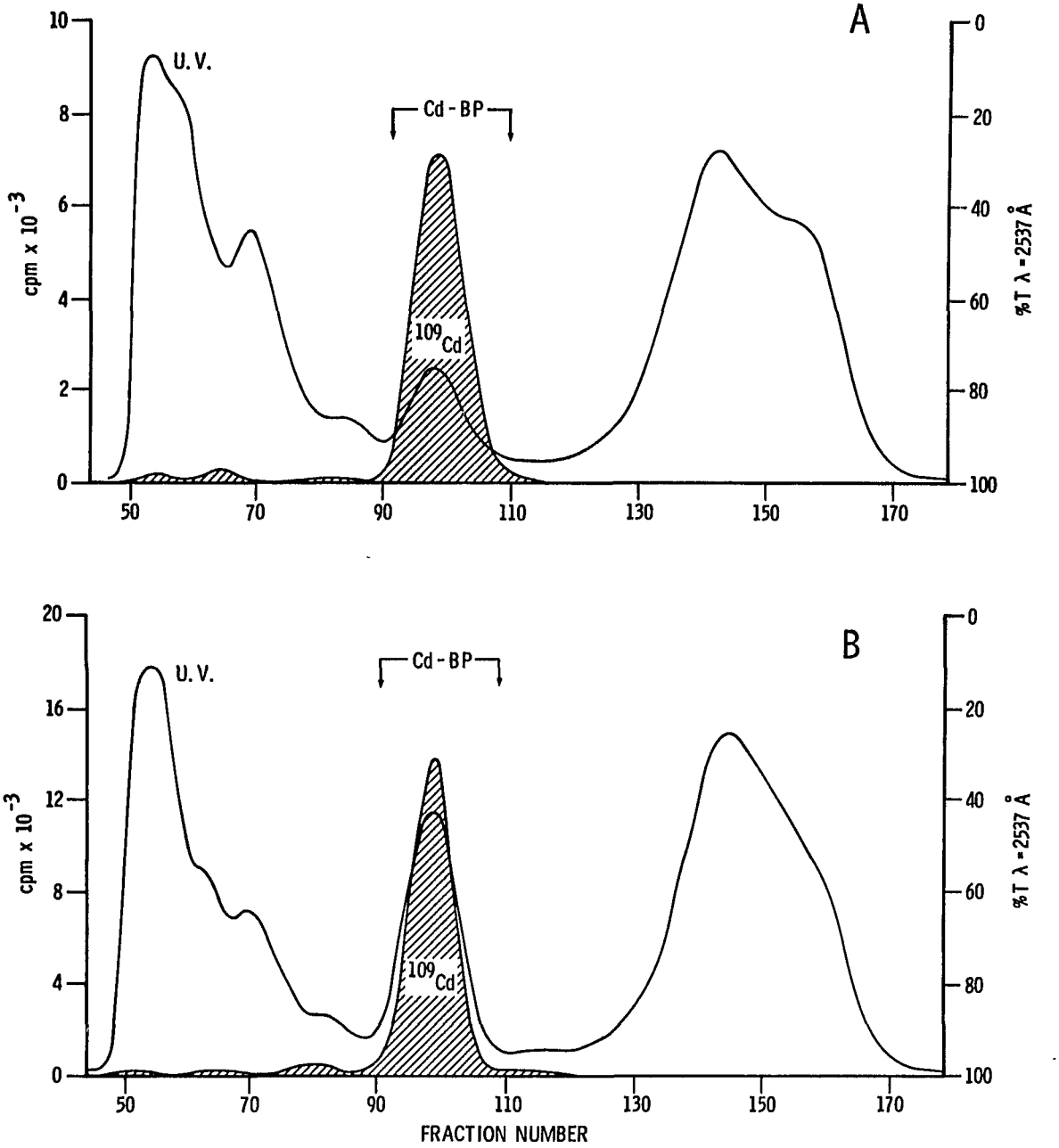


FIGURE 12. Effect of cadmium on rat liver Cd-BP. A, after a single dose of 0.09 mmole $CdCl_2$/Kg and B, after 9 doses of 0.01 mmole $CdCl_2$/Kg/day. Sephadex G-75, 2.5 x 90 cm column; flow rate, 19.65 ml/hour (A) and 18.75 ml/hour (B).

Cd-BP was noticed in all cases. Figures 13A and B represent two examples of these. In both experiments, 5.5 ml of the soluble fraction was chromatographed. The sample for experiment 1 (Figure 13A) was prepared from the kidneys of a rat which had received 0.03 mmole $CdCl_2$/Kg/day, for 3 days. As observed in Figure 13A, a small UV-absorbing component was eluted in the Cd-BP region. Its protein content was estimated to be 4.3 mg by the method of Lowry et al. (1951). Figure 13B illustrates the separation of the kidney soluble fraction from a rat which was injected with 0.01 mmole $CdCl_2$/Kg/day, for 9 days. The UV-absorption in the Cd-BP region was increased to a greater extent than in the first sample, and it accounted for 6.5 mg of Folin-Ciocalteu-reactive protein.

As far as it is known, the absorption of cadmium from the gastrointestinal tract is poor. Nevertheless, this is the most frequent route of cadmium absorption as opposed to the parenteral administration. Studies were undertaken to determine the effect of ingested cadmium on rat liver Cd-BP. A group of 6 rats was maintained from 1 to 12 weeks on drinking-water containing 0.05 mM $CdCl_2$. The daily consumption of cadmium-containing water by each rat was recorded and the total quantity of cadmium ingested was calculated. Two male rats were used as controls; one was killed at the beginning of the experiment and the other after 12 weeks. The control animals received distilled water for drinking. Rats maintained on $CdCl_2$ solution were killed after 1, 2, 3, 6, 8, and 12 weeks. To label the Cd-BP, all animals were given

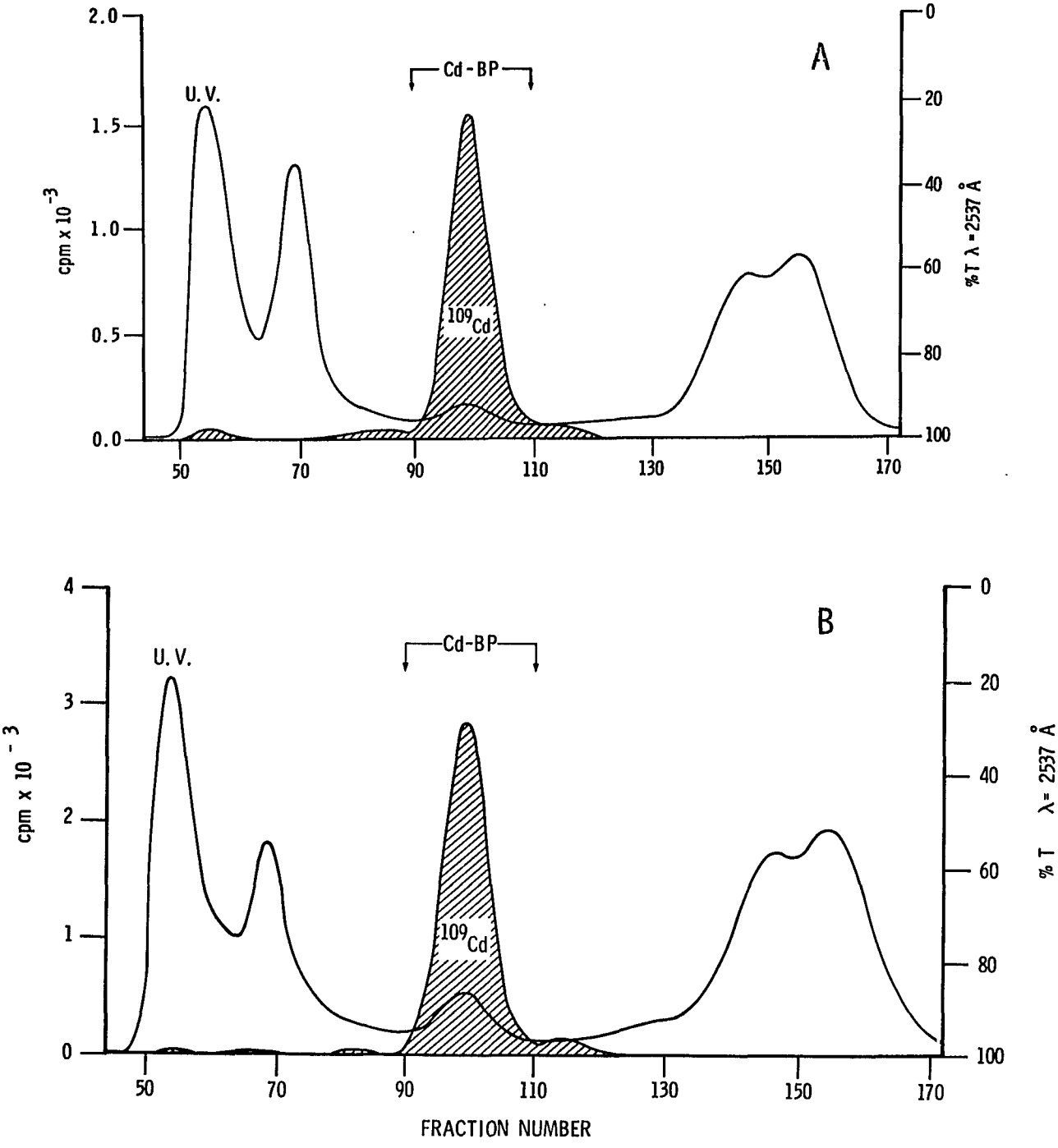


FIGURE 13. Effect of cadmium on rat kidney Cd-BP. A, after 3 injections of 0.03 mmole $CdCl_2$/Kg/day, and B, after 9 injections of 0.01 mmole $CdCl_2$/Kg/day. Sephadex G-75, 2.5 x 90 cm column; flow rate, 18.9 ml/hour (A) and 19.2 ml/hour (B).

a subcutaneous injection of carrier-free $^{109}CdCl_2$, 24 hours before they were killed.

The liver soluble fractions were analyzed on Sephadex G-75 column. Table XV summarizes the results obtained. An increase in the liver Cd-BP was observed in animals which ingested $CdCl_2$ for 1 to 12 weeks. During this exposure to cadmium, the rats looked healthy and gained weight.

Gel filtration patterns of liver soluble fractions obtained from rats after ingestion of $CdCl_2$-containing water are shown in Figures 14A and B. An increase in UV-absorption in the Cd-BP region is evident. Apparently sufficient cadmium for the stimulation of the Cd-BP synthesis was absorbed from the alimentary canal and reached the liver. In these experiments, only the liver tissue was analyzed.

5E. Purification of ^{109}Cd-labeled Proteins:

Kägi and Vallee (1961) purified crude equine kidney metallothionein by ion-exchange chromatography on DEAE-cellulose and isolated a protein component containing 37.8% of the total cadmium applied to the column. The total recovery of cadmium from the column was 49.1%. Later, Pulido et al. (1966) achieved purification of human kidney metallothionein by gel filtration on Sephadex G-50 and G-75, followed by polyacrylamide P-20. The isolated metallothionein was further resolved by Porath column electrophoresis into two cadmium-containing fractions accounting for 87.5% of the total applied cadmium.

TABLE XV. Effect of Ingested $CdCl_2$ on Rat Liver Cd-BP

Weeks	Total Cd Ingested		Cd-BP* (G-75 column)	Weight of Animal	
				Beginning	End
	mmoles	mg	mg/g liver	g	g
0 (control)	----	---	2.8	217	217
1	0.06	11.4	3.1	218	248
2	0.17	31.1	3.5	211	290
3	0.24	44.4	4.0	224	327
6	0.44	81.6	4.1	219	383
8	0.52	95.8	4.0	210	358
12	0.80	147.0	3.6	209	432
12 (control)	----	---	2.7	220	450

*Determined by Lowry's method in one animal.

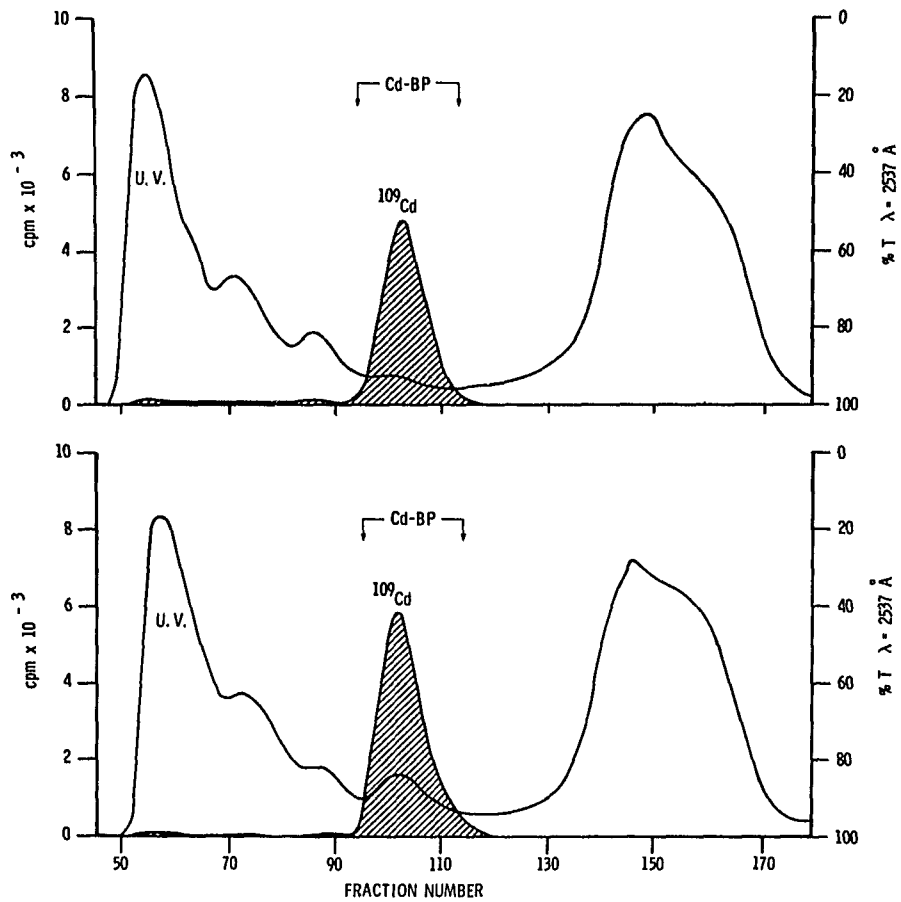


FIGURE 14. Rat liver soluble fractions, after exposure of the animals to 0.5 mM $CdCl_2$ in drinking water for 1 (A) and 8 (B) weeks. Sephadex G-75, 2.5 x 90 cm column; flow rate, 18.6 ml/hour (A) and 18.9 ml/hour (B).

To further purify the rat liver Cd-BP isolated by Sephadex G-75 gel filtration, ion-exchange chromatography was performed. As a first step towards purification, the dried protein labeled with ^{109}Cd was desalted on a Sephadex G-25 (Fine), 1.5 x 85 cm column, using distilled water as elutant. Protein fractions containing 95% of the applied ^{109}Cd were eluted in the void volume of the gel, which had an exclusion limit of 70,000 molecular weight. The ^{109}Cd-containing fractions were pooled and freeze-dried.

Further purification of Cd-BP was done on DEAE-Sephadex A-25 and A-50, using several Tris-HCl, pH 8.6 buffer systems. These included: stepwise change of the concentration from 0.001 to 0.25 and then to 0.5 M Tris, in the presence of 0.02% sodium azide; a continuous gradient from 0.001 to 0.25 M Tris, containing 0.02% sodium azide, 0.1% Chloretone (β,β,β-trichloro-tert. butanol, K & K Laboratories, N. Y.), or without any added preservative; continuous 0.25 M NaCl gradient in 0.05 M Tris-HCl buffer, in the presence of 0.02% sodium azide, 0.1% Chloretone, or without any added preservative.

For chromatography, 10 mg of Cd-BP was dissolved in 1 ml of the starting buffer and applied to a 1.5 x 27 cm column, which was then eluted with one of the buffer systems described above. The best resolution of Cd-BP was achieved on a DEAE-Sephadex A-25 column, using a continuous 0.001 to 0.25 M Tris gradient containing 0.02% sodium azide. The elution diagram is shown in Figure 15. In all, four UV-

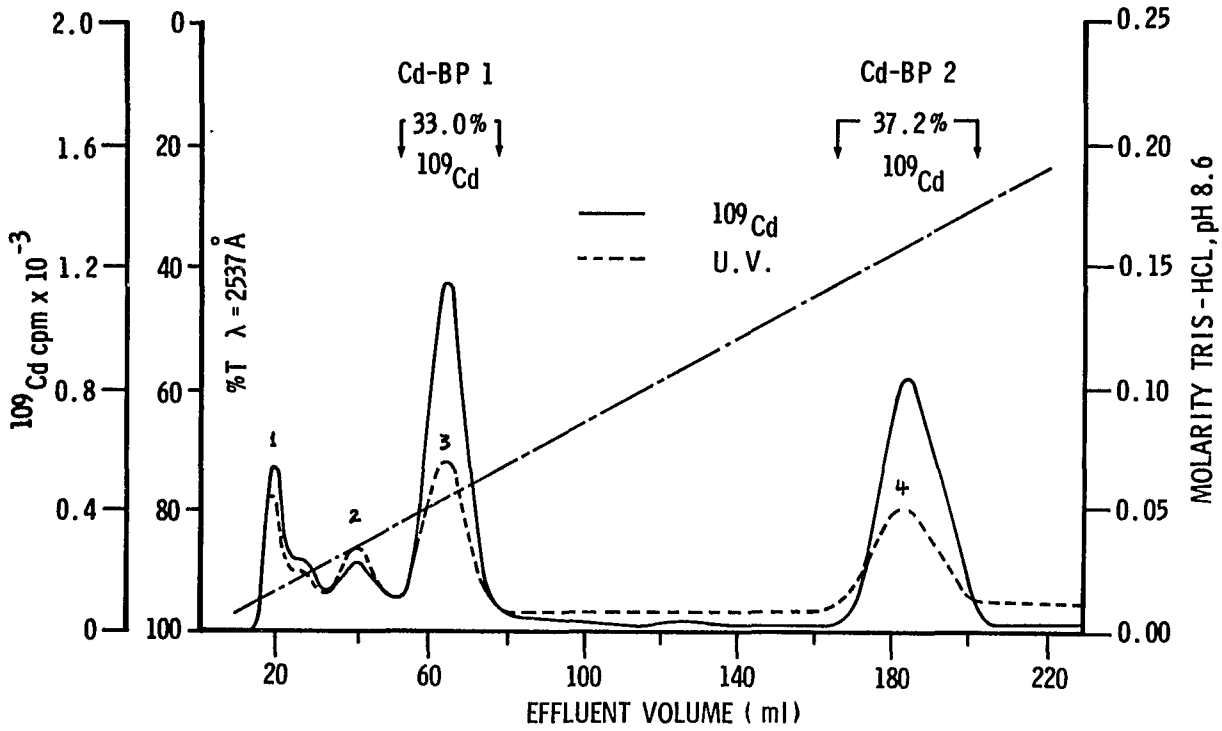


FIGURE 15. Chromatography of rat liver Cd-BP. DEAE-Sephadex A-25, 1.5 x 27 cm column; flow rate, 13.14 ml/hour (2.19 ml/fraction).

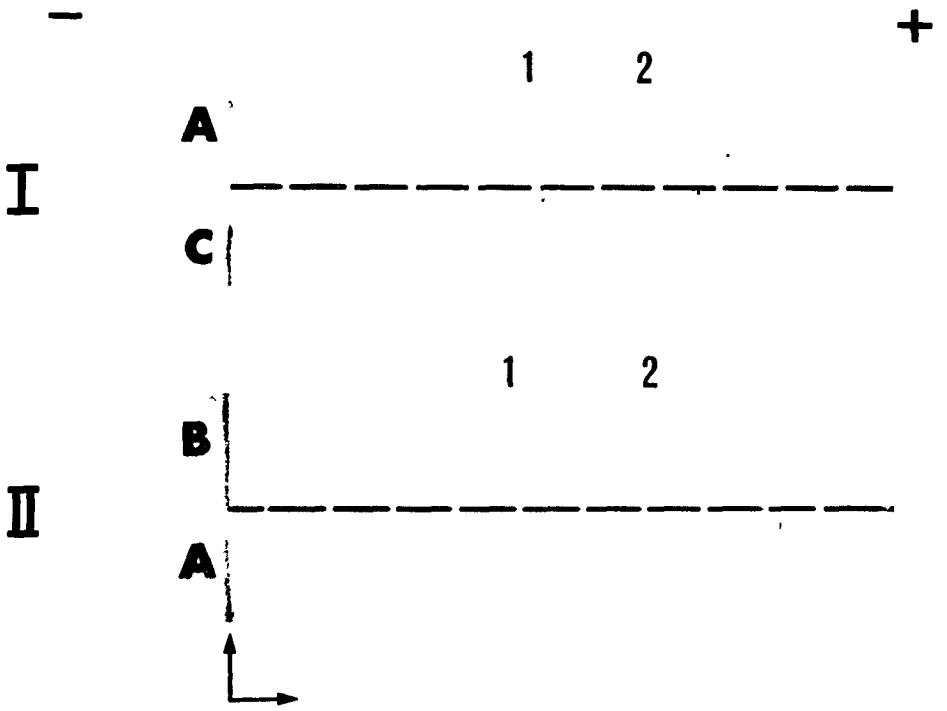
absorbing components were eluted from the column. The total ^{109}Cd recovered in 210 ml of the effluent was 85% of that applied to the column. The distribution of ^{109}Cd in column fractions corresponded to the UV-absorption. Of the total radioactivity recovered from the column, 33% was associated with component 3 and 37.2% was bound to the component eluted last. These two components were named as Cd-BP 1 and Cd-BP 2 respectively. The combined recovery of ^{109}Cd in Cd-BP 1 and Cd-BP 2 was 66.7% of the total applied to the column. This value was higher than that reported for metallothionein by Kägi and Vallee (1961), on a DEAE-cellulose column. Fractionation of rat kidney Cd-BP was also performed on DEAE-Sephadex and it revealed the presence of Cd-BP 1 and Cd-BP 2 in the kidney tissue as well.

5F. Criteria of Purity:

Ion-exchange chromatography had resolved the crude Cd-BP into two major components. It was not known whether these components were homogenous. The homogeneity of the desalted Cd-BP 1 and Cd-BP 2 was verified by electrophoresis on cellulose acetate and also on polyacrylamide gel. Along with these isolated proteins, the crude protein was also subjected to electrophoresis under identical conditions.

Figure 16 shows the photograph of stained cellulose acetate strips after electrophoresis of the crude and purified Cd-BP. The electrophoretic run was carried out in 0.08 M sodium barbital, pH 8.6 buffer. Two protein bands

FIGURE 16. Cellulose acetate electrophoresis of rat liver cadmium-binding proteins. Two protein samples were applied on strip I and two on strip II. Sample A was crude Cd-BP isolated from Sephadex G-75 column. Protein samples B and C were Cd-BP 1 and Cd-BP 2 isolated by ion-exchange chromatography. The protein bands are numbered 1 and 2 according to their electrophoretic mobility.


−
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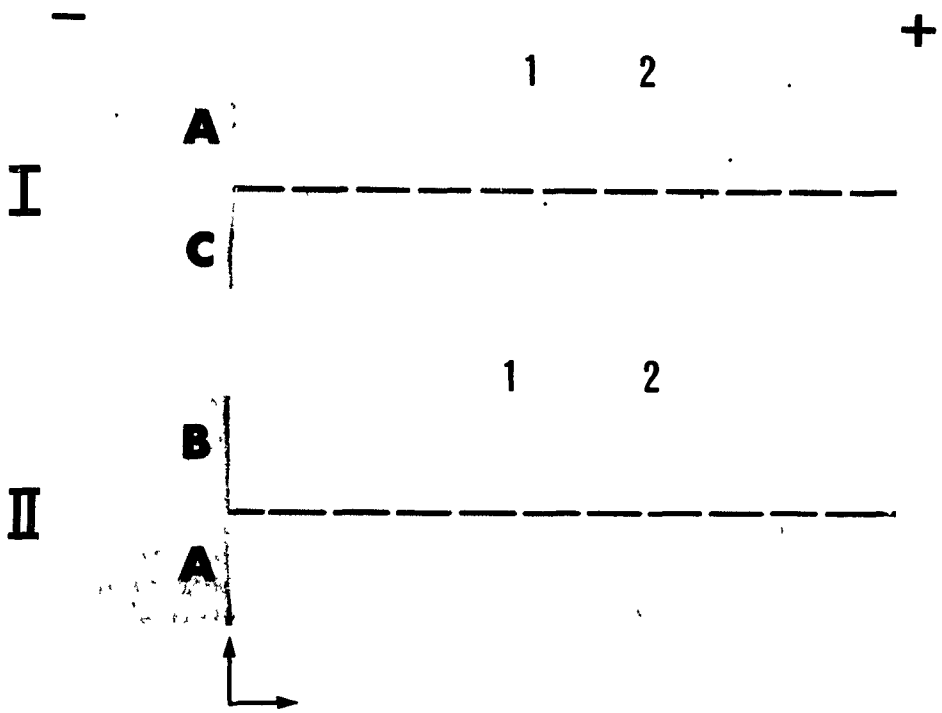
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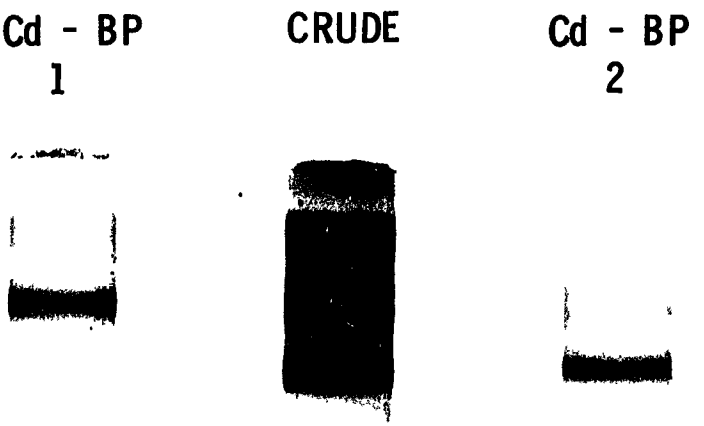
A

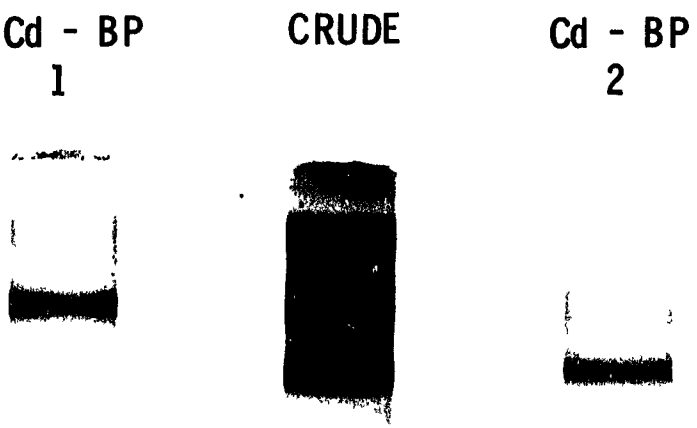
were obtained from crude Cd-BP, while purified Cd-BP 1 and Cd-BP 2 migrated as single bands without further resolution. The mobility of Cd-BP 2, on cellulose acetate, was greater than that of Cd-BP 1. The former protein probably had a higher net negative charge than the latter, since it was eluted from the DEAE column by a buffer of higher ionic strength. On comparison with the crude Cd-BP, the mobility of Cd-BP 1 band coincided with the slow moving band of crude Cd-BP. Similarly Cd-BP 2 had the same mobility as the fast moving component of the crude protein.

Disc electrophoresis of the proteins was carried out in 7% polyacrylamide, at pH 9.5. Between 10 and 15 μl of aqueous solution containing 20-25 μg of protein was mixed with the loading gel layer before polymerization of the acrylamide. Electrophoresis was performed at room temperature for 40 minutes in tris-glycine, pH 9.5 buffer. On staining the gel columns, sharp protein bands were obtained. The crude protein sample produced three stained components (Figure 17). Further resolution of Cd-BP 1 was not achieved, and it moved at the same rate as the band of intermediate mobility separated from the crude Cd-BP. In comparison, Cd-BP 2 had a greater mobility in the electric field than Cd-BP 1 and it corresponded to the fast moving band of the crude Cd-BP. To determine the distribution of ^{109}Cd, unstained gels were sliced and assayed. The results revealed that radioactivity was present in the region of the two fast moving bands, representing Cd-BP 1 and Cd-BP 2. In addition,

FIGURE 17. Polyacrylamide gel electrophoresis of rat liver cadmium-binding porteins. The crude sample was obtained from Sephadex G-75 column. Cd-BP 1 and Cd-BP 2 were purified by ion-exchange chromatography.


Cd - BP
1
CRUDE
Cd - BP
2

(+) (+) (+)


Cd - BP
1
CRUDE
Cd - BP
2
(+)
(+)
(+)

the slow moving band of crude Cd-BP also contained 10% of the total ^{109}Cd.

5G. Isolation:

The experimental observations obtained during the purification of Cd-BP were integrated and a simplified procedure for isolation of Cd-BP was devised to replace the method of Pulido et al. (1966). This procedure is outlined in Figure 18. One of its basic features is that it involves only mild treatments of the protein molecules during isolation, and avoids the ethanol-chloroform precipitation repeatedly used by Kägi and Vallee (1960; 1961) and Pulido et al. (1966).

To perform physicochemical analyses of Cd-BP 1 and Cd-BP 2, more proteins were needed than could be isolated from normal rats. Therefore, the synthesis of Cd-BP was first enhanced in rats by repeated injections of $CdCl_2$ and later, the proteins were isolated by the procedure described in Figure 18. Table XVI gives the analyses of cadmium, Folin-Ciocalteu-reactive protein, nitrogen, and the dry weight of various fractions during isolation of Cd-BP. For this particular batch mentioned in Table XVI, four male rats were injected subcutaneously with 0.01 mmole $^{109}CdCl_2$/Kg daily, for 10 days. On the 11th day, the animals were killed and the liver tissue from all four animals was pooled. Thirteen grams of liver tissue was homogenized in 2 volumes of 0.25 M sucrose and centrifuged to obtain the soluble fraction. The residue obtained after centrifugation was

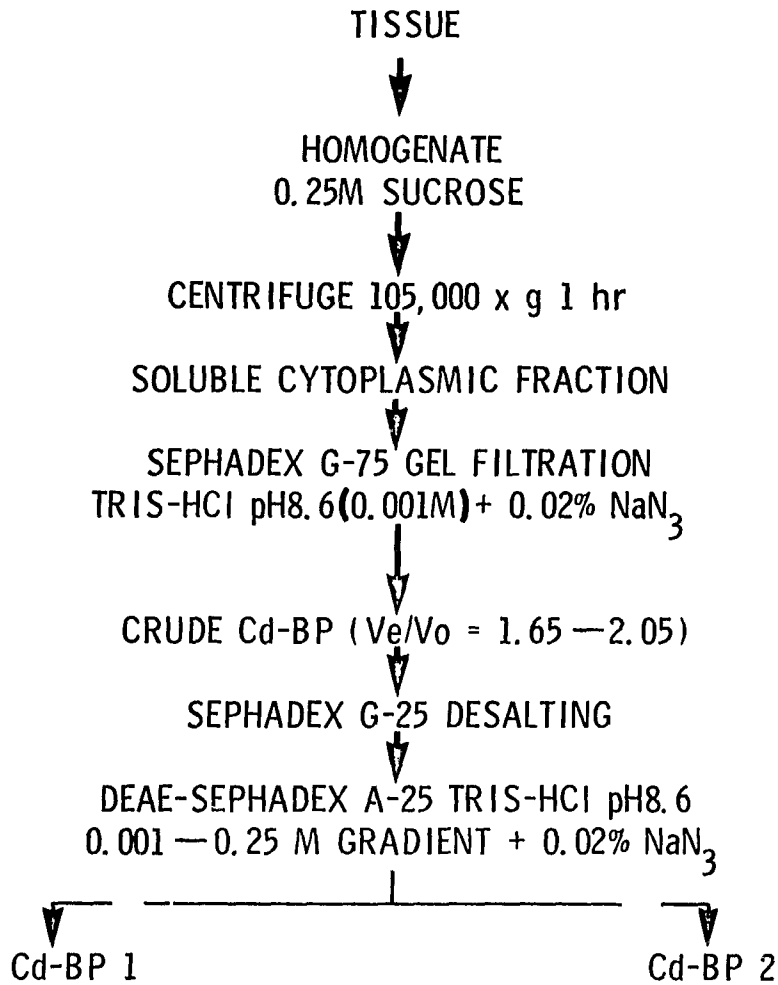


FIGURE 18. Scheme for the isolation of cadmium-binding proteins.

TABLE XVI. Representative Yield During Isolation of Cadmium-binding Proteins from Rat Liver*

Fraction	Cd^{2+}		Protein**		Nitrogen		N/Prot.**	Specific Activity	
	(μg)	(%)	(mg)	(%)	(μg)	(%)	(%)	$mgCd^{2+}$/g Prot.**	$mgCd^{2+}$/g F.D.P.≠
Liver Homogenate	208	100.0	***	***	***	***	***	***	***
Soluble Fraction	148	71.1	63.00	100.0	***	***	***	2.3	***
Crude - Cd-BP (G-75 column)	129	62.0	***	***	***	***	***	***	***
Cd-BP after Desalting	125	60.1	6.27	9.9	519	100.0	8.1	19.9	28.0
Cd-BP 1	50	24.0	1.09	1.7	56	10.8	5.1	45.9	68.5
Cd-BP 2	53	25.5	1.25	2.0	79	15.2	6.3	42.4	60.9

* Results are expressed per gram of liver tissue.

** Determined by the method of Lowry et al. (1951).

≠ Freeze-dried protein.

*** Not determined.

discarded without being washed. Twenty-five ml of the soluble fraction was used for gel filtration on a Sephadex G-75 column. The fractions containing ^{109}Cd (Ve/Vo 1.65 to 2.05) were pooled and freeze-dried. Before ion-exchange chromatography, the crude Cd-BP was desalted by a Sephadex G-25 column and lyophilized. The above procedure was repeated several times until all of the liver tissue was utilized. The freeze-dried protein was then purified on a 2.5 x 34 cm column packed with DEAE-Sephadex A-25. About 50 mg of crude Cd-BP was dissolved in 2 ml of 0.001 M Tris-HCl, pH 8.6 buffer and applied to the column. The gradient for this column was formed by mixing 0.5 M Tris-HCl, pH 8.6 buffer with 500 ml of 0.001 M Tris-HCl, pH 8.6 buffer, at a rate of 23 ml/hour. The ^{109}Cd-containing fractions of Cd-BP 1 and Cd-BP 2 regions were pooled separately and freeze-dried. The lyophilized proteins were desalted on a 2.5 x 56 cm Sephadex G-25 column, and stored in the dry state. Cadmium was estimated from the specific activity of the reference $^{109}CdCl_2$ solution used for injection. Assuming that the cadmium stores of the control animals were negligible as compared to the heavy loads injected, no corrections for the endogenous cadmium values were applied in the calculations. It was observed that Cd-BP 1 contained 45.9 mg Cd^{2+}/g of protein, or 24% of the total cadmium in the liver (Table XVI). Similarly, Cd-BP 2 had 42.4 mg Cd^{2+}/g of Folin-ciocalteu-reactive protein, or 25.5% of the total liver cadmium. The total recovery of cadmium in Cd-BP 1 and Cd-BP 2 was 49.5%

of that originally present in the tissue homogenate, or 64% of that found in the soluble fraction. After gel filtration and ion-exchange chromatography of the soluble fraction an 18 to 20-fold enrichment of the isolated protein fractions was achieved.

The nitrogen to Folin-Ciocalteu-reactive protein ratio was rather low (0.083). A possible cause of this was the over-estimation of protein content by Lowry's method (1951). Comparison of the protein content determined by Lowry's method (1951) with the dry weight of the lyophilized and desiccated preparation (uncorrected for the moisture content) showed that the ratio between the two values was in the order of 1.5. Despite the higher values for proteins estimated by the former method, it was retained in practice to keep uniformity in results. As shown in Table XVI, in terms of dry weight, the cadmium content of Cd-BP 1 was 6.85% and that of Cd-BP 2 was 6.09%.

5H. Physicochemical Properties:

The amino acid compositions of rat liver Cd-BP 1 and Cd-BP 2, purified by DEAE-Sephadex chromatography, were determined. Performic acid-oxidized and native protein samples were hydrolyzed in HCl and chromatographed on an amino acid analyzer. Alkaline hydrolysis for tryptophan was not carried out. It was apparently not present since the proteins had no absorption peak between 275 and 280 nm. In all, 13

different amino acids were estimated in both Cd-BP 1 and Cd-BP 2. Only traces of arginine, histidine, tyrosine, and phenylalanine were found. The results of the amino acid analysis of Cd-BP are expressed as the number of amino acid residues per 100 residues in Figure 19 and Table XVII.

Both cadmium-binding proteins were rich in half-cystinyl residues, which accounted for nearly one-third of the total amino acid residues. Lysyl and seryl residues together constituted another quarter of the total amino acids.

In Cd-BP 1, other amino acids in decreasing order of abundance were: glycine, aspartic acid, threonine, alanine, proline, glutamic acid, valine, and methionine. The amino acid distribution in Cd-BP 2 was in the following order: alanine, aspartic acid, glutamic acid, glycine, proline, threonine, valine, and methionine. About one residue of each of isoleucine and leucine was also present in both proteins (Table XVII). The amino acid composition per 100 residues of Cd-BP 1 differed only slightly from Cd-BP 2.

The mean residue weight of the apoproteins calculated from the amino acid composition was 100.71 for Cd-BP 1 and 100.58 for Cd-BP 2. Using these values and applying corrections for NH_3, the minimum molecular weights of apoprotein 1 and apoprotein 2 were determined to be 10,310 and 10,263 respectively. Estimation of nitrogen from the amino acid composition revealed that the proteins contained 15.7 and 15.8 % nitrogen, respectively. The sulfur content of apoprotein 1 was found to be 11.1% and that of apoprotein 2 was 9.8%.

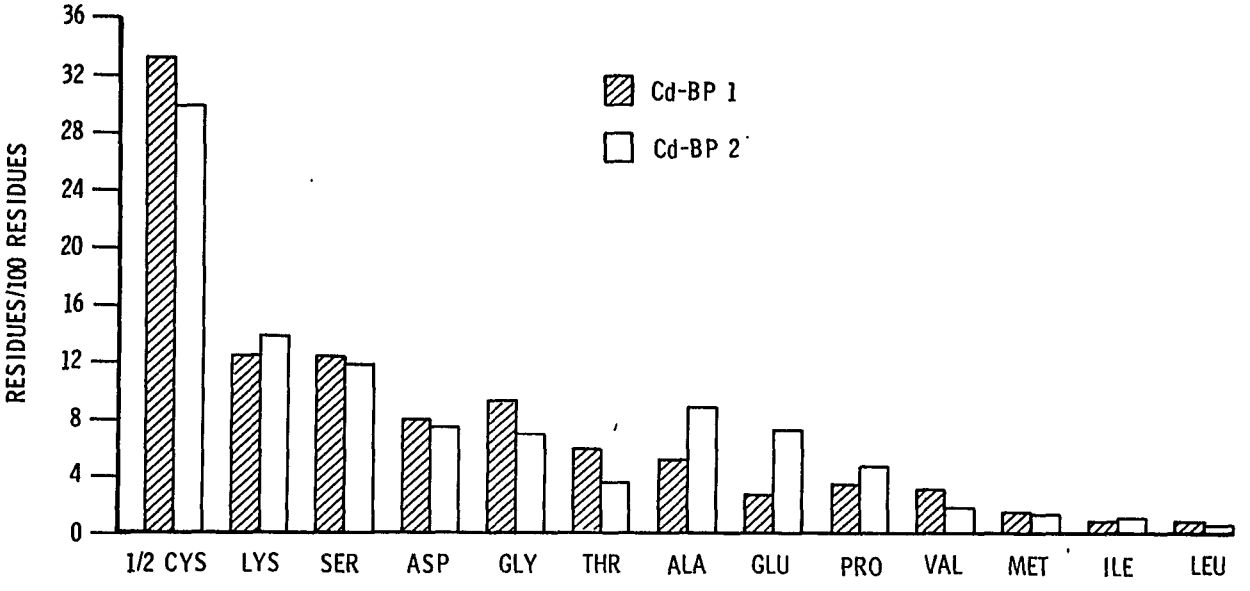


FIGURE 19. Comparison between amino acid compositions of rat liver Cd-BP 1 and Cd-BP 2.

TABLE XVII. Amino Acid Analysis of Rat Liver Cd-BP

	(no. of residues/100 residues)*			
Amino Acid	Cd-BP 1		Cd-BP 2	
Lysine	12.6	(13)**	13.8	(14)
Aspartic Acid	8.0	(8)	7.6	(7)
Threonine	6.0	(6)	3.7	(4)
Serine	12.3	(12)	11.8	(12)
Glutamic Acid	2.8	(3)	7.4	(7)
Proline	3.6	(4)	4.7	(5)
Glycine	9.4	(9)	7.0	(7)
Alanine	5.2	(5)	8.9	(9)
Half Cystine	33.3	(33)	29.8	(30)
Valine	3.2	(3)	2.0	(2)
Methionine	1.7	(2)	1.6	(1)
Isoleucine	1.0	(1)	1.1	(1)
Leucine	1.0	(1)	0.7	(1)

* Average of 2 determinations.

**Nearest whole number.

Used for further calculations

The molecular weights of Cd-BP 1 and Cd-BP 2 were also determined by Sephadex gel filtration. A 1.5 x 85 cm Sephadex G-75 column was standardized with known molecular weight markers by determining the elution volumes of reference proteins. These results were plotted against the respective molecular weights of the proteins on a semi-logarithmic scale. As shown in Figure 20, a straight line relationship was obtained for most reference proteins. Only bovine serum albumin (MW 67,000) and equine heart cytochrome C (MW 12,400) deviated from this relationship. Their apparent molecular weights estimated from the graph were 74,000 and 15,000, respectively. In two separate observations, the molecular weight of Cd-BP 1 was determined to be 11,300 and 11,500 (average 11,400), and that of Cd-BP 2 as 12,000 (Table XVIII). In addition to the elution peaks of purified Cd-BP 1 and Cd-BP 2, a higher molecular weight ^{109}Cd-containing component also appeared as a shoulder to the main elution peak. The molecular weight of this component was approximately twice that of the main protein. It was suspected, therefore, that the higher molecular weight proteins were putative dimers of the parent molecules.

The cadmium content of Cd-BP was estimated from the specific activities of the proteins and the molecular weights obtained by the gel filtration method. In Table XVI, the value of Cd/g of freeze-dried protein is stated to be 68.5 mg/g for Cd-BP 1 and 60.9 mg/g for Cd-BP 2. Applying these figures to the molecular weights of the metalloproteins, it

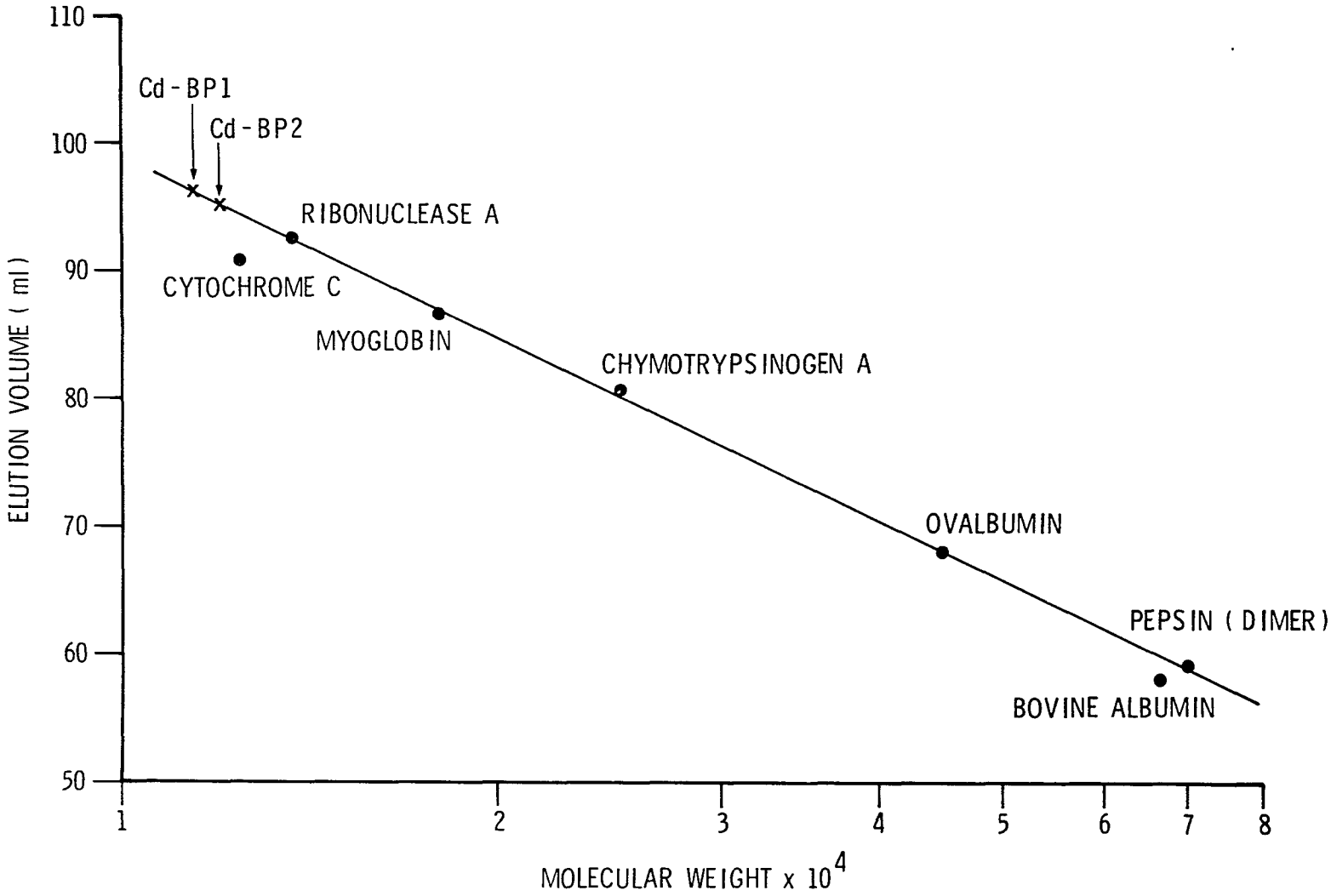


FIGURE 20. Relationship between molecular weights of proteins and their elution volumes on Sephadex G-75.

TABLE XVIII. Gel Filtration Behaviour of Molecular Weight Markers and Cd-BP on Sephadex G-75

Material	Source	Elution Volume (ml)	Molecular Weight
Pepsin (dimer)	Porcine Stomach	59.00	71,000
Albumin	Bovine Serum	58.00	67,000
Albumin	Ovine	68.00	45,000
Chymotrypsinogen A	Bovine Pancreas	80.75	25,000
Myoglobin	Sperm Whale	86.50	17,800
Ribonuclease A	(Unknown)	92.60	13,700
Cytochrome C	Equine Heart	90.80	12.400
Cd-BP 1*	Rat Liver	95.95	11,400
Cd-BP 1 (dimer)*	Rat Liver	83.00	21,800
Cd-BP 2*	Rat Liver	95.05	12,000
Cd-BP 2 (dimer)*	Rat Liver	82.00	23,000
Blue Dextran*	---	51.50	2,000,000
Sucrose	---	134.55	342

*Average of two determinations.

was found that Cd-BP 1 had 6.9 g-atoms of Cd/11,400 molecular weight, and Cd-BP 2 had 6.5 g-atoms of Cd/12,000 molecular weight. The ratio of the number of possible -SH groups/atom of Cd for both proteins was calculated to be 5.1. Subtraction of the metal content from the molecular weights of metalloproteins revealed that the molecular weight of apoprotein 1 was 10,620 and that of apoprotein 2 was 11,270. These values were very close to those calculated from the amino acid composition of the proteins.

5I. Metabolism:

The biosynthesis as well as degradation of Cd-BP was studied *in vivo*. For this purpose rats were injected with an aqueous solution containing uniformly labeled cystine - ^{14}C and the incorporation of ^{14}C into the liver Cd-BP was determined. In some experiments uniformly labeled threonine-^{14}C and lysine-2-^{14}C were also used.

To study the biosynthesis of Cd-BP, male rats were divided into two groups. The animals of the first group received 1 ml of uniformly labeled cystine-^{14}C (17.95 nmoles, 4.2 μCi) subcutaneously, and these served as controls. In the other group each animal was injected with a single subcutaneous dose of 0.03 mmole $CdCl_2$/Kg and 1 ml of cystine-^{14}C (17.95 nmoles, 4.2 μCi). The rats were killed 2, 5, 12, and 24 hours after injection. Liver soluble fraction from each animal was prepared by homogenization of the tissue

(20% w/v) in 0.25 M sucrose followed by ultracentrifugation. Fourteen ml of the soluble fraction from each rat liver was fractionated by gel filtration on a 2.5 x 90 cm column packed with Sephadex G-75. The total ^{14}C recovered in the Cd-BP region was calculated as a percent of the soluble fraction radioactivity. The distribution of radioactivity in the Cd-BP region of control specimens was compared with that of cadmium-treated animals. Table XIX shows the results obtained from both groups. The incorporation of ^{14}C into Cd-BP increased continuously during the 24 hours of study in the control and in the treated animals. In the rats injected with $CdCl_2$, the increased rate of incorporation of ^{14}C into Cd-BP was accompanied by a simultaneous elevation in UV-absorption in the Cd-BP region. A plot of ^{14}C radioactivity found in the Cd-BP region versus the time elapsed after injection of the amino acid is presented in Figure 21. It reveals that the biosynthesis of Cd-BP accelerated between 5 and 12 hours after $CdCl_2$ challenge. At 24 hours the Cd-BP region of a cadmium-exposed animal had concentrated some 3.4 times more ^{14}C than that of the control rat.

The gel filtration patterns of liver soluble fractions from the control and cadmium-treated rats are illustrated in Figures 22A and B. Column chromatography resolved the soluble fraction into several ^{14}C-labeled components. In the case of the liver soluble fraction from the control animal, 47.9% of ^{14}C appeared in the molecular weight region greater than Cd-BP. The Cd-BP region (Ve/Vo 1.65 - 2.05) contained only

TABLE XIX. Incorporation of ^{14}C Derived from Cystine-^{14}C Into Rat Liver Cd-BP

Hours After Injection	0.03 mmole $CdCl_2$/Kg + 17.95 nmoles Cystine-^{14}C*			17.95 nmoles Cystine-^{14}C*		
	Soluble Fraction	Cd-BP (Ve/Vo 1.65-2.05)		Soluble Fraction	Cd-BP (Ve/Vo 1.65-2.05)	
	dpm/g liver	dpm/g liver	% total dpm	dpm/g liver	dpm/g liver	% total dpm
	x 10^{-3}	x 10^{-3}		x 10^{-3}	x 10^{-3}	
2	44.58	0.94	2.1	47.89	0.81	1.7
5	52.95	1.26	2.4	30.66	0.76	2.5
12	35.70	2.45	6.8	N.D.**	N.D.**	N.D.**
24	32.52	5.56	17.1	21.39	1.05	5.0

* 4.76 μCi/rat
**Not determined.

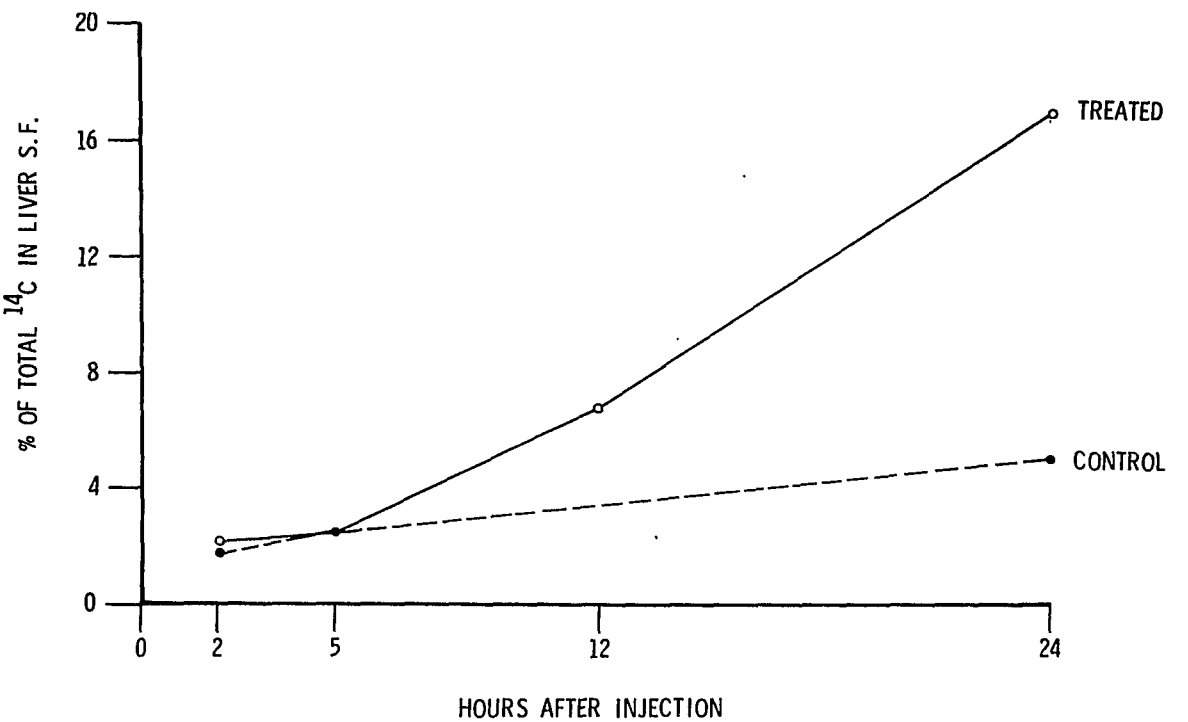


FIGURE 21. Rate of biosynthesis of Cd-BP *in vivo*. The control rats received 17.95 nmoles cystine-^{14}C and the test animals were injected with 0.03 mmole $CdCl_2$/Kg and 17.95 nmoles cystine-^{14}C.

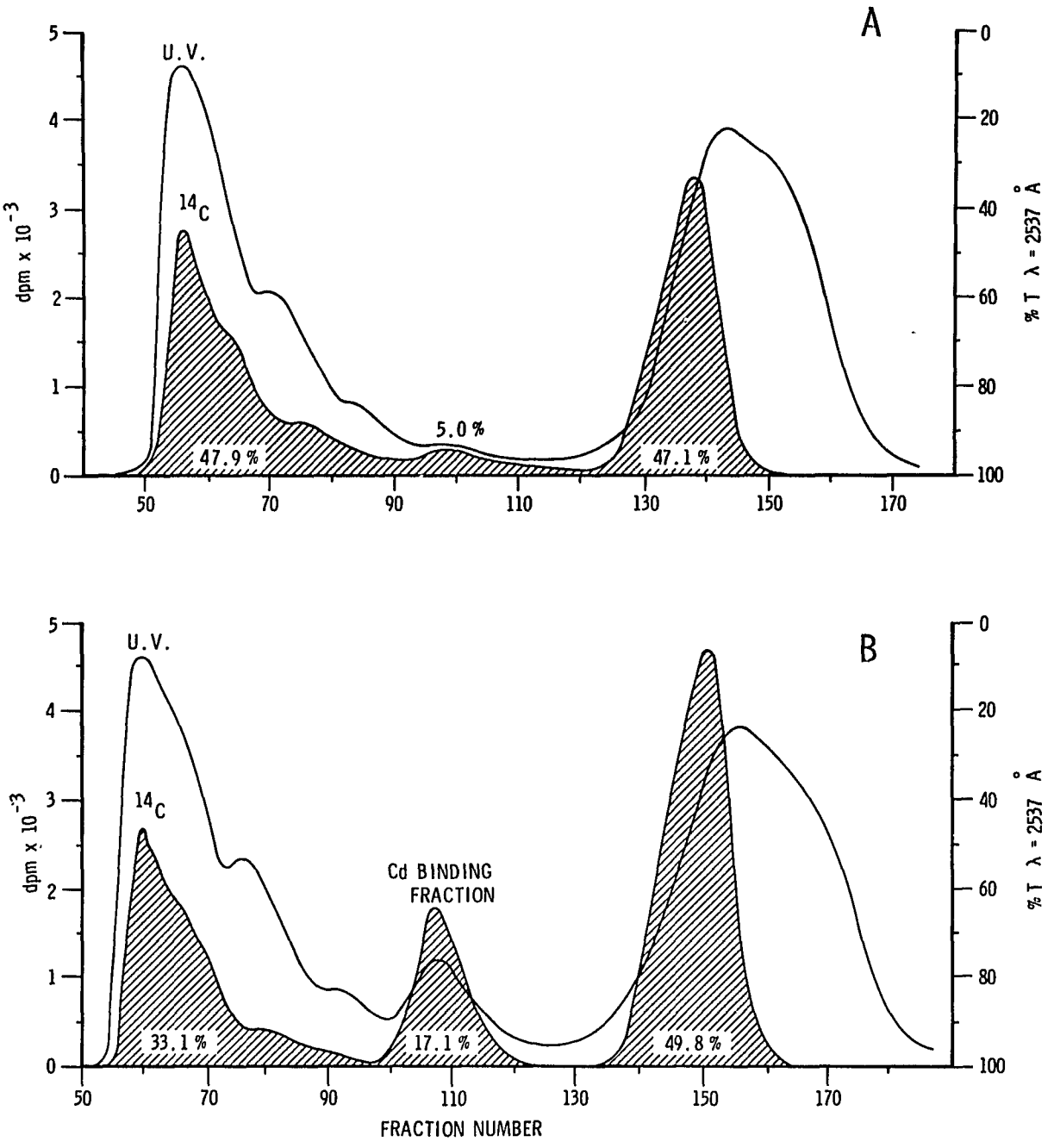


FIGURE 22. Distribution of ^{14}C derived from cystine-^{14}C in liver soluble fraction of control (A) and test (B) rat, 24 hours after injection. Sephadex G-75, 2.5 x 90 cm column; flow rate, 19.8 ml/hour (A), and 18.0 ml/hour (B).

5% of the radioactivity. The remaining ^{14}C was associated with smaller molecular weight compounds eluted between Ve/Vo 2.28 and 2.78. Liver soluble fraction from the cadmium-treated animal (Figure 22B) showed a notable increase in ^{14}C in the Cd-BP region, in comparison with the control. In this liver preparation the total ^{14}C associated with higher molecular weight components (Ve/Vo $>$ 1.65) was less than in the control (Figure 22A). However, the ^{14}C content of the small molecular weight component was in the same order of magnitude as in the control.

The column fractions were tested for their reactivity with Folin-Ciocalteu reagent (Lowry et al., 1951). All ^{14}C-containing fractions including those in the Ve/Vo region 2.28 to 2.78 produced a positive reaction. This suggested that the small molecular weight ^{14}C-containing component might comprise of low molecular weight peptides, and other metabolites of cystine. The presence of taurocholic acid in this region was also suspected. To verify these suggestions a qualitative test of cysteine, glutathione, polylysine, and taurocholic acid was carried out with the Folin-Ciocalteu reagent. A blue color was obtained in all cases, which varied in decreasing order of intensity as: cysteine $>$ glutathione $>$ polylysine $\gg$ taurocholic acid. The first three compounds also gave color in the absence of Cu^{2+}. It was observed that the reaction of polylysine and taurocholic acid with Folin-Ciocalteu reagent was enhanced by pretreatment with alkaline Cu^{2+} solution, while cysteine and glutathione showed a decrease in color

intensity. One of the above mentioned compounds, the taurocholic acid, was also chromatographed on a Sephadex G-75 column. This bile acid (100 mg) was eluted as a diffuse peak with a maximum at Ve/Vo 2.61. No further studies on the characterization of the small molecular weight ^{14}C-labeled components were undertaken.

In addition to the incorporation of ^{14}C into rat liver proteins, its turnover in these proteins was also investigated. A total of three rats were used. One rat was injected subcutaneously with 17.95 nmoles of cystine-^{14}C and the other two rats were given a subcutaneous dose of cystine-^{14}C (17.95 nmoles) together with $CdCl_2$ (0.03 mmole/Kg). The control and a cadmium-treated animal were killed one week after injection. A remarkable decrease in ^{14}C radioactivity in the liver soluble fraction was observed (Table XX). In the liver of the control animal, no radioactivity was detected in the Cd-BP region. The Cd-BP in the $CdCl_2$-treated rat contained 24.4% of the total ^{14}C present in the soluble fraction. A further decrease in the ^{14}C-content was noted in the soluble fraction of the cadmium-treated rat killed after 2 weeks. On column chromatography, the loss of ^{14}C from all fractions was evident. However, of the total radioactivity left in the soluble fraction, 14.5% was associated with the Cd-BP region (Table XX).

To establish whether the level of Cd-BP was maintained constant by a fresh supply of newly synthesized protein molecules, the following experiment was designed. First of all

TABLE XX. Utilization of ^{14}C from Cystine-^{14}C for the Biosynthesis of Rat Liver Cd-BP

$CdCl_2$*	Cystine-^{14}C**	Animal Killed	Soluble Fraction	Cd-BP (Ve/Vo 1.65 - 2.05)	
Dose (mmole)	Day	Day	dpm/g liver (x 10^{-3})	dpm/g liver (x 10^{-3})	% of total dpm
none	1	2	21.39	1.05	5.0
0.03	1	2	32.52	5.57	17.1
0.03	7	8	24.40	3.95	16.2
none	1	8	3.98	N.R.D.***	0.0
0.03	1	8	9.83	2.40	24.4
0.03	1	15	3.02	0.44	14.5
0.03	7	36	0.48	0.05	11.3

* Injected on day 1.

** 17.95 nmoles, 4.76 μCi/rat.

*** No radioactivity detected.

the synthesis of Cd-BP was stimulated by subcutaneous administration of 0.03 mmole $CdCl_2$/Kg into each of 2 rats. One week later 17.95 nmoles of cystine-^{14}C were injected subcutaneously into both animals. The rats were killed 24 hours and 29 days following cystine injection. Liver soluble fraction of each animal was obtained by centrifugation, and it was chromatographed on a Sephadex G-75 column.

The results of the above experiment are included in Table XX. On gel filtration of the soluble fraction from the rat killed after 24 hours, the Cd-BP region was found to contain 16.2% of the total ^{14}C (Figure 23A). This value was very similar to that obtained from the rat killed 24 hours after a simultaneous dose of $CdCl_2$ and cystine-^{14}C (Figure 22B, Table XX). It indicated that apparently the degradation and biosynthesis of Cd-BP were taking place at an equal rate and a steady state was maintained. The same results were observed for the higher molecular weight compounds eluted from the column before Cd-BP. Collectively, these large molecules contained 34.7% of the total ^{14}C. This observation added further support to the suggestion that the Cd-BP were metabolized at a rate comparable to that of other cytoplasmic macromolecules. In the second rat which was kept for 29 days following cystine injection, most of the ^{14}C-labeled protein was degraded and only 480 dpm/g of liver were found in the liver cytoplasm (Table XX). Analysis of the soluble fraction by gel filtration, as shown in Figure 23B, revealed that 11.3% of the total radioactivity was

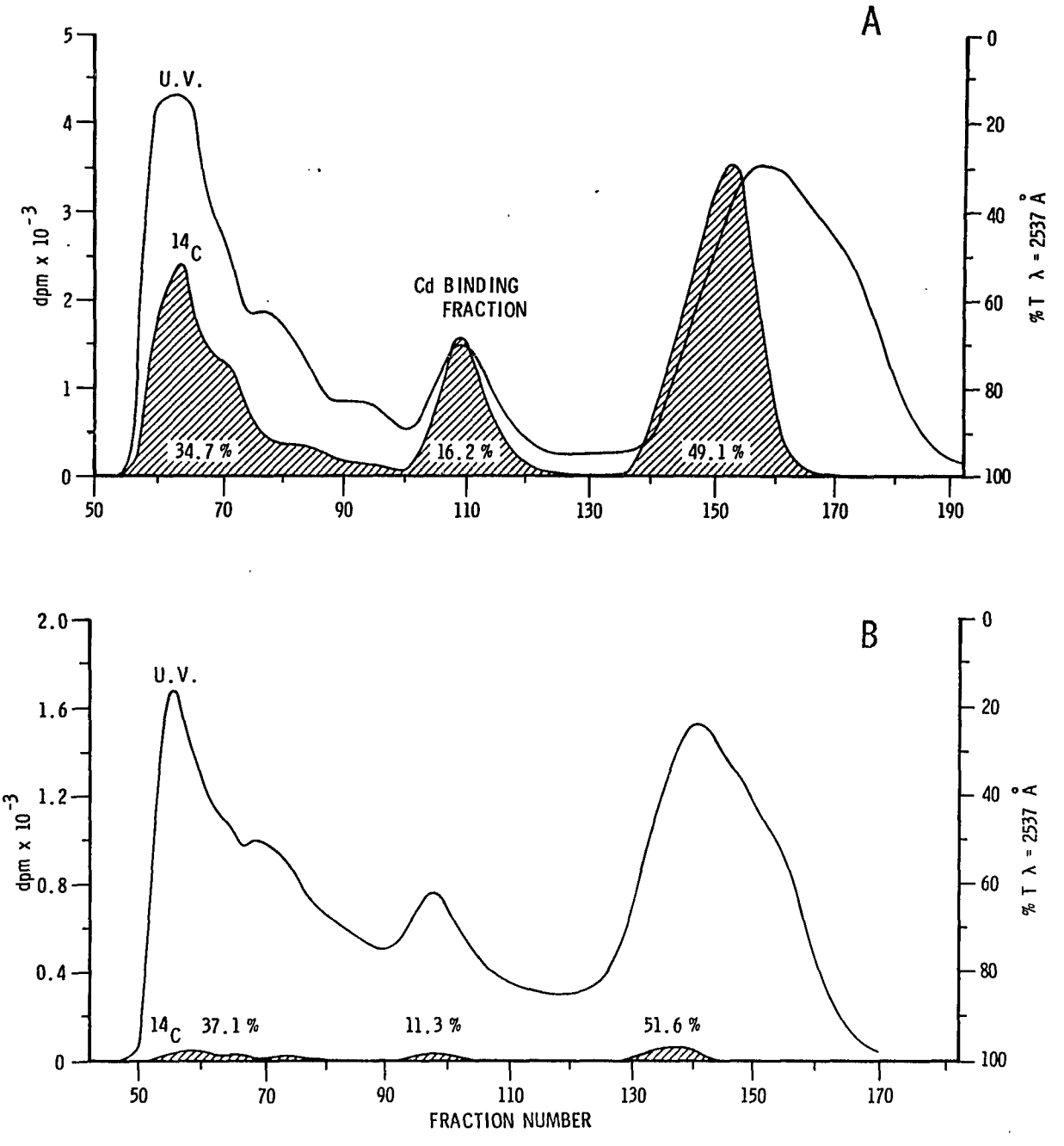


FIGURE 23. Turnover of rat liver Cd-BP. Rats were injected with 0.03 mmole $CdCl_2$/Kg and 6 days later with 17.95 nmoles of cystine-^{14}C. Liver soluble fraction was obtained 24 hours (A) and 29 days (B) after administration of cystine. Sephadex G-75 column; flow rate, 18.0 ml/hour (A) and 19.8 ml/hour (B).

present in the Cd-BP region. A simultaneous decrease in the UV-absorption or protein content of Cd-BP region did not occur. This indicated that apparently the body burden of cadmium maintained a high level of Cd-BP synthesis in vivo.

In addition to cystine-^{14}C, the incorporation of ^{14}C from uniformly labeled threonine-^{14}C and lysine-^{14}C into Cd-BP was also investigated. Their contributions toward the biosynthesis of rat liver Cd-BP in normal and $CdCl_2$-treated rats were compared. For this experiment 4 male rats were used. Two rats were injected with 62.2 nmoles (10.8 μCi) of threonine-^{14}C and the other two received 2.99 μmoles (9.75 μCi) of lysine-^{14}C. One animal from each group served as a control and the other was given a subcutaneous dose of 0.03 mmole $CdCl_2$/Kg. All animals were killed after 24 hours. The liver tissue from each rat was excised, homogenized, and centrifuged. The soluble fraction was chromatographed on a Sephadex G-75 column. The fraction of the radioactivity associated with the Cd-BP region in control and cadmium-treated rats is tabulated in Table XXI. It shows that the percentage incorporation of ^{14}C derived from threonine-^{14}C or cystine-^{14}C into the Cd-BP region of control soluble fractions was less than that from lysine-^{14}C. In animals treated with $CdCl_2$ the utilization of ^{14}C from cystine-^{14}C was the highest followed by lysine-^{14}C and threonine-^{14}C. Figure 24 shows a plot of radioactivity derived from all three amino acids recovered in the Cd-BP region. It suggested that cadmium treatment accelerated the utilization of ^{14}C

TABLE XXI. Relative Incorporation of ^{14}C Derived from ^{14}C-Labeled Amino Acids into Rat Liver Cd-BP

Group	Threonine-^{14}C*			Lysine-^{14}C**			Cystine-^{14}C***		
	Soluble Fraction	Cd-BP		Soluble Fraction	Cd-BP		Soluble Fraction	Cd-BP	
	dpm/g liver	dpm/g liver	% of total dpm	dpm/g liver	dpm/g liver	% of total dpm	dpm/g liver	dpm/g liver	% of total dpm
	$x10^{-3}$	$x10^{-3}$		$x10^{-3}$	$x10^{-3}$		$x10^{-3}$	$x10^{-3}$	
Control	29.23	1.46	5.0	18.37	1.26	6.9	21.39	1.05	5.0
Treated (0.03 mmole $CdCl_2$/kg)	22.19	1.48	6.7	20.37	1.78	8.8	32.52	5.57	17.1

* 62.20 nmoles, 10.8 µCi/rat
** 2.99 µmoles, 9.75 µCi/rat
*** 17.95 nmoles, 4.76 µCi/rat

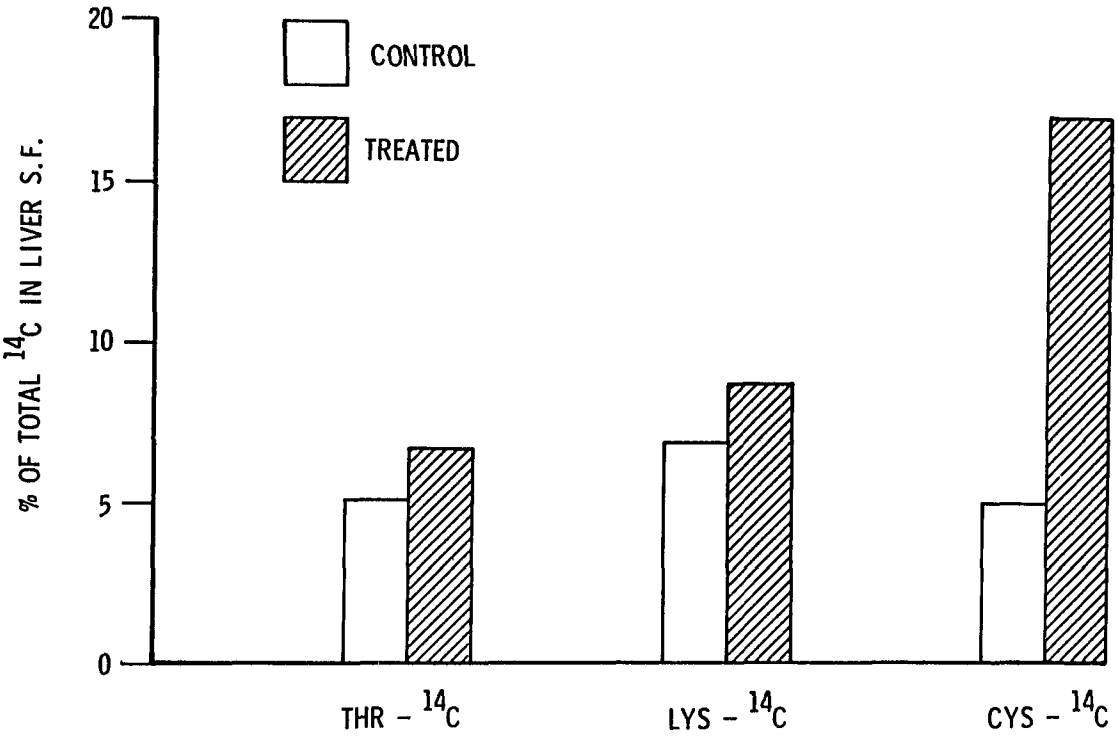


FIGURE 24. Incorporation of ^{14}C derived from labeled amino acids into rat liver Cd-BP, 24 hours after subcutaneous injection. The control animals were injected with 62.2 nmoles threonine-^{14}C, 2.99 μmoles lysine-^{14}C, or 17.95 nmoles cystine-^{14}C. The treated group received in addition to the radioactive amino acids, 0.03 mmole $CdCl_2$/Kg body weight.

from the radioactive amino acids for the biosynthesis of Cd-BP. The relative efficiency of the amino acids to provide ^{14}C-label was in the order: cystine$\gg$ lysine $\geqslant$ threonine.

5J. Purification of ^{14}C-labeled Proteins:

The Cd-BP isolated by gel filtration of the soluble fraction of rats exposed to a $CdCl_2$ load and uniformly labeled cystine-^{14}C were further analyzed by ion-exchange chromatography. The desalted protein equivalent to 12 mg of Folin-Ciocalteu-reactive protein was applied to a DEAE-Sephadex A-25 column. The protein was eluted with a 0.001-0.25 M Tris concentration gradient. As shown in Figure 25, the crude Cd-BP separated into 4 UV-absorbing components. Assay of ^{14}C radioactivity in the column fractions revealed that all UV-absorbing fractions contained ^{14}C. Of the total radioactivity applied to the column, 72% was recovered in 200 ml of the effluent. The two major components labeled as Cd-BP 1 and Cd-BP 2 together accounted for 67.4% of the total ^{14}C recovered. Comparison of the elution patterns of ^{14}C-labeled Cd-BP and the ^{109}Cd-labeled Cd-BP (Figure 15) showed a striking resemblance between the distribution of the two isotopes in Cd-BP 1 and Cd-BP 2. Estimation of Folin-Ciocalteu-reactive protein in the column fractions indicated that the total protein content of Cd-BP 1 was 1.77 mg and that of Cd-BP 2 was 1.91 mg. The specific activities of these proteins were higher than that of the crude Cd-BP. The

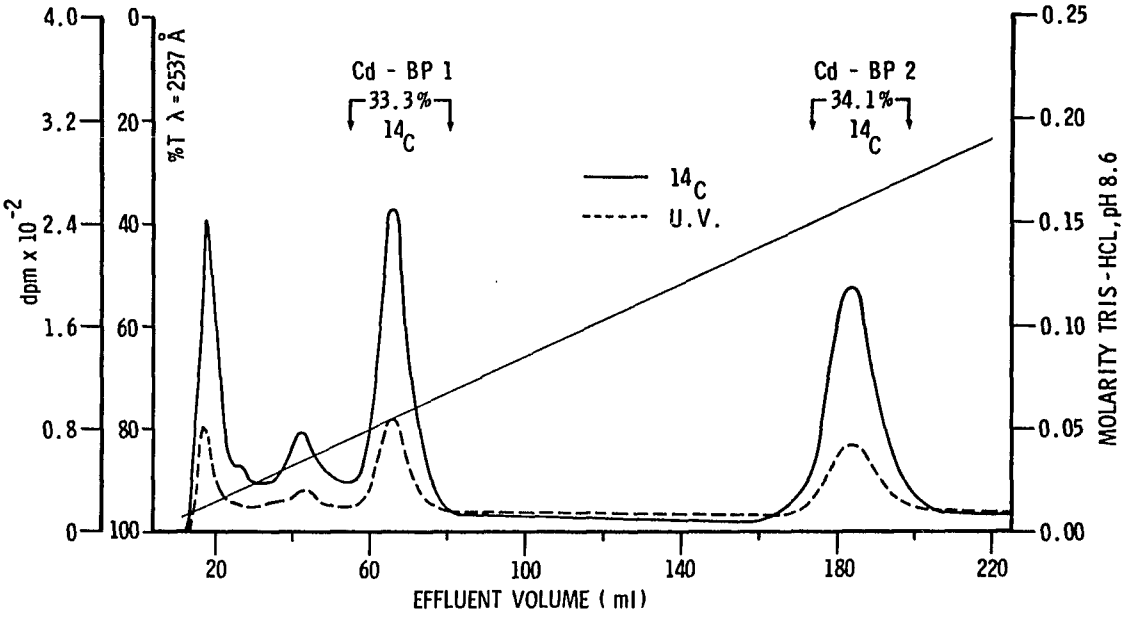


FIGURE 25. Chromatography of rat liver Cd-BP labeled in vivo with ^{14}C. DEAE-Sephadex A-25, 1.5 x 27 cm column; flow rate, 11.25 ml/hour (1.87 ml/fraction).

specific activity of the protein isolated from Sephadex G-75 column was 500 dpm/mg of Folin-Ciocalteu-reactive protein, while the purified Cd-BP 1 contained 820 dpm/mg and Cd-BP 2 contained 780 dpm/mg of Folin-Ciocalteu-reactive protein.

5K. Rodents:

The experiments in Section 3 with rats and mice injected with $^{109}CdCl_2$ have shown that ^{109}Cd is rapidly cleared from the blood and deposited in the tissues. The highest affinity for ^{109}Cd was demonstrated by the liver and kidneys. In these tissues of rats ^{109}Cd was localized in the soluble fraction. It was further shown to be bound to specific Cd-BP. The fate of ^{109}Cd was also traced in other rat tissues using the gel filtration technique. In addition to the rat tissues, the liver and kidneys of mice were also studied under similar conditions.

Since rat spleen and pancreas exhibited a notable affinity towards ^{109}Cd, attempts were made to separate Cd-BP from these tissues. Six male rats were injected daily with 0.02 mmole $^{109}CdCl_2$/Kg, subcutaneously, over a period of 10 days. From each animal the spleen and the pancreas were excised and the organs were pooled separately. The pooled tissue was homogenized in 0.25 M sucrose and the soluble fraction was separated by centrifugation. Of the total ^{109}Cd present in the splenic tissue, 74% was associated with the soluble fraction. In the case of the pancreatic tissue

86% of ^{109}Cd was in the soluble fraction. This pattern of ^{109}Cd distribution was very similar to that found in the liver and kidney tissues of rats (Table XII).

For gel chromatography 20 ml of splenic soluble fraction obtained from a 20% (w/v) tissue homogenate in 0.25 M sucrose was used. The results are shown in Figure 26. Two ^{109}Cd-containing components were separated, one near the exclusion limit of the gel and the other in the Ve/Vo region between 1.63 and 2.18. Total recovery of ^{109}Cd from the column was 92%. The Cd-BP contained 72.2% of this radioactivity. In terms of elution volume ratio the Cd-BP of the spleen was similar to that found in rat liver and kidneys.

The gel filtration pattern of the pancreatic tissue preparation is depicted in Figure 27. It was obtained after chromatography of 9.5 ml of the soluble fraction prepared from a 14.8% (w/v) homogenate in 0.25 M sucrose. Total ^{109}Cd recovered from the column was 82%. The Ve/Vo 1.63 to 2.18 region contained 90.2% of the recovered radioactivity. These results indicated that rat pancreas also contained Cd-BP, which on Sephadex G-75 behaved similarly to the Cd-BP isolated from the liver, kidneys, and spleen.

Investigations on Cd-BP were extended to rat placental tissue. Aqueous solution of carrier-free $^{109}CdCl_2$ (643,500 cpm) was injected subcutaneously into an animal on the 21st day of gestation. The animal was killed 24 hours later and a total of 12 placentae were removed from the uterus. The pooled placental tissue containing 4.67% of the injected dose was

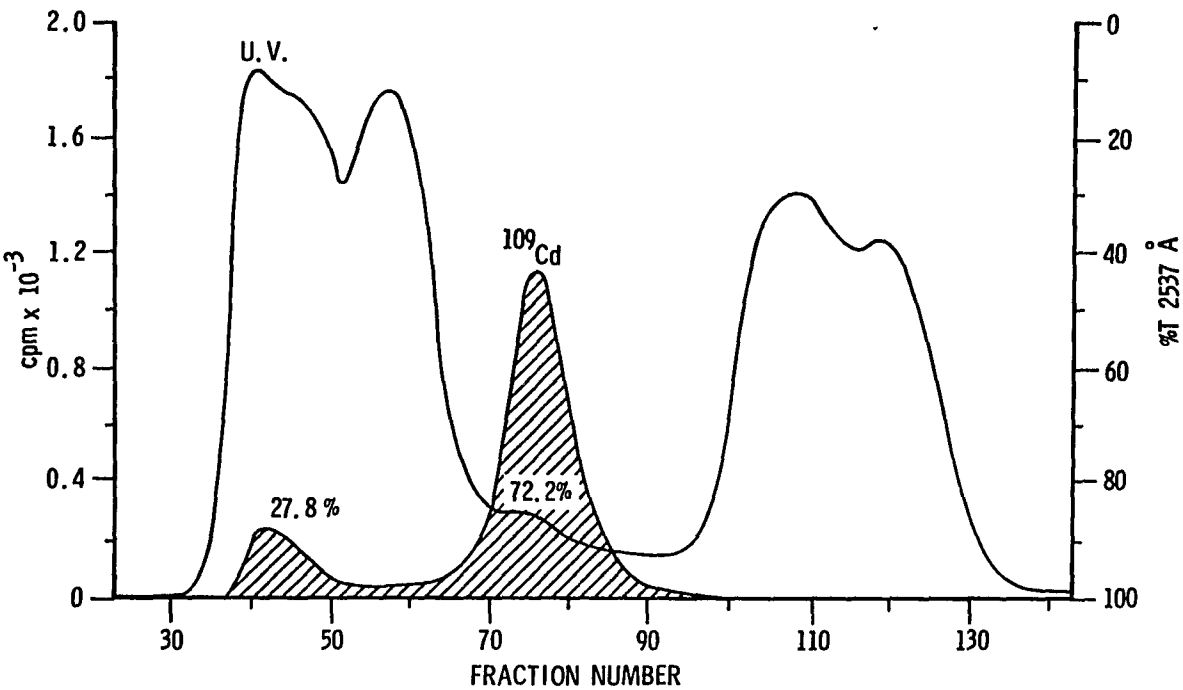


FIGURE 26. Distribution of ^{109}Cd in rat spleen soluble fraction. The rats received 0.02 mmole $^{109}CdCl_2$/Kg daily, for 10 days. Sephadex G-75, 2.5 x 90 cm column; flow rate, 27.0 ml/hour (4.5 ml/fraction).

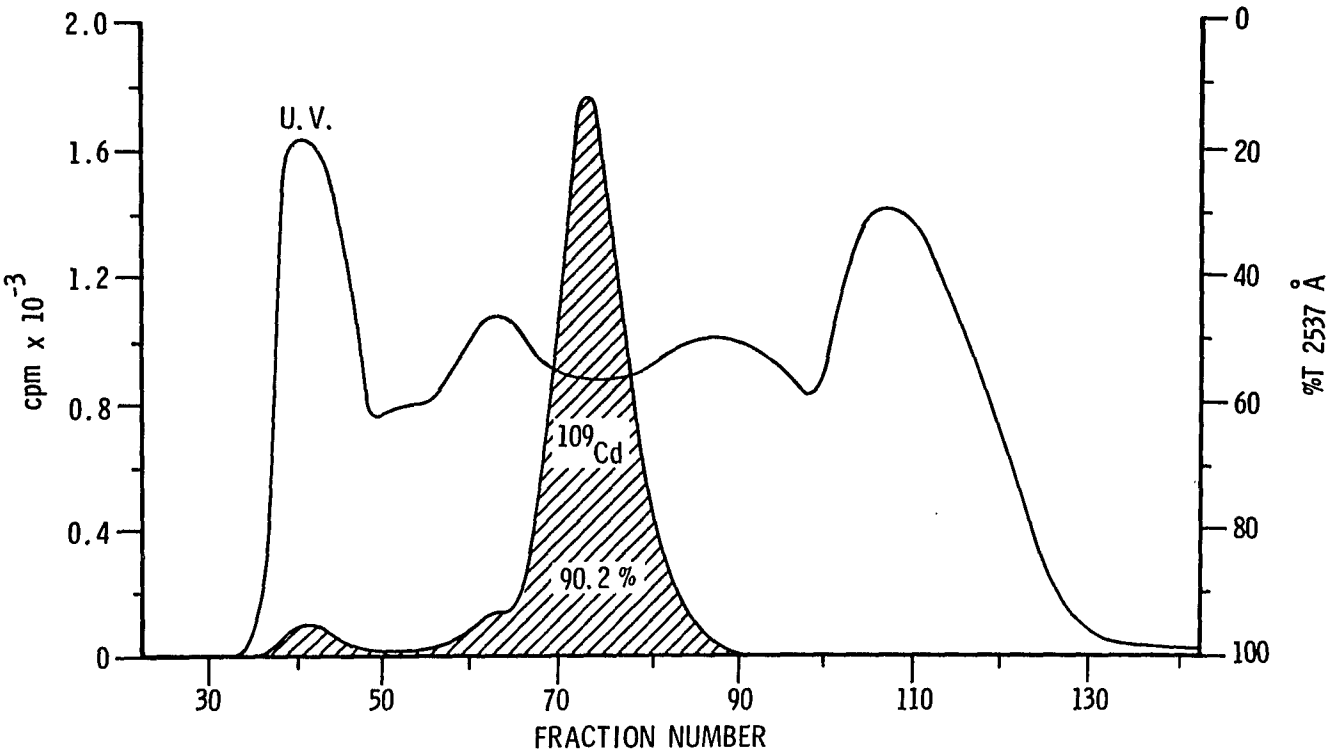


FIGURE 27. Distribution of ^{109}Cd in rat pancreas soluble fraction. The rats received 0.02 mmole $^{109}CdCl_2$/Kg daily, for 10 days. Sephadex G-75, 2.5 x 90 cm column; flow rate, 26.8 ml/hour (4.47 ml/fraction).

homogenized (20%, w/v) in 0.25 M sucrose. The homogenate was then centrifuged at 105,000 x g for 1 hour to obtain the soluble fraction. This fraction accounted for 51.6% of the total radioactivity. On a Sephadex G-75 column the soluble fraction (14 ml) separated into two ^{109}Cd-containing components (Figure 28). One of the components was eluted in the void volume of the gel and the other was eluted between Ve/Vo 1.66 and 2.2. In terms of elution volume ratio this latter component was similar to the Cd-BP described previously in other rat tissues. The ^{109}Cd distribution was, however, different from that observed in other tissues, and only 38.9% of the radioactivity was associated with the Cd-BP region. Further characterization of the placental cadmium-binding components was not carried out.

In Section 3 of this chapter, a comparison was made between the distribution and turnover of ^{109}Cd and ^{65}Zn in rats and mice. Differences in the results were noted. Cadmium-109 was primarily found in the liver and kidneys of these two mammalian species. Further analyses of rat tissues showed that ^{109}Cd was mainly associated with the Cd-BP, while ^{65}Zn was distributed among several Zn-binding components. To determine the intracellular localization of cadmium and zinc, the liver and kidneys of mice were also analyzed.

Three male mice were injected subcutaneously with 10 nmoles of $^{109}CdCl_2$ and 10 nmoles of $^{65}ZnCl_2$. The animals were killed after 24 hours and the liver and kidneys were pooled separately. A tissue homogenate (20%, w/v) was

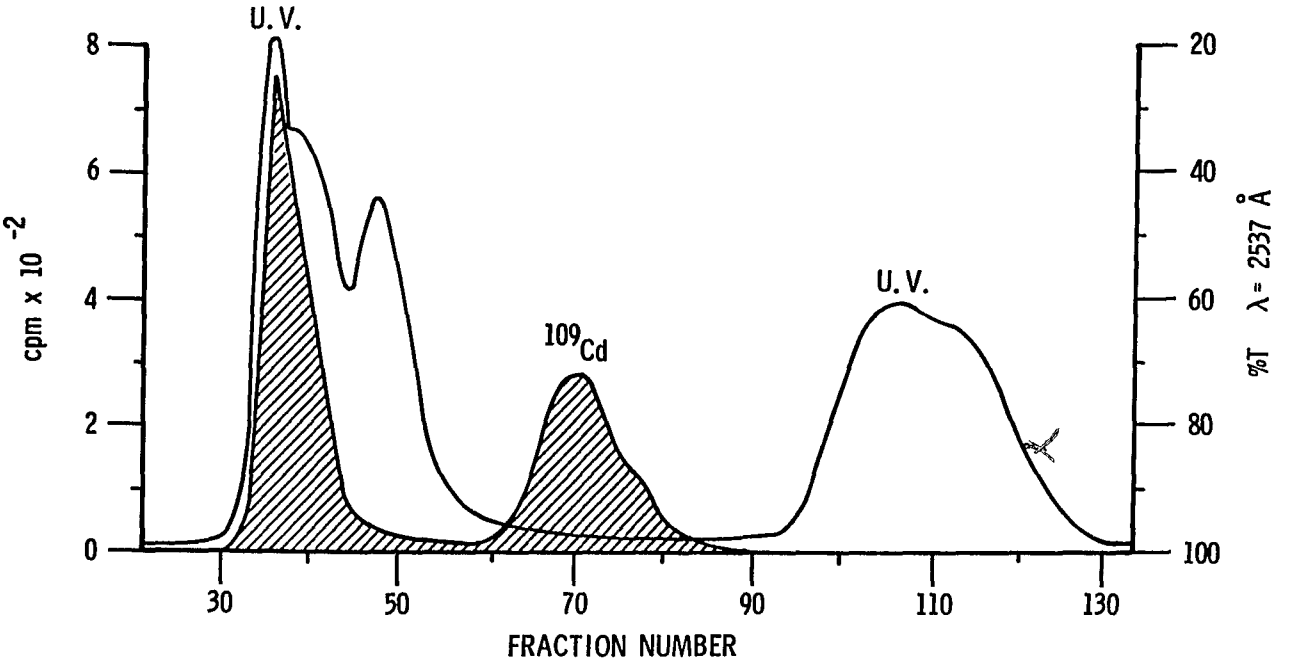


FIGURE 28. Distribution of ^{109}Cd in rat placenta soluble fraction. A pregnant rat was injected with carrier-free $^{109}CdCl_2$ on 21st day of gestation and placentae were removed 24 hours later. Sephadex G-75, 2.5 x 90 cm column; flow rate, 27.5 ml/hour (4.6 ml/fraction).

prepared in 0.25 M sucrose and centrifuged at 105,000 x g for 1 hour. The soluble fraction obtained from mouse liver contained 72.1% of ^{109}Cd and 65.0% of ^{65}Zn originally present in the total homogenate. For gel filtration, 14 ml of the soluble fraction was used. Figure 29A shows the UV-abosrption and the radioactivity distribution in column fractions. Five ^{65}Zn-containing and three ^{109}Cd-containing components were isolated. In the Cd-BP region (Ve/Vo 1.65-2.05) 88% of the ^{109}Cd and 10% of the ^{65}Zn was eluted. No UV-absorbing protein peak was detected in this region.

In mouse kidney homogenate, 57.5% of ^{109}Cd and 52.2% of ^{65}Zn was associated with the cytoplasmic soluble fraction. A total of 8.3 ml of mouse kidney soluble fraction was used for chromatography. As illustrated in Figure 29B, the distribution of isotopes among column fractions resembled that in the liver preparation. Gel filtration profiles of the mouse liver and kidney preparations had striking similarities with those of rat tissues, and ^{109}Cd was found in the Cd-BP region.

5L. Primates:

Reports from Harvard Medical School presented evidence for the existance of cadmium-binding proteins "metallothioneins" in equine and human kidney cortex (Kägi and Vallee, 1960; 1961; Pulido et al., 1966). The molecular weights of these proteins ranged between 10,000 and 10,500. To confirm these observations tissue specimens from Rhesus monkey liver, human liver, and

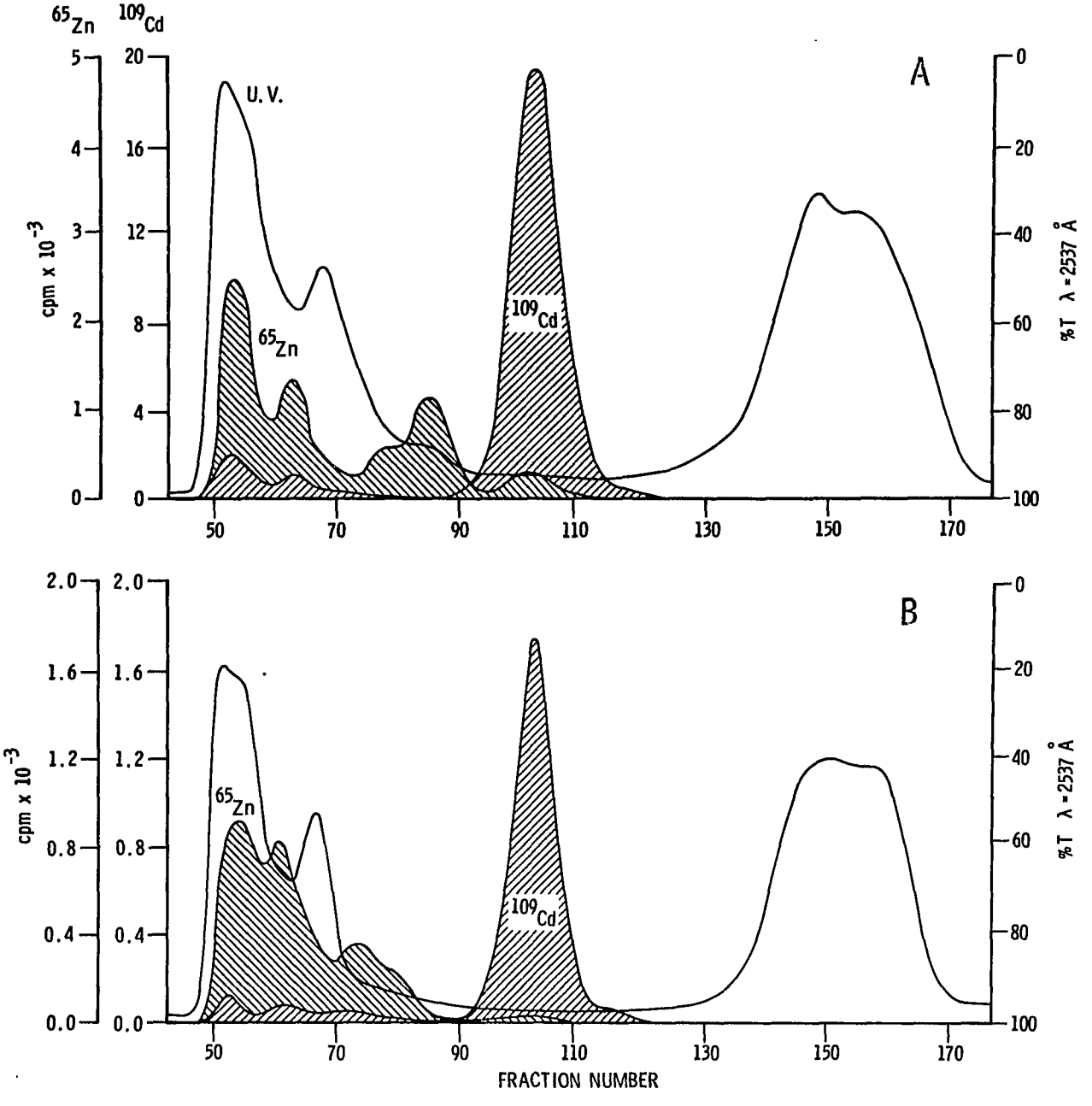


FIGURE 29. Distribution of ^{109}Cd and ^{65}Zn in mouse liver (A), and kidney (B) soluble fractions, 24 hours after injection. Sephadex G-75, 2.5 x 90 cm column; flow rate, 18.76 ml/hour (A) and 18.75 ml/hour (B).

human kidney were analyzed by gel chromatography. The elution patterns obtained were compared with those of rat and mouse tissues.

Figure 30 shows the results obtained from the monkey liver soluble fraction. The tissue was homogenized (20% w/v) in 0.25 M sucrose and 14 ml of the soluble fraction obtained after ultracentrifugation was applied on the Sephadex G-75 column. Separation of a UV-absorbing component with Ve/Vo 1.65 to 2.05 was observed. The gel filtration pattern of this component was similar to that of the rat liver Cd-BP. This substance present in monkey liver was not further identified.

The human tissues were homogenized and fractionated as described for the monkey liver. Figure 31A shows the gel filtration recording of human liver soluble fraction. A UV-absorbing component with Ve/Vo 1.65 to 2.05 was separated. It was in the same molecular weight region as the rat liver Cd-BP. The extent of UV-absorption by human liver cadmium-binding component was greater than in the monkey tissue. Estimation of protein content by means of the Folin-Ciocalteu reagent indicated that human liver contained 3.4 mg protein in the Cd-BP region, per gram of wet tissue.

As shown in Figure 31B, a human kidney preparation when chromatographed on Sephadex G-75 column revealed a component in the Cd-BP region which had marked UV-absorption. The protein content of this component was 4.7 mg per gram of wet tissue. Both liver and kidney tissues were from the

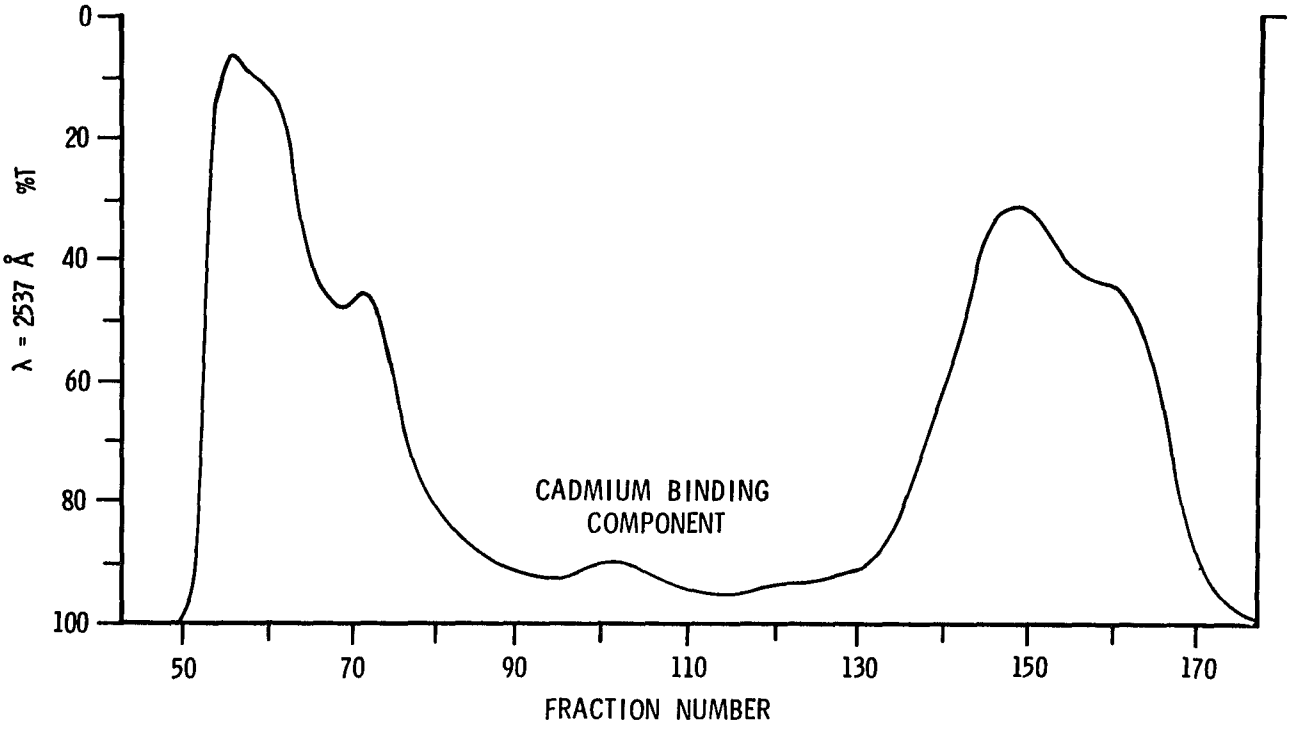


FIGURE 30. Gel filtration of monkey liver soluble fraction. Sephadex G-75, 2.5 x 90 cm column; flow rate, 18.9 ml/hour (3.15 ml/fraction).

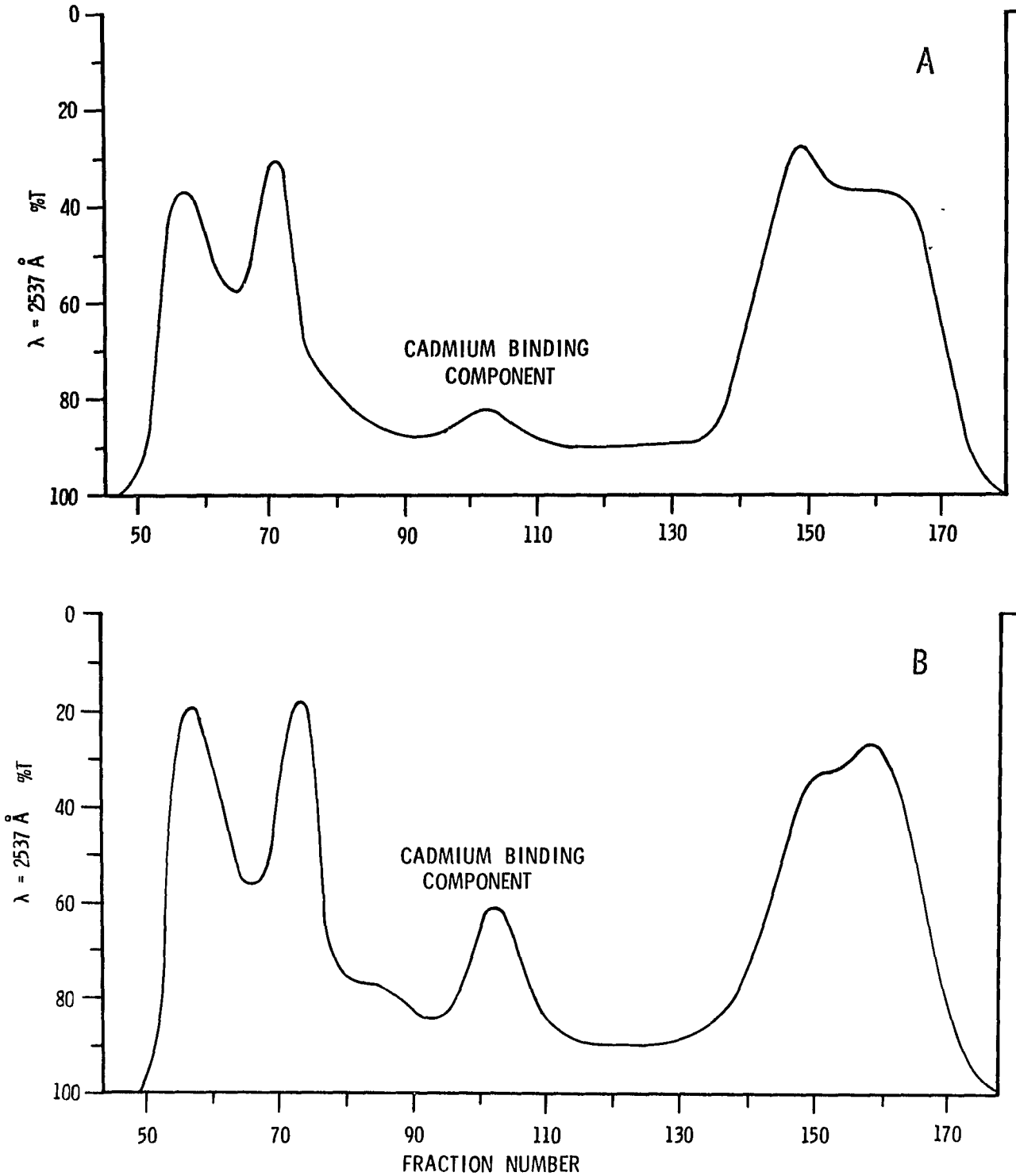


FIGURE 31. Gel filtration of human liver (A) and kidney (B) soluble fractions. Sephadex G-75, 2.5 x 90 cm column; flow rate, 18.9 ml/hour (A) and 18.6 ml/hour (B).

same individual. A difference in the relative UV-absorption and protein content of the Cd-BP region perhaps reflected the tissue cadmium level. Kidneys are known to contain more cadmium than the liver (Schroeder and Balassa, 1961).

5M. Labeling with ^{109}Cd in vitro:

The in vitro binding studies were carried out with whole liver soluble fraction of rats as well as with isolated human kidney Cd-BP. The objective of these investigations was to establish the affinity of various macromolecules for ^{109}Cd. For the first experiment 5 g of fresh rat liver was homogenized in 20 ml of 0.25 M sucrose and 0.001 M Tris-HCl, pH 8.6. The homogenate was centrifuged at 105,000 x g for 1 hour to obtain the soluble fraction. To 11.8 ml of this preparation, 2.2 ml of the solution containing 0.58 μCi of carrier-free $^{109}CdCl_2$ was added and the mixture was stirred at room temperature for 10 minutes. It was then applied to a Sephadex G-75 column and eluted with 0.001 M Tris-HCl, pH 8.6 buffer. Total ^{109}Cd recovered from the column was 86%. As shown in Figure 32A, only 15% of ^{109}Cd was bound to Cd-BP. The remaining radioactivity was associated with higher molecular weight compounds. This indicated that in the in vitro system Cd^{2+} may form metal-protein complexes with molecules other than Cd-BP. However, in the in vivo system cadmium was preferentially associated with the Cd-BP. Binding of ^{65}Zn (carrier-free) to rat liver soluble fraction was also studied under

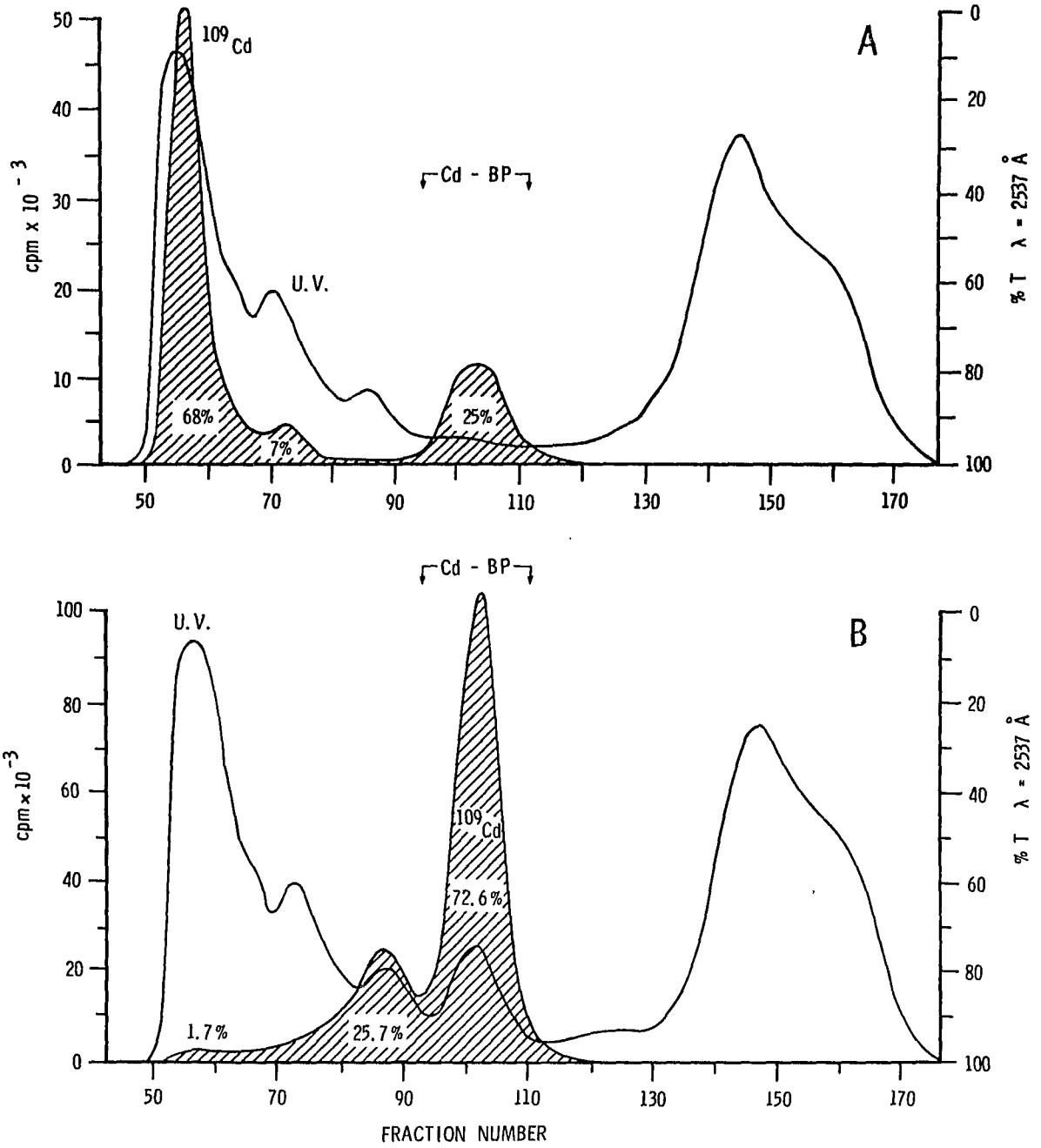


FIGURE 32. The in vitro labeling of Cd-BP. A, rat liver soluble fraction mixed with $^{109}CdCl_2$; B, human kidney Cd-BP incubated with $^{109}CdCl_2$, mixed with rat liver soluble fraction. Sephadex G-75, 2.5 x 90 cm column; flow rate, 18.9 ml/hour (A) and 19.2 ml/hour (B).

similar conditions. This isotope did not appear in the Cd-BP region after column chromatography; instead, 92% of the total ^{65}Zn applied on the column was recovered from a component eluted in the void volume of the gel.

In another experiment, human kidney Cd-BP was isolated from a Sephadex G-75 column. The protein was desalted and 12 mg of the freeze-dried protein was dissolved in 2 ml of 0.001 M Tris-HCl, pH 8.6 buffer. This solution was combined with 0.2 ml of $^{109}CdCl_2$ (1.1 μCi, carrier-free) and incubated in a water-bath at 37°C, for 30 minutes. After labeling with ^{109}Cd, the Cd-BP solution was added to 11.8 ml of rat liver soluble fraction and the mixture was chromatographed on a Sephadex G-75 column. The results are shown in Figure 32B. Human kidney Cd-BP was eluted in the Ve/Vo region 1.65 - 2.05 which was evident from the increase in UV-absorption. Total recovery of ^{109}Cd from the column was 99%. Of the total ^{109}Cd recovered, 72.6% appeared in the Cd-BP region. The remaining radioactivity was present in a higher molecular weight component. The nature of this component was not determined, but it may have originated by dimerization of human kidney Cd-BP.

For further fractionation of the human kidney Cd-BP, a crude sample was obtained by Sephadex G-75 gel filtration. It was labeled with carrier-free $^{109}CdCl_2$ as described above, and freeze-dried. The labeled Cd-BP was redissolved in 1 ml of distilled water and applied to a DEAE-Sephadex A-25 column. For the elution of Cd-BP a Tris concentration gradient

(0.001-0.25 M) was used. A simultaneous purification of 15 mg of rat liver Cd-BP was also carried out. Elution profiles of the two Cd-BP preparations and the distribution of ^{109}Cd in various fractions are compared in Figure 33. Three ^{109}Cd containing components were isolated from human kidney Cd-BP. By contrast, rat liver Cd-BP was separated into four components. Of these, the components labeled as Cd-BP 1 and Cd-BP 2 were the two dominant ones. These proteins, in rat liver preparation, accounted for 40.3 and 36% of the total eluted radioactivity. Human kidney Cd-BP 1 contained 35.6% of ^{109}Cd and human kidney Cd-BP 2 had 22.9% of the total ^{109}Cd recovered from the column. These observations suggested that Cd-BP in rat and human tissues were apparently very similar molecules.

6. Tissue Distribution and Intracellular Localization of ^{203}Hg and ^{109}Cd:

Zinc, cadmium, and mercury are all related chemically. Biologically, zinc has beneficial effects on life; the other two elements do not share this property. Metabolism of zinc and cadmium was compared earlier during this study and striking differences in the distribution, turnover, and binding of these elements in various tissues were observed. The object of this thesis was to focus attention mainly on cadmium and to find out as much as possible about the biological fate of this trace element. Towards the conclusion of this report, however, it was considered of interest to

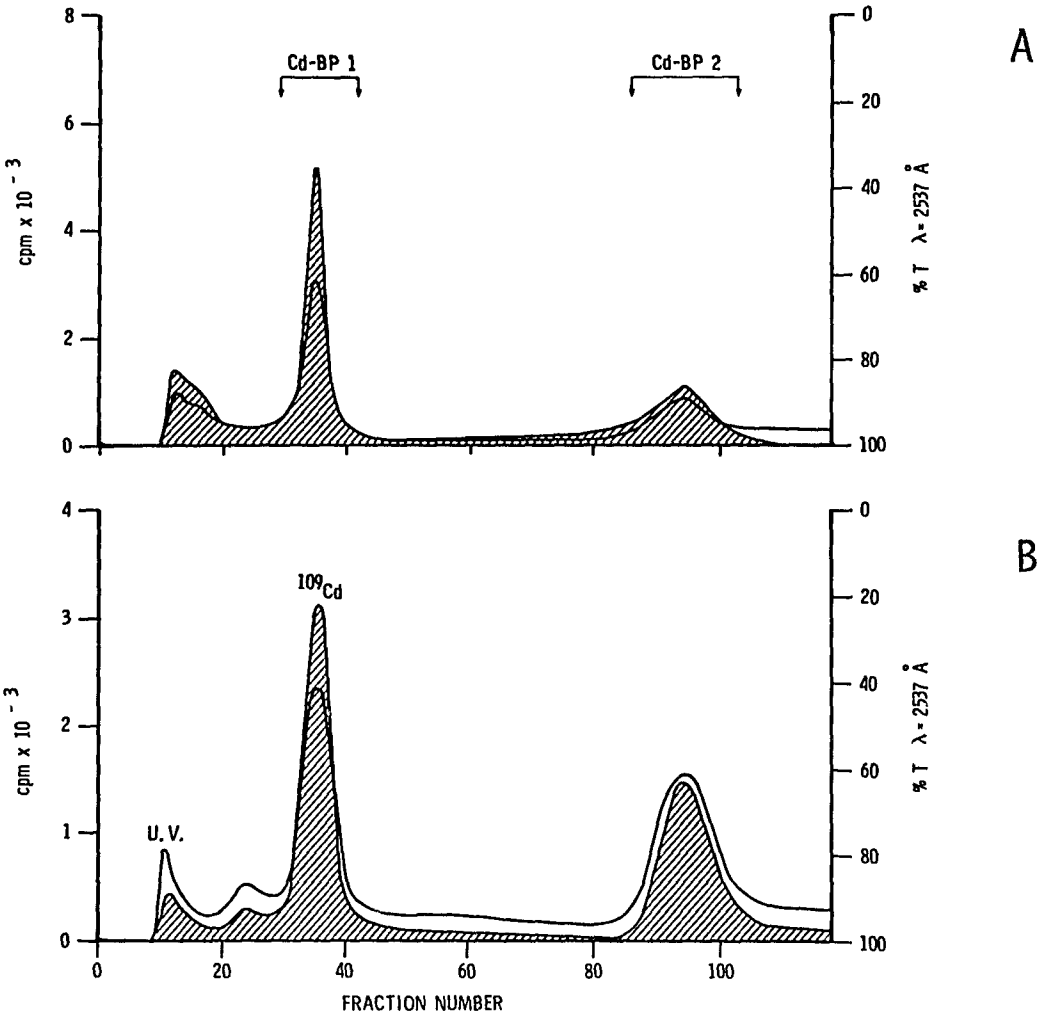


FIGURE 33. Chromatography of human kidney Cd-BP labeled *in vitro* (A) and rat liver Cd-BP labeled *in vivo* (B). DEAE-Sephadex A-25, 1.5 x 27 cm column; flow rate, 11.7 ml/hour (1.95 ml/fraction).

compare the tissue distribution of ^{203}Hg and ^{109}Cd. It was known at this time that $ZnCl_2$ or $HgCl_2$ injected subcutaneously in male rats did not stimulate the biosynthesis of Cd-BP. Moreover, the affinity of ^{65}Zn for Cd-BP was less than that of ^{109}Cd. These observations led to an investigation of ^{203}Hg binding to the liver and kidney Cd-BP in vivo.

An adult male rat was injected subcutaneously with 0.03 mmole $CdCl_2$/Kg to increase the Cd-BP content of the tissues. Twenty-three hours later, the animal was given an intravenous dose of a mixture of $^{109}CdCl_2$ (carrier-free) and $^{203}HgCl_2$ (2.1 nmoles) in 1 ml of physiological saline. The animal was killed 1 hour later, and its tissues were assayed for ^{109}Cd and ^{203}Hg. The tissue distributions of the isotopes are listed in Table XXII. The highest accumulation of ^{203}Hg, per unit weight of tissue, was found in the kidneys. This was followed in decreasing order by spleen, blood, liver, lungs, urinary bladder, prostate, epididymides, testes, heart, seminal vesicles, pancreas, and skeletal muscle. In contrast, the order of tissue affinity for ^{109}Cd was as follows: liver, kidneys, pancreas, heart, spleen, lungs, seminal vesicles, urinary bladder, prostate, epididymides, and skeletal muscle.

A comparative picture of the ^{203}Hg and ^{109}Cd content of the tissues is shown in Figure 34. Circulating levels of ^{203}Hg and ^{109}Cd detected in the plasma were higher than those found in the skeletal muscles. Both plasma and the blood cells contained more ^{203}Hg than ^{109}Cd. In addition, the testes, lungs, spleen, and kidneys had a higher ^{203}Hg content

TABLE XXII. Distribution of ^{109}Cd and ^{203}Hg in Rat Tissues 24 Hours after Injection

Tissue	(Fraction of dose/g) x 10^3	
	^{109}Cd	^{203}Hg
Blood	0.8	13.9
Skeletal Muscle	0.6	1.2
Urinary Bladder	1.8	3.7
Prostate	1.7	3.4
Seminal Vesicles	2.0	2.4
Testes	1.0	2.6
Epididymides	1.6	3.0
Lungs	2.9	5.6
Heart	4.0	2.6
Spleen	3.8	22.6
Pancreas	10.8	2.2
Kidneys	14.8	94.0
Liver	65.6	9.2

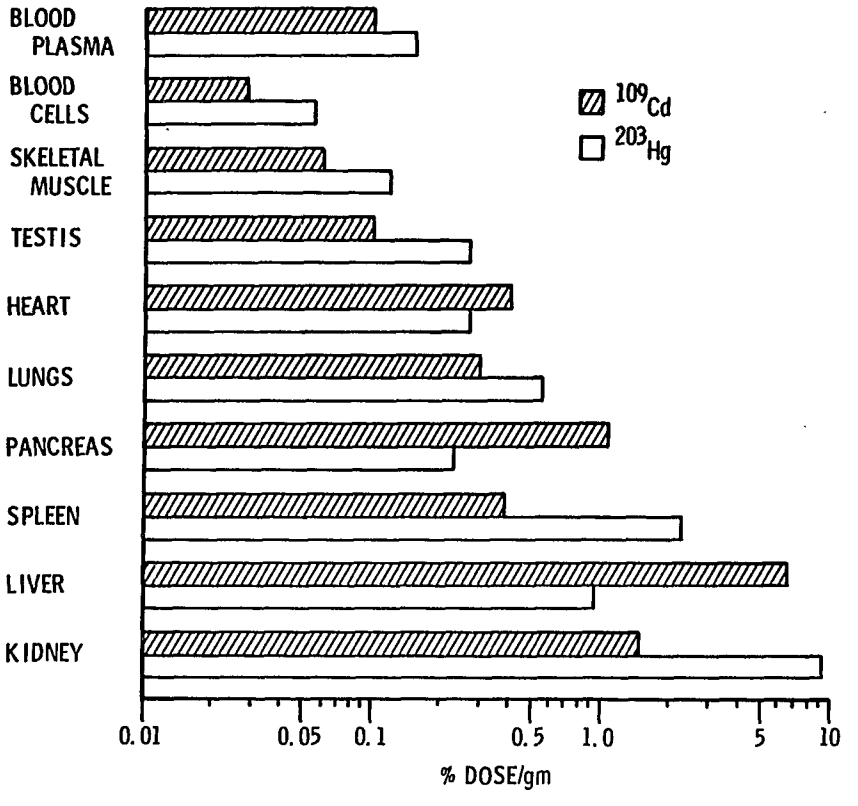


FIGURE 34. Distribution of intravenously injected ^{203}Hg and ^{109}Cd in rat tissues.

than ^{109}Cd. On the other hand, heart, pancreas, and liver showed greater affinity for ^{109}Cd.

To establish the intracellular localization, gel filtration studies were carried out on the liver and kidney preparations. The soluble fraction of the liver, obtained from 20% (w/v) homogenate in 0.25 M sucrose, contained 85% of ^{109}Cd and 49.7% of ^{203}Hg originally present in the homogenate. Figure 35A shows the gel filtration profile of 14 ml of liver soluble fraction on a Sephadex G-75 column. Over 97% of ^{109}Cd recovered from the column was eluted in a single peak with Ve/Vo 1.65 to 2.05. Of the total ^{203}Hg recovered, 71.1% was present in the Cd-BP region. The mid points of the two radioactive peaks deviated from each other, and ^{203}Hg was shifted one fraction towards the lower molecular weight side.

For fractionation of rat kidneys, a 12% (w/v) homogenate of the tissue was prepared in 0.25 M sucrose. The soluble fraction contained 70% of ^{109}Cd and 64% of ^{203}Hg. A total of 9.9 ml of the soluble fraction was chromatographed on a Sephadex G-75 column. As shown in Figure 35B, both ^{109}Cd and ^{203}Hg separated into two components. The Cd-BP region had most of the eluted radioactivity, and 87% of ^{109}Cd and 80% of ^{203}Hg were present in this component. The shift of the ^{203}Hg peak towards the lower molecular weight region was more pronounced in the kidney preparation than that seen in the liver soluble fraction. These results indicated that ^{203}Hg was associated with some biological macromolecules which had

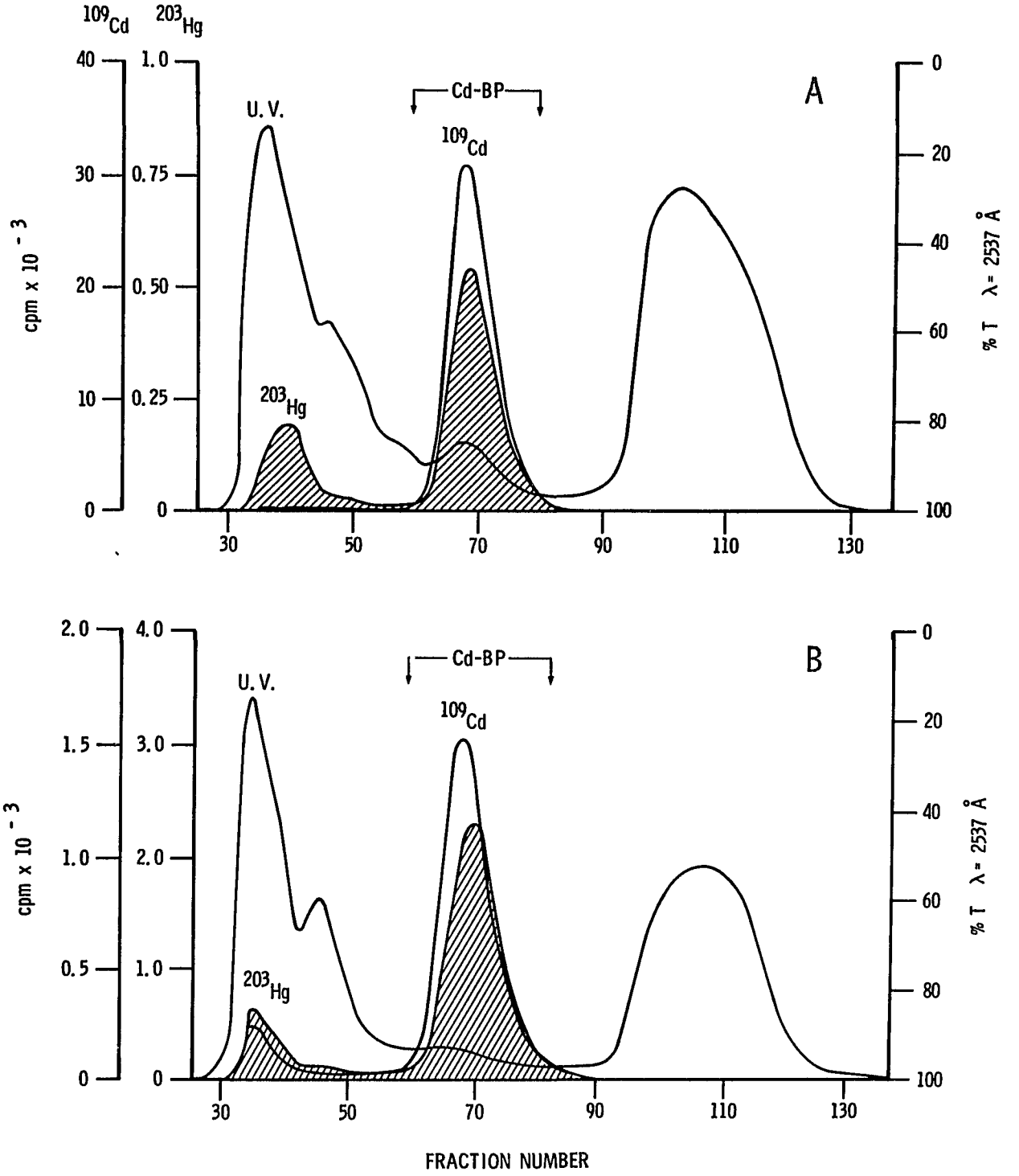


FIGURE 35. Distribution of ^{109}Cd and ^{203}Hg in rat liver (A) and kidney (B) soluble fractions, 1 hour after intravenous injection. Sephadex G-75, 2.5 x 90 cm column; flow rate, 25.0 ml/hour (A and B).

gel filtration characteristics similar to those of Cd-BP. The difference in the elution maximum of ^{203}Hg and that of ^{109}Cd suggested that ^{203}Hg may be bound to a component of slightly lower molecular weight; most probably Cd-BP 1. This possibility was not further explored.

CHAPTER IV. DISCUSSION

1. METABOLISM OF ^{109}Cd, ^{65}Zn, AND ^{203}Hg

1A. Gastrointestinal Absorption and Excretion:

In man, dietary cadmium intake, according to a revised estimate (McCaull, 1971), ranges between 50 and 80 μg/day depending on the composition of the food. Of the total ingested cadmium, only 3 to 4% is absorbed by the organism and nearly all of the absorbed cadmium is retained (McCaull, 1971). Experiments with animals reported in this thesis also indicated a poor intestinal absorption of ^{109}Cd and a rapid elimination of the ingested dose in feces. These observations were in agreement with other workers who used ^{109}Cd or ^{115}Cd as tracer, in mice (Cotzias et al., 1961b) or in rats (Decker et al.,1957). Isotopic cadmium absorbed from the intestinal tract concentrated in detectable quantity in the liver as well as in the kidneys of rats. The accumulation of absorbed ^{115}Cd in the liver and kidneys of rats was also reported by Decker et al. (1957). In comparison with cadmium, the intestinal absorption of dietary zinc in man (Underwood, 1971b) and experimental animals (Cotzias et al., 1962) was more efficient. In rats, the absorbed cadmium was concentrated in the liver, kidneys, pancreas, and other tissues (Ballou and Thompson, 1961). Similar observations were made in rats, during this study (Figure 1 and Table I).

The alimentary canal, in man and animals, functions

as a major route for the absorption and excretion of both cadmium and zinc. Cotzias and Selleck (1960) observed that in mice a balance between the absorbed and excreted ^{65}Zn was maintained by a homeostatic mechanism. This control over zinc metabolism was operative both at the absorption as well as at the excretion levels (Cotzias et al., 1962). In mice injected with ^{109}Cd and ^{65}Zn the dietary loads of stable zinc caused an acceleration in the turnover of endogenous ^{65}Zn. By contrast, similar doses of cadmium did not affect ^{109}Cd turnover (Cotzias et al., 1962). According to Cotzias and Selleck (1960) the absorption of cadmium was not controlled by a negative feed-back and continued irrespective of the total body burden of this element. Feeding the mice a diet containing 0.4 - 1.2 μmoles of Cd/100 g resulted in deceleration of zinc excretion (Cotzias et al., 1962). The interference of cadmium in zinc turnover led Cotzias et al. (1961b) to believe that the metabolism of cadmium and zinc was very similar. They also proposed that cadmium acted as an antimetabolite of zinc. In the light of the results of the present study, it could be argued that the decrease in total zinc excretion was probably caused by an indirect effect of cadmium on zinc metabolism. Intracellular cadmium could have stimulated the synthesis of Cd-BP in the tissues. This protein, by virtue of its zinc-binding capacity, could bind some of the tissue zinc and thereby decrease the total amount of zinc excreted. Further support to this suggestion was provided by a later study by Cotzias and Papavasiliou (1964).

It was found that after feeding cadmium in the diet, the concentration of ^{65}Zn in subcellular organells decreased, while in the soluble fraction the concentration of ^{65}Zn was increased. The suggestion of Cotzias et al. (1961b) that cadmium acts as an antimetabolite of zinc in vivo does not seem to be correct, since the gastrointestinal absorption and tissue uptake of ^{109}Cd was considerably less and its turnover remarkably slower than that of the ^{65}Zn (Table I).

A high uptake of both ^{109}Cd and ^{65}Zn by rat small intestine was noted within 24 hours (Table I). The exact mechanism by which cadmium and zinc were transported across the mucosa was not investigated. It was reported by Sahagian et al. (1966) that in intact strips of rat intestine in vitro, the highest uptake of cadmium and mercury per unit weight of tissue was in duodenum and that of zinc was in the ileum. Further, the transmural transport of cadmium and zinc across rat small intestine in vitro was not found to be energy dependant or occurring against a concentration gradient (Sahagian et al., 1967). However, the in vitro incubation studies with rat small intestine strips showed that the uptake of one metal was affected by the presence of another in the medium (Sahagian et al., 1966). Higher than equimolar concentrations of cadmium, zinc or mercury, with few exceptions, reciprocally enhanced the uptake of one another from jejunum and ileum. The uptake of cadmium was enhanced by twice molar concentration of zinc, but higher quantities of zinc, and all concentrations of mercury depressed cadmium uptake (Sahagian et al., 1966).

In addition to interacting among themselves, the metals of the II-B group also affected copper uptake by ligated intact segments of rat duodenum. At a molar ratio of 1:150 to 1:600, the uptake of ^{64}Cu was depressed by cadmium and zinc, but not by mercury (Van Campen, 1966). According to Van Campen and Mitchell (1965), in rats zinc was absorbed readily from the duodenum, and copper was absorbed from both duodenum and stomach. While studying intestinal absorption in chicks, Starcher (1969) observed that the duodenal absorption of ^{64}Cu was reduced by orally administered cadmium, zinc, or mercury. This investigator also observed that in the duodenal tissue ^{64}Cu was localized in the mucosa. By analysis of the duodenal homogenate, Starcher (1969) found that ^{64}Cu was bound to a protein of approximately 10,000 molecular weight. The amount of the isotope bound to the protein was decreased by both cadmium and zinc but not by mercury, when these elements were administered orally. By Sephadex gel filtration, ^{64}Cu, ^{115}Cd, and ^{65}Zn, were all found to be bound to the same protein in the duodenum. From these observations, Starcher (1969) concluded that the decrease in copper absorption by cadmium and zinc in chicks was due to competition for the same binding sites on the "copper-transporting" protein.

A similar copper-binding protein identified as metallothionein was recently isolated from bovine duodenal tissue (Evans et al., 1970a). It was suspected by these workers that cadmium and zinc interfered with copper absorption

by competing for binding at sulfhydryl groups of the duodenal metallothionein. To elucidate this further, a partially purified preparation of metallothionein (14 -SH groups/molecule) was incubated with ^{64}Cu in the presence of twice as much Cd^{2+} or 15 times more Zn^{2+} on a molar basis. Their results showed that in the presence of either Cd^{2+} or Zn^{2+}, approximately 40% less ^{64}Cu was bound to the protein. It is not known whether the same protein functions for the transport of cadmium, zinc, and mercury, as well as copper in the in vivo system. At the present state of knowledge, therefore, the mechanism of cadmium and zinc absorption is still not clearly understood.

1B. Transport of ^{109}Cd and ^{65}Zn in Blood:

In mammalian blood iron and copper are transported by specific serum proteins known as transferrin and ceruloplasmin respectively. Similar transporting proteins for zinc or cadmium have not been clearly identified. In the present investigation, the analyses of ^{109}Cd and ^{65}Zn in rat plasma showed that within 10 minutes after intravenous injection both isotopes were bound to plasma proteins. Okunewick et al. (1962) also found that more than 90% of the ^{65}Zn was bound to rat plasma proteins as early as 3 minutes after intravenous administration. As reported in this thesis, upon electrophoretic separation of the plasma, the labeled components migrated as α- and β-globulins (Figure 2). The cadmium- and zinc-transporting proteins apparently

resided in these globulin fractions. Dennes et al. (1962) also performed electrophoretic separation of rabbit plasma proteins. Their results showed that 5 minutes after intravenous injection, most of the plasma ^{65}Zn was in the α-globulin region (59 - 83%); albumin and β-globulins contained lesser quantities. An increase in the ^{65}Zn content of β-globulins was observed by Dennes et al. (1961) 60 minutes after injection. The experiments in rats (Figure 2) showed that plasma ^{109}Cd and ^{65}Zn were bound predominently to α- and β-globulins respectively. It is possible that the distribution ratio of zinc between various plasma globulins in rats and rabbits may be different and β-globulins of rat plasma have greater affinity for ^{65}Zn than those of the rabbit plasma. Dennes et al. (1962) suggested that in rabbits ^{65}Zn was apparently transported by α-globulins. Recently, an α_2-macroglobulin was isolated from human serum by Parisi and Vallee (1970). This globulin accounted for 30-40% of the total zinc present in human serum; the remaining zinc was bound to albumin. Attempts to label this protein in vitro with ^{65}Zn were unsuccessful, suggesting that zinc was firmly bound to the protein molecules (Parisi and Vallee, 1970). The binding of cadmium to human α_2-macroglobulin was not studdied. Rapid depletion of injected ^{109}Cd from rat plasma (Figure 3) and absence of circulating levels of ^{109}Cd in the plasma after 48 hours, do not favor the concept that ^{109}Cd may be bound strongly to a protein.

Another compartment of blood which might take part in the transport and storage of zinc was suspected by Dennes et al. (1962). They showed that the addition of erythrocytes labeled with ^{65}Zn to plasma in vitro resulted in the appearance of the isotope in the plasma. From the apparently free exchange of zinc between the erythrocytes and the plasma, it was hypothesized by Dennes et al. (1962) that loosely-bound zinc in the former compartment of blood might represent an additional pool for the storage and transport of this element.

1C. Distribution and Turnover of Parenterally Injected Isotopes:

In a number of studies which were conducted to elucidate the toxic effects of cadmium in animals, the subcutaneous route of administration of cadmium salts was preferred (Nilsson, 1970). In order to be consistent with the toxicological studies, subcutaneous injection was utilized to determine the fate of ^{109}Cd and ^{65}Zn in rats and mice. The tissue distribution of ^{109}Cd and ^{203}Hg was compared after intravenous injection; the purpose was to label rapidly the metal-binding components of the tissues.

As reported in this thesis, timed experiments in rats after subcutaneous administration of ^{109}Cd and ^{65}Zn showed that both isotopes appeared in the plasma within 30 minutes. At this time, the concentration of ^{109}Cd was

nearly equal to that of ^{65}Zn (Table II). Follow-up of animals showed that ^{109}Cd was cleared from the plasma faster than ^{65}Zn. Perry et al. (1970) also made a similar observation in rats, after intravenous injection of the two isotopes. According to Johnson and Miller (1970), the maximum concentration of ^{109}Cd in rat blood was reached in 10 minutes, following a subcutaneous dose. Lucis et al. (1969) reported a continuous depletion of ^{109}Cd from rat plasma between 0.5 and 336 hours. In dogs, almost all of the intravenously injected ^{115}Cd was found to leave the plasma within one hour after injection (Walsh and Burch, 1959). On the other hand, subcutaneously administered ^{65}Zn took a longer time to leave the vascular compartment and 90% of the injected dose was cleared from the blood in 3 hours (Dennes et al., 1962). In the present study, ^{203}Hg, when injected intravenously, was also not cleared from the plasma as rapidly as simultaneously administered ^{109}Cd (Table XXII). Due to continuous absorption from the site of injection, the results of subcutaneous injection of the isotopes cannot be compared with those of the intravenous studies. Nevertheless, the results of these experiments do point towards differences in handling of the elements of II-B subgroup by rat plasma.

The blood cells took up subcutaneously injected ^{109}Cd and ^{65}Zn with time, but showed different trends for each isotope. The concentration of ^{65}Zn continuously increased in the cells between 0.5 and 48 hours after injection. Rapid uptake of ^{65}Zn by the blood cells, after

intravenous administration of the isotope into dogs, was also observed by Robertson and Burns (1963). They reported that a maximum level was reached within 24 hours which was followed by slow disappearance of ^{65}Zn. In contrast to the rapid accumulation of ^{65}Zn, the association of ^{109}Cd with erythrocytes became accelerated only after 12 hours. A suggestion of delayed uptake of ^{109}Cd by the erythrocytes was also provided by the data of Lucis et al. (1969). The cause of this delayed appearance of increased quantity of cadmium is not known, although it is possible that the metal deposited in erythropoetic cells of the bone marrow is being further incorporated into the newly formed erythrocytes. This is supported by the observation of Carlson and Friberg (1957) that after long-term exposure almost all of the blood cadmium is found in the erythrocytes. These workers also reported that in red blood cells of rabbits, isotopic cadmium was bound to the hemoglobin molecules.

The uptake of subcutaneously injected ^{109}Cd and ^{65}Zn by rat and mouse tissues was rapid. In all tissues except blood the concentration of ^{65}Zn declined after reaching the maximum, whereas that of ^{109}Cd remained almost unchanged for 25 days. The concentration of ^{109}Cd per unit weight of tissue was the highest in liver, followed by kidneys, pancreas, salivary glands, spleen, and a smaller fraction in other tissues as well. In most tissues, the maximal level of ^{109}Cd was attained within 12 hours. Similar reports have been made for isotopic cadmium, by other workers in

subcutaneously injected rats (Lucis et al., 1969) and rabbits (Friberg, 1952). The former investigators found that in female hooded rats the uptake of ^{109}Cd, per unit weight, was greater by the kidneys than by the liver. This discrepancy could be due to the difference in strains of rats. The testes of various inbred strains of mice are known to show statistically significant differences in the uptake of ^{109}Cd (Lucis and Lucis, 1969).

In this thesis, the analyses of rat and mouse tissues for the appearance of radioactivity were performed beginning at 30 minutes after injection; earlier events were not investigated. Recently, Johnson and Miller (1970) who studied the initial uptake of ^{109}Cd by rat tissues reported gradual increase in radioactivity for 40-80 minutes following subcutaneous injection.

In rats and mice subcutaneously injected ^{65}Zn was concentrated, shortly after administration, in the pancreas more than in any other soft tissue. By contrast, after intravenous injection of ^{65}Zn to rats (Ballou and Thompson, 1961; Rubini et al., 1961), mice (Stand et al., 1962), or dogs (Robertson and Burns, 1963), the liver tissue contained more radioactivity per unit weight, during 4-6 hours, than the pancreas. It is interesting to note that, while parenterally administered ^{109}Cd was distributed in the tissues in the same order regardless of the route (Potts et al., 1950; Friberg, 1952; Decker et al., 1957; Berlin and Ullberg, 1963; Lucis et al., 1969; Lucis and Lucis, 1969), the distribution

of ^{65}Zn was dependant on whether it was injected subcutaneously or intravenously. In most tissues the maximum concentration of ^{65}Zn was reached within 4-6 hours after injection, which is in accord with published reports (Rubini et al., 1961; Ballou and Thompson, 1961; Stand et al., 1962).

As reported in the present investigation, slow but continuous uptake of ^{65}Zn was observed in the testes and epididymal tissue of rats and mice. Stand et al. (1962) similarly noted a continuous increase of ^{65}Zn in testes of mice, lasting for 24 hours. Rat brain showed a discrimination between ^{109}Cd and ^{65}Zn. A small fraction of ^{109}Cd (0.007% of the dose) was found in the brain during the first 2 hours after subcutaneous injection. After this time interval ^{109}Cd remained virtually unchanged, although the isotope was present in the plasma at a higher concentration for up to 10 hours (Table II). In comparison with ^{109}Cd, the uptake of ^{65}Zn by the brain was slow and it continued for 48 hours. The results of Ballou and Thompson (1961) showed that ^{65}Zn, deposited in rat brain after intravenous administration, remained unchanged between 1 and 10 days.

In rat kidneys, ^{109}Cd and ^{65}Zn were primarily concentrated in the cortex. Similar reports were made for isotopic cadmium by Berlin and Ullberg (1963) in mice, by Gunn and Gould (1957) in rats, and by Friberg (1952) in rabbits. Bergman and Söremark (1968) found that ^{65}Zn was also localized in the cortical tissue of mouse kidneys.

Striking differences were observed between the distribution of intravenously injected ^{109}Cd and ^{203}Hg (Table XXII). The accumulation of ^{109}Cd was predominantly in the liver, while that of ^{203}Hg was mainly in the kidneys. The distribution patterns of mercury in rat tissues were comparable to those reported in guinea pigs (Nordberg and Serenius, 1969), rats, rabbits, and monkeys (Berlin et al., 1969). According to Perry et al. (1970), the uptakes of intravenously injected II-B group elements in rat liver were in the order : Cd > Zn > Hg. For the kidneys this sequence, however, was reversed.

In mice, ^{109}Cd and ^{65}Zn were determined in the stomach, small intestine, and large intestine, along with their contents. The radioactivity was detected in these organs already 30 minutes after subcutaneous injection. Berlin and Ullberg (1963) using whole body sagittal section autoradiography found that the intravenously injected ^{109}Cd in mice appeared in the gastric and intestinal mucosa as well as the stomach and intestinal contents, within 20 minutes after injection. According to Bergman and Söremark (1968), mice injected with ^{65}Zn intraperitoneally showed a continuous uptake of the isotope by the gastric and intestinal mucosa. The uptake of ^{65}Zn was demonstrable between 4 and 60 minutes after injection. Moreover, the mucosa of the small intestine had higher radioactivity than that of the large intestine. This is also suggested by the results shown in Tables VI and X where the small intestine had more ^{65}Zn and also ^{109}Cd

per unit weight than the large intestine. It was shown by Bergman and Söremark (1968) that ^{65}Zn was present in the intestinal contents 32 minutes after intraperitoneal injection. From these observations it was evident that although the mucosal lining contained ^{109}Cd and ^{65}Zn the isotopes took more than 20 minutes to appear in the intestinal lumen. A decrease in ^{109}Cd content of the intestinal mucosa was noticed by Berlin and Ullberg (1963) in mice, 24 hours after intravenous injection. Sixteen days later, intestinal wall had greater activity than the mucosal lining. According to Bergman and Söremark (1968), the uptake of ^{65}Zn by the intestinal wall in mice was noticeable between 2 and 4 hours after intraperitoneal injection. As indicated in Tables VI and X, both ^{109}Cd and ^{65}Zn approached their maximum concentration in the stomach, small intestine, and the large intestine at different times. In the stomach, the highest concentration of the isotopes was after 24 hours, while in the small and large intestines it was after 6 and 18 hours respectively. Time delay in the accumulation of ^{109}Cd in the large intestine was apparently due to reabsorption of the isotope released in the intestinal, pancreatic, and biliary secretions. Lucis et al. (1969) who studied the turnover of ^{109}Cd in the intestinal tract of the rats reported a gradual increase in the ^{109}Cd in the wall as well as in the contents of the large intestine, over a period of 6 to 24 hours following a subcutaneous dose. It was suggested by these workers that the large intestine may be involved in a partial reabsorption of the isotope.

1D. Excretion of Subcutaneously Injected ^{109}Cd and ^{65}Zn:

After subcutaneous administration in rats, ^{109}Cd and ^{65}Zn were eliminated mainly through feces. Due to active turnover of ^{65}Zn in the organism the total excretion of ^{65}Zn during 5 days was more than three times greater than that of ^{109}Cd. In the feces, ^{109}Cd probably originated from gastric and intestinal secretions, as well as disquamated mucosa. That injected cadmium is excreted primarily in the feces has been previously reported in rats (Decker et al., 1957; Gunn and Gould, 1957; Lucis et al., 1969) and dogs (Potts et al., 1950; Burch and Walsh, 1959).

After subcutaneous injection of ^{109}Cd and ^{65}Zn in rats, a total of 17.1% of the ^{65}Zn was excreted in 5 days by way of gastrointestinal tract. The possible sources of the fecal ^{65}Zn were the gastric, intestinal, biliary, and the pancreatic secretions (Montgomery et al., 1943; Robertson and Burns, 1963). Significant depletion of ^{65}Zn was noticed in the gastrointestinal, liver, and pancreatic tissues of rats and mice (Tables VIII and X). Other studies in mice have also shown that intestinal excretion is more efficient than renal filtration in maintaining a balance of zinc in the organism (Rubini et al., 1961; Stand et al., 1962; Cotzias et al., 1962). Obstruction of the anus in mice for 10 days did not increase urinary excretion of ^{65}Zn (Cotzias et al., 1962). It is likely that zinc derived from dietary sources replaces the isotopic zinc (but not cadmium) bound to the

tissues. The mobilized ^{65}Zn combines with the total zinc pool of the body and is eliminated via feces, the rate of excretion depending on the dietary load (Cotzias et al., 1962).

1E. Intracellular Binding in Tissues:

The livers of rats and mice accumulate about half of the injected dose of ^{109}Cd and one-fourth of the total ^{65}Zn. By differential centrifugation of rat liver homogenate, the greatest quantity of intracellular ^{109}Cd and ^{65}Zn was found to be associated with the soluble fraction, 24 hours after injection. Lesser quantities of both isotopes were present, in a decreasing order, in mitochondria, microsomes, and nuclei and cell debris. In all subcellular fractions the concentration of ^{109}Cd was higher than that of ^{65}Zn. Johnson et al. (1970) working with ^{109}Cd showed that in rat liver the soluble fraction contained 60-70% of the radioactivity. A similar report was also made by Gubb and Kench (1971) in chicks. The data of Thiers and Vallee (1957) showed that in rat liver 43.2% of the total zinc was in the soluble fraction, but their value for the nuclei and unbroken cells was rather high (37.2%). An incomplete homogenization might have been a contributing factor. Cotzias et al. (1961b) were the only investigators who studied the simultaneous distribution of ^{109}Cd and ^{65}Zn in mouse liver subcellular fractions, 1 hour after intraperitoneal administration.

Their results showed 68% of ^{109}Cd and 62% of ^{65}Zn in the soluble fraction. These values are in accord with those reported in Table XI.

The soluble fraction obtained from rat liver, 24 hours after subcutaneous injection, was further fractionated by gel filtration. Sephadex G-75 gel chromatography of the soluble fraction revealed that ^{109}Cd was mainly associated with a particular low molecular weight protein component. Of the ^{65}Zn present, 12.1% was in this component, while the rest was bound to fractions of higher molecular weight. Timed experiments showed that ^{65}Zn initially associated with ^{109}Cd-binding region decreased to an undetectable level within 1 week. In other ^{65}Zn-containing components, the content of this isotope was progressively depleted with time. On the contrary, the binding of ^{109}Cd to the protein component seemed to be firm, since its concentration in the Cd-BP region or its distribution in the soluble fraction was not altered with time. Cadmium-109 was not detected in the blood samples analyzed 1 week or 2 weeks after injection. The movement of ^{109}Cd from the intracellular compartment to the blood circulation was probably inhibited by the strong binding of the isotope to Cd-BP.

Since the turnover of ^{109}Cd was not only slow in the liver, but also in other tissues of rats, the possibility existed that ^{109}Cd-binding proteins might be present in those tissues as well. The analyses of the soluble fractions from rat kidneys, spleen, pancreas, and placentae by gel filtration

method resolved ^{109}Cd-binding components in a pattern similar to that of the rat liver Cd-BP.

Analysis of rat pancreatic tissue showed that 90.2% of ^{109}Cd present in the soluble fraction was eluted in the Cd-BP region. Since the pancreatic tissue contains various peptidases, it was expected that some enzymatic degradation of Cd-BP during the separation procedure could occur. Results obtained, however, indicated that Cd-BP separated from the pancreatic tissue had physiochemical characteristics similar to those of Cd-BP separated from other tissues. This suggested that under the experimental conditions used, the pancreatic Cd-BP was resistant to enzymatic degradation.

The presence of Cd-BP in the soluble fraction of rat placentae was of interest, since essential trace elements and other nutrients are transferred from the mother to the fetuses through this organ. Zinc is known to cross the placental barrier in rats (Feaster et al., 1955). Similarly, isotopic cadmium injected into the maternal organism was found in the fetal tissue of golden hamsters (Ferm et al., 1969). It is possible that a limited quantity of cadmium can be bound to the placental Cd-BP. If the concentration of cadmium exceeds the placental threshold, this element may reach the fetus and cause damage. As far as it is known, this threshold level for cadmium has not been determined. The teratogenic effects reported in hamsters by Ferm and Carpenter (1967; 1968) and Mulvihill et al. (1970) were observed after an intravenous load of 2 mg of cadmium sulfate

per kilogram body weight. This dose greatly exceeded the cadmium content which is normally derived from dietary sources. Purina Laboratory chow analyzed by Schroeder et al. (1967) contained 0.63 μg Cd/g.

In addition to rat tissues, the liver and kidneys of mice were also fractionated by centrifugation and gel filtration. The distribution of ^{109}Cd and ^{65}Zn in the soluble fraction was comparable to that of the rat liver and kidney soluble fractions. A ^{109}Cd-binding component, similar in its chromatographic properties to the Cd-BP of rat tissues, was separated from both liver and kidney tissues of mice.

2. THE CADMIUM-BINDING PROTEINS

2A. Isolation:

In the present investigation, the cadmium-binding proteins from rat liver and kidney homogenates were first separated by the procedure of Pulido et al. (1966). It was found that during fractionation steps involving ethanol and chloroform treatment and subsequent dialysis against distilled water resulted in a loss of ^{109}Cd as well as ^{65}Zn. Of the total isotopes present in the tissues, only 33 to 43.5% of the ^{109}Cd and 6.4 to 8.3% of the ^{65}Zn were recovered (Table XII). In terms of cadmium and zinc recoveries, these results were similar to those of Pulido et al. (1966) who fractionated human kidney tissue. It was suspected that poor recoveries of the isotopes might have been caused by solvent-

treatment of the tissue homogenates. In subsequent experiments, these steps were eliminated and a new procedure was developed.

From the observations of Table XI it was evident that the major portion of the ^{109}Cd was present in the cytoplasm and obviously the Cd-BP were cytoplasmic proteins. In the first step towards developing a new isolation procedure, the tissue was homogenized in 0.25 M sucrose solution and centrifuged at 105,000 x g to obtain the soluble fraction. This avoided mitochondrial and microsomal contamination in the tissue extract as obtained after centrifugation at 3,000 rpm (Pulido et al., 1966).

The soluble fraction so obtained was analyzed by gel filtration, without salt or organic solvent fractionation to ensure an optimal recovery. By trial and error, Sephadex G-75 gel packed in a 2.5 x 90 cm or in a 1.5 x 85 cm column, was found to separate the Cd-BP from other soluble components. From this procedure, 72.7% of the total ^{109}Cd and 7.9% of the ^{65}Zn present in rat liver were recovered in the Cd-BP (Table XIII). In contrast, Pulido et al. (1966) could recover only 11.0% of the total cadmium and 2.2% of the zinc, in the crude metallothionein, after Sephadex G-75 gel filtration.

The Cd-BP isolated from rat liver by gel chromatography was not a homogenous preparation. Kägi and Vallee (1961) had utilized DEAE-cellulose chromatography to further purify equine metallothionein. This technique was, however, discontinued by Pulido et al. (1966) who replaced it with

Porath column electrophoresis. The crude rat liver Cd-BP isolated by Sephadex gel filtration was fractionated further using ion-exchange chromatography on DEAE-Sephadex A-25. A stepwise increase in the molarity of Tris buffer from 0.001 M to 0.25 M eluted a single fraction containing 43.7% of ^{109}Cd. If, however, a continuous gradient (0.001 M to 0.25 M) was used, the Cd-BP was resolved into two main components. The total ^{109}Cd content of Cd-BP 1 and Cd-BP 2 accounted for 70.2% of that applied on the column (Figure 15). An interesting observation was that the resolution of the two cadmium-binding components was improved by the presence of 0.02% sodium azide in the eluting buffer. The sodium azide was used accidentally for DEAE chromatography and it contradicted the instructions of the manufacturer, since it is apparently adsorbed on the ion-exchanger. When sodium azide was omitted from the buffers or when it was replaced with Chloretone as a preservative, the resolution between the components of Cd-BP was poor. For all further experiments in isolation of Cd-BP 1 and Cd-BP 2 by DEAE-Sephadex, only buffer solutions containing sodium azide were used.

In order to prove that Cd-BP 1 and Cd-BP 2 were not artefacts, these proteins along with the Cd-BP fraction from Sephadex G-75 were subjected to electrophoresis. Cellulose acetate electrophoresis indicated that the crude Cd-BP contained two detectable components which electrophoretically corresponded to the purified Cd-BP 1 and Cd-BP 2.

Electrophoretic separation on polyacrylamide gel confirmed these observations. The studies of Pulido et al. (1966) also indicated that human renal metallothionein contained two electrophoretically distinct components. Similarly two components were also reported by Piscator (1964) for rabbit liver metallothionein.

Azide is a well known chelating agent for metal ions and it is used for inhibiting the activities of metalloenzymes (Vallee and Wacker, 1970c). In the buffers it was added as a preservative against microbial growth. On Sephadex columns equilibrated with buffers containing sodium azide, metal ions would have been chelated by the azide, if those were not bound strongly to some other ligands. The recovery of 95% or more of the total applied ^{109}Cd from Sephadex G-75 columns indicated that the metal ions were strongly bound to the Cd-BP.

The tight binding of ^{109}Cd was further indicated by other observations. Under conditions of low ionic strength Sephadex gel is known to carry a net negative charge and behaves as a weak cation-exchanger (Fischer, 1969). When eluted with either 0.001 M Tris-HCl buffer or distilled water, neither ^{109}Cd nor Cd-BP were retained on the gel. Greater losses of the isotope were encountered on dialysis in Visking (20/32) tubing. Desalting of the Cd-BP was therefore performed by a Sephadex G-25 column equilibrated with distilled water; the average yield in this procedure was 95%.

The desalted and freeze-dried proteins did not change their chromatographic properites for at least six months, when sotred in a desiccated state at 4°C.

2B. Physicochemical Properties:

Metallothionein was first discovered by Margoshes and Vallee in 1957. The complete amino acid composition of metallothionein from horse liver has been published recently by Kägi (1970). When its amino acid composition was compared to those of rat liver Cd-BP 1 and Cd-BP 2, it was observed that the horse liver metallothionein and the rat liver cadmium-binding proteins had a very similar distribution of amino acids. In Cd-BP 1 and Cd-BP 2, as well as in hepatic metallothionein, cysteinyl residues accounted for about one-third of the total amino acid residues. According to Kägi (1970), cysteine was distributed uniformly in the hepatic metallothionein and in certain peptides it was regularly spaced by two other amino acids. The cysteine contents of horse and human kidney metallothioneins were described as 25 to 27% of the total amino acids (Kägi and Vallee, 1961; Pulido et al., 1966). In the metallothioneins all of the half-cystine was present as cysteine, while in rat liver Cd-BP some cystine was always detected. It could not be distinguished whether the cystine originated from air-oxidation or whether it was a part of the native protein. To solve this problem the analyses of sulfhydryl groups of the Cd-BP are essential.

From the amino acid compositions of Cd-BP 1 and Cd-BP 2 the nitrogen contents of apoprotein 1 and apoprotein 2 were calculated as 15.7 and 15.8% respectively. These were within the range of values described for horse kidney metallothionein (Kägi and Vallee, 1961). Lower values for nitrogen/Folin-Ciocalteu-reactive protein were probably due to overestimation of protein content by Lowry's method (1951). The Folin-Ciocalteu reagent can not only be reduced directly by tyrosine, tryptophan, cysteine, and to some extent by histidine, but also by cystine and peptide bonds of protein molecules reacted with alkaline copper solution (Chu and Goldstein, 1960). The abundance of sulfur-containing amino acids in Cd-BP had perhaps contributed considerably in the reduction of phosphomolybdic-phosphotungstic acid complex. Furthermore, bovine serum albumin was used as a reference protein, which has an entirely different composition of Folin-Ciocalteu-reactive amino acids as compared to Cd-BP. As a result of these considerations, the values of protein content measured by the method of Lowry et al. (1951) cannot be regarded as absolute. Nevertheless, these values may serve to compare the relative protein content of Cd-BP isolated during different experiments.

The sulfur contents of the apoproteins of Cd-BP 1 and Cd-BP 2, as determined from their amino acid compositions, were 11.1 and 9.8% respectively. Similarly sulfur contents of different metallothioneins were also high. The purest preparations of horse and human kidney thioneins (apoproteins)

contained 9.3 and 8.1% sulfur respectively (Kägi and Vallee, 1961; Pulido et al., 1966). The hepatic variety of thionein had the highest value, 11.3% (Kägi, 1970). Until recently, glutathione was the only other natural compound known to contain 10.4% sulfur. Porter (1971) isolated from mitochondria of newborn calves a copper-storage protein (neonatal hepatic mitochondrocuprein) which contained 35% half-cystine residues.

The molecular weights of Cd-BP 1 and Cd-BP 2 were estimated by a gel chromatographic method as well as from the amino acid composition. The values obtained by both methods were very similar. By gel filtration, the molecular weight of Cd-BP 1 was 11,400 and that of Cd-BP 2 was 12,000. According to Andrews (1964; 1970) the accuracy of these measurements is within ± 10% of the actual values determined by the chemical composition. The molecular weights of rat liver Cd-BP 1 and Cd-BP 2 were in the same order of magnitude as those of the metallothioneins isolated by Kägi and Vallee (1961) from horse kidneys (10,000 ± 260) and by Pulido et al. (1966) from human kidneys (10,500 ± 1,050). For the hepatic variety of the metallothionein, Kägi (1970) reported a lower molecular weight (6,600).

In terms of dry weight, the cadmium content of Cd-BP 1 was 6.85% and that of Cd-BP 2 was 6.09%. As compared to Cd-BP, the cadmium contents of horse and human kidney metallothioneins were 5.9 and 4.2% respectively (Kägi and Vallee, 1961; Pulido et al., 1966). Since elemental analyses of Cd-BP were not performed, total metal content

of these proteins could not be evaluated. On a molar basis, Cd-BP 1 had 6.9 g-atoms of cadmium and Cd-BP 2 had 6.5 g-atoms. The cadmium content of horse kidney metallothionein (5.2 g-atoms; total metal, 8.9 g-atoms) was close to rat liver Cd-BP. The human kidney metallothionein had a lower cadmium content than rat liver Cd-BP. Pulido et al. (1966) estimated 3.9 g-atoms Cd (total metal 8.9 g-atoms) in "component B" and 3.7 g-atoms Cd (total metal 7.9 g-atoms) in "component C" of human kidney metallothionein. It was reported that the metal ions were bound to these proteins through mercaptide linkages, involving 3 sulfhydryl groups for each bound metal ion (Kägi and Vallee, 1961; Pulido et al., 1966; Kägi, 1970). The stoichiometry of cadmium binding to the Cd-BP was not studied.

2C. The Binding of Cadmium in vitro:

Kägi and Vallee (1961) carried out in vitro binding experiments with isolated horse kidney metallothionein. This protein contained both cadmium and zinc. Addition of 20 times molar excess of Cd^{2+} to metallothionein replaced all zinc which was bound to the protein. To displace cadmium, however, a 20,000 times higher concentration of Zn^{2+} was required. This indicated that cadmium binding to the protein in vitro was stronger than the binding of zinc. Kägi and Vallee (1961) also calculated the cumulative association constants for metal:ligand (1:3) complexes to be $K_{3Cd} = 10^{25.5}$ and $K_{3Zn} = 10^{21}$.

In the *in vitro* binding experiments reported in chapter III (Section 5M), the soluble fraction of rat liver and the isolated human kidney metallothionein were labeled with carrier-free ^{109}Cd. In the soluble fraction only 25% of the isotope was bound to the Cd-BP and the rest of the label was associated with higher molecular weight components. Similar observations were made by Wisniewska-Knypl and Jablonska (1970). Several explanations are possible for these findings. The cadmium-binding sites on Cd-BP may be saturated and only limited binding of ^{109}Cd and/or exchange between the bound and free metal ions can occur. A replacement of zinc ions may also be suspected. It is also possible that the fraction of ^{109}Cd which binds with Cd-BP attaches itself to the protein molecules through non-specific binding sites. In the presence of inconclusive evidence, all of these possibilities have to be taken into consideration, since these may all be operative, singly or collectively, in the *in vitro* system. Binding of ^{109}Cd to Cd-BP in the *in vivo* system appears to be selective as well as specific, and as shown in the preceding chapter, a major portion of the total ^{109}Cd in the soluble fraction was associated with the Cd-BP. The binding of ^{65}Zn only to the large molecular weight components *in vitro* suggested that either no zinc-binding site was free on Cd-BP or ^{65}Zn could not exchange with zinc (and cadmium) already bound to the protein.

When a crude preparation of human kidney Cd-BP was incubated with carrier-free ^{109}Cd, almost all of the label was bound to the protein. Whether this involved an exchange reaction, replacement of other metal ions, additional binding to the unsaturated sites or simply non-specific interaction with the protein molecules, could not be evaluated from the present data. The labeled human kidney preparation did not lose its ^{109}Cd after mixing with rat liver soluble fraction, indicating that other metal-binding ligands could not displace cadmium bound to Cd-BP *in vitro*.

3. INDUCTION OF CADMIUM-BINDING PROTEIN SYNTHESIS

3A. Effect of Cadmium:

It has been shown that in animals a tracer dose of cadmium was retained in the tissues and the element was eliminated at a very slow rate. Since the tracer ^{109}Cd was bound to intracellular Cd-BP, it was of interest to establish if the quantity of Cd-BP in the cells was limited or it could be augmented if the body burden of cadmium was increased. The investigations carried out in this regard explored both the so called "normal" route (oral) as well as the subcutaneous injection of a soluble salt of cadmium, to observe an effect on the tissue Cd-BP.

The experiments with an oral tracer dose of ^{109}Cd showed that only a small fraction of the dose was absorbed through the gastrointestinal tract. The absorbed ^{109}Cd was

localized mainly in the liver and kidneys (Table I). The intake of cadmium chloride-containing drinking water (0.05 mM $CdCl_2$) by rats was, therefore, expected to result in an increase in the total cadmium content of the tissues. When Cd-BP content of the liver soluble fraction was measured 1 to 12 weeks after ingestion of $CdCl_2$, a consistent increase in this protein fraction was observed. This increase in Cd-BP was dependant on the length of exposure to cadmium. Thus it seems that a small fraction of cadmium, which was absorbed, was capable of regulating the level of intracellular Cd-BP. Cadmium intake at this dose level apparently did not impair weight gain in rats.

The increase in Cd-BP content was more pronounced after subcutaneous injection of $CdCl_2$ than after gastrointestinal absorption. This was obviously due to more cadmium reaching the tissue in the former case. It was shown that the effect of the injected $CdCl_2$ on Cd-BP was dose dependant. A single injection of 0.03 to 0.09 mmole $CdCl_2$/Kg was less effective in increasing Cd-BP, than the same amount of cadmium administered at smaller dose levels, over a prolonged period of time. From the observations it became apparent that the acceleration of the biosynthesis of Cd-BP was not dependant on sex. In addition, the testicular necrosis induced by administration of $CdCl_2$ in normal rats was probably not a factor in the acceleration of the Cd-BP production in other organs; the castrated animals also showed an increase in hepatic Cd-BP. After

long-term exposure (unspecified) to cadmium a similar increase in a metallothionein-like protein, containing 5% cadmium, was also reported by Piscator (1964) in rabbit liver. While the present work was in progress, two other independant reports described a similar phenomenon in the liver tissue of rats (Wisniewska and Jablonska, 1970) and mice (Nordberg et al., 1970), after repeated injections of cadmium salts.

As reported in this thesis, the observed increase in Cd-BP content after $CdCl_2$ injection was not restricted to rat liver; the kidneys also showed an elevation in the Cd-BP content. Piscator (1964) suggested that metallothionein was synthesized in the liver and that it might be deposited later in the renal tubules. According to this investigator, an increase in the Cd-BP of the kidneys was linked with a transport of Cd-BP originally synthesized in the liver. Experiments described in the present investigation failed to support this hypothesis. In all animals, after ^{109}Cd injection, the blood plasma contained barely detectable radioactivity after 24 hours.

In the light of the observations that human kidney metallothionein contained zinc and mercury, in addition to cadmium (Pulido et al., 1966), the effects of these and some other heavy-metal salts (water soluble) on rat liver Cd-BP were explored. Subcutaneous injection of a solution of either zinc, mercury, nickel, or cobalt chloride, or lead acetate, equivalent to 0.03 mmole/Kg, could not produce an

increase in the rat liver Cd-BP, 24 hours after injection. This protein was apparently produced specifically in response to cadmium.

After cadmium exposure, the Cd-BP was isolated not only from the liver and kidneys of rats, but also from rat pancreas, spleen, and placentae. Other organs were not analyzed but it is probable that Cd-BP may be present in all tissues which tend to accumulate this element.

3B. Biosynthesis and Turnover:

From the foregoing discussion it follows that the amount of Cd-BP in the tissues is related to the intracellular level of cadmium. One may argue that cadmium was merely attaching itself to a 10-11,000 molecular weight cysteine-rich peptide which might have originated during the biosynthetic or degradative pathway of some larger molecular weight protein. An attempt to find an answer was made by studying the incorporation of ^{14}C-labeled amino acids into Cd-BP and the disappearance of the ^{14}C-label from the protein molecules. This technique has been widely used in studies of the biosynthesis and turnover of mammalian proteins (Schimke, 1969).

Due to an abundance of half-cystine residues in Cd-BP (Table XVII), the uptake of uniformly labeled cystine-^{14}C was utilized as a tool to study the biosynthesis of this protein. After incorporation into the Cd-BP, the same

^{14}C-label served as an indicator for the in vivo degradation of Cd-BP. In mammalian tissues the proteins are continuously being synthesized as well as degraded and a steady state is maintained. In the presence of a stimulus, the increased level of a protein may be regulated by either the synthetic, or degradative, or by both pathways, operating at altered rates to produce a new steady state (Schimke, 1969; Schimke and Doyle, 1970).

The incorporation of ^{14}C-label into rat liver Cd-BP, under normal conditions, during 24 hours, was 5% of the total radioactivity in the soluble fraction. By increasing the body burden of cadmium (0.03 mmole/Kg), a 3.4-fold increase in ^{14}C associated with the Cd-BP was observed within 24 hours. This observation indicated that the isotopic label was being utilized for the accelerated biosynthesis of Cd-BP. The distribution pattern of ^{14}C-radioactivity in liver soluble fraction of control and cadmium-treated rats (Figure 22A and B) suggested that the ^{14}C-containing fractions could be devided into three components; the Cd-BP, and two others comprising a heavier (Ve/Vo = 1.0-1.65) and a lighter (Ve/Vo = 2.28-2.78) molecular weight component. Under altered steady state, the amount of radioactivity in the lighter molecular weight component did not change. The competition for the labeled amino acids seemed to be between the Cd-BP and the larger molecular weight proteins. Under conditions of cadmium-stimulated biosynthesis of Cd-BP, more of the ^{14}C was diverted towards the Cd-BP.

It was not likely that the Cd-BP was a stabilization product of metal-protein interaction, which was rendered by cadmium ineffective to further enzymatic breakdown. In fact it was demonstrated that the Cd-BP was constantly being synthesized and degraded like other cellular proteins. The results of Figure 23B and Table XX showed that a considerable amount of ^{14}C derived from exogenous cystine-^{14}C was lost with time from the soluble fraction of rat liver. In the soluble fraction from a control animal no ^{14}C could be detected in the Cd-BP region after one week. A loss of radioactivity was also evident in the Cd-BP of a cadmium-treated rat; nevertheless, some ^{14}C-label was associated with the Cd-BP even after 29 days.

It is well known that the rates of degradation of different proteins in vivo are not equal and marked variations exist (Schimke, 1969; Schimke and Doyle, 1970). For example, the half-life of δ-aminolevulinate synthetase is 1 hour, of tryptophan oxygenase is 2 to 3 hours, of catalase is 30 hours, and that of ferritin is 3 days (Schimke and Doyle, 1970). The half-life of Cd-BP was not determined, however, it was shown that the synthesis and degradation of Cd-BP, like other proteins, was a continuous process. As illustrated in Figure 23B, the loss of ^{14}C from the Cd-BP of a cadmium-treated rat was not accompanied by a decrease in Cd-BP. That Cd-BP was maintained at a constant level was also shown by an experiment in which the synthesis of the Cd-BP was stimulated for six days by a

single injection of $CdCl_2$. Administration of cystine-^{14}C at this stage resulted in the uptake of 16.2% of the total liver soluble fraction label in Cd-BP within 24 hours (Figure 23A). This fraction of radioactivity incorporated into the Cd-BP was close to the figure obtained when $CdCl_2$ and cystine-^{14}C were injected together (Figure 22B).

Separation of crude Cd-BP by DEAE chromatography indicated that ^{14}C label was about equally distributed between Cd-BP 1 and Cd-BP 2. In rats treated with cadmium, an increased incorporation of ^{14}C-label from two other amino acids, lysine and threonine, could also be demonstrated.

The control on protein synthesis in mammalian systems may be exerted at the nuclear level by regulating the replication of the gene (DNA), transcription of the gene into mRNA, stabilization of the mRNA, or by facilitating its transfer to the cytoplasm. Protein synthesis may also be controlled in the cytoplasm by changes in the stability of the mRNA, and formation of polysomes, in addition to several other controlling factors operating at this level (Tomkins, 1968; Schimke, 1969). The ideal technique to study these mechanisms would be in vitro studies in tissue culture, with the use of appropriate site-specific inhibitors of protein synthesis.

In the present work, however, the time-course of Cd-BP biosynthesis was studied in the liver of the intact rat to elucidate the overall events. A comparison was made between the incorporation of ^{14}C from uniformly labeled

cystine into the liver Cd-BP of control and cadmium-treated rats during the first 2 to 24 hours. The results revealed that a marked increase in the uptake of the ^{14}C label into Cd-BP of the Cd-treated rat occured between 5 and 12 hours after subcutaneous injection of $CdCl_2$ and cystine-^{14}C.

From the ^{109}Cd distribution studies, it is known that the subcutaneously injected isotope reaches the liver tissue of rat rapidly and within 2 hours more than 40% of the maximum level is achieved (Table IV). Williamson and Clark (1966) have reported that the appearance of subcutaneously injected cystine-^{35}S in rat liver proteins is also a rapid process and the highest specific activity of the proteins is observed 1 hour after the injection. The lack of increase in Cd-BP content, for the first 5 hours after $CdCl_2$ and cystine-^{14}C injection, suggests that the synthesis of Cd-BP apparently requires the production of new mRNA. If this is the case, then it may be suspected that cadmium "induces" (Jacob and Monod, 1961) the biosynthesis of Cd-BP.

It has been shown by other investigators that the synthesis of certain metal-binding proteins can be regulated by their respective binding ions. Two examples, those of ferritin and ceruloplasmin, are cited here as a comparison to the Cd-BP.

Ferritin is an iron-storage protein and its synthesis is stimulated by iron. Drysdale (1968) using leucine-^{14}C incorporation into rat liver ferritin demonstrated that maximum response occurred between 2 and 3 hours after

intraperitoneal injection of ferric ammonium sulfate. The apparent lag period was shortened if the iron salt was given intravenously. Chu and Fineberg (1969) demonstrated that in cultured HeLa cells the effect of added ferric chloride on leucine-^{14}C incorporation into ferritin could be observed in less than 10 minutes. An interesting observation was that actinomycin D did not inhibit ferritin synthesis; instead it had a stimulatory effect on leucine-^{14}C incorporation into ferritin (Drysdale, 1968; Chu and Fineberg, 1969). On the other hand, the production of ferritin was completely inhibited by cyclohexamide (Chu and Fineberg, 1969). From these observations, a translational-level control for ferritin synthesis was proposed, which was sensitive to intracellular iron concentration (Drysdale, 1968; Chu and Fineberg, 1969).

The induction of a copper-transporting protein, ceruloplasmin, by copper sulfate was demonstrated by Evans et al. (1970b). They reported that the appearance of ^{14}C-label derived from lysine-^{14}C was increased in the circulating ceruloplasmin, 24 hours after injection of a dose of 2.5 mg Cu/Kg into rats. Both actinomycin D and cycloheximide were inhibitory to the uptake of ^{14}C-label into the ceruloplasmin, suggesting that the effect of copper on the biosynthesis of this protein was at the transcriptional level. The investigations of Neifakh et al. (1969) in monkeys showed that the effect of copper was dose dependant. After a single dose of 1-1.5 mg Cu/Kg of body weight, the ceruloplasmin content

of the serum was increased 1.5-fold within 24 hours. Repeated injections of 3.2 mg Cu/Kg, at 24 hour intervals, decreased the concentration of ceruloplasmin in the serum within 2 to 3 days. On the contrary, the effect of single or repeated injections of $CdCl_2$ (1.12 -22.48 mg/Kg) on rat liver and kidney Cd-BP was always stimulatory and no inhibition of Cd-BP synthesis by this dose was observed during the present study.

4. ROLE OF CADMIUM-BINDING PROTEINS IN MAMMALIAN TISSUES

A protein which exists in normal animal and human cells or extracellular fluids must have some physiological function. Kägi and Vallee (1960), who first published a detailed report on the isolation of matallothionein, attributed detoxification as one of the probable functions to this protein. Piscator (1964) also proposed a similar role for metallothionein and added that with increasing exposure to cadmium larger quantities of this protein are produced by the organism. As evaluated by Nilsson (1970), Piscator's theory lacked experimental evidence.

According to the data of this thesis, a detoxifying mechanism appears to operate in cadmium-exposed rats. Since Cd-BP is responsible for prolonged retention of cadmium within the cells, this may very well represent the biological sequestration of the toxic Cd^{2+} ions. The observation that

intracellular Cd-BP can also bind mercury further adds to the physiological importance of this protein whose synthesis appears to be regulated by cadmium. Jakubowski et al. (1970) independently observed that in rats, after injection of $^{203}HgCl_2$ (0.1 mg Hg/Kg), the isotope was bound in the kidneys and liver to a protein component of 11,000 molecular weight and also to higher molecular weight proteins. They found that after injection of a dose of 3 mg Hg/Kg, the same amount of ^{203}Hg was bound to the former component, while the latter component had increased mercury content. Toxic effects of mercury were reported by Wisniewska et al. (1970) after injecting the rats with a dose of 0.3-0.5 mg Hg/Kg. These workers suggested that the mercury-binding capacity of the "metallothionein" was limited and when it was exceeded, toxic effects due to mercury resulted.

It was shown in Figures 35A and B that ^{203}Hg was predominantly bound to a protein fraction emerging from a Sephadex G-75 column in a region where ^{109}Cd-binding component was also eluted. Subcutaneously injected $HgCl_2$ was ineffective in increasing the concentration of Cd-BP. This was in accord with the findings of Jakubowski et al. (1970) that at either 0.1 or 3.0 mg ^{203}Hg/Kg, the same total of the isotope was associated with the 11,000 molecular weight protein. Thus, the Cd-BP content of the tissues seems to be a limiting factor in the binding of mercury. As compared with cadmium or zinc, mercury binding to metallothionein is known to be stronger and mercury is not displaced from this protein even at pH 2.0 (Pulido et al., 1966).

A recent report by Evans et al. (1971) have added another element to the list of cations which can bind to a protein of very similar molecular weight. This metallothionein-like protein isolated from rat, bovine, and human liver, as well as bovine duodenum, is described as a copper-storage protein. The effect of copper on the biosynthesis of this protein has not been investigated.

In rat and mouse liver and kidneys ^{65}Zn was also found associated with the ^{109}Cd-binding component whose production was, however, not accelerated by subcutaneous injection of $ZnCl_2$. It is likely that the Cd-BP is synthesized in the tissues to bind Cd^{2+} ions, the binding of other metal ions to this protein may reflect either unsaturation of the protein molecules with cadmium or displacement of the bound Cd^{2+} ions.

In addition to the observations made during the present study, the reports from other laboratories also suggested a protective role for the Cd-BP. Terharr et al. (1965), for example, reported that in rats following an oral dose of 0.55 mmole $CdCl_2$/Kg, the testicular damage was prevented if the animals were given orally a single dose of 0.11 mmole $CdCl_2$/Kg, at least 7 hours prior to the larger dose. Oral administration of as little as 0.05 μmole $CdCl_2$/Kg, 24 hours before the testis-toxic dose, also prevented testicular necrosis. According to Terharr et al. (1965) orally administered 1.1-2.2 mmoles of $CdCl_2$/Kg body weight were lethal to rats. However, the rats survived the

toxic load if they had received 0.11 mmole/Kg orally, 24 hours earlier.

High incidence of mortality (90%) was also observed by Gabbiani et al. (1967a), when female rats were injected intravenously with 0.04 mmole $CdCl_2$/Kg. On autopsy, these rats exhibited lesions in sensory ganglia and ovaries, as well as acute liver necrosis. If, however, the animals were pretreated with 0.01 mmole $CdCl_2$/Kg intravenously, once daily for 5 days, the toxic dose of $CdCl_2$ on the 6th day was well tolerated without any histological changes. Further, the ganglionic lesions produced by 0.05 mmole $CdCl_2$/Kg were also prevented by subcutaneous injection of 4-fold molar excess of $CoCl_2$, but not by $FeCl_2$ or $NiCl_2$. Unlike the permanent resistance acquired by rats after pretreatment with smaller doses of $CdCl_2$, the protection offered by $CoCl_2$ was temporary and 16 days later a second dose of cadmium caused ganglionic lesions. This was apparently due to the inability of cobalt to stimulate Cd-BP synthesis, as it has been reported in this thesis. In the case of pretreatment with $CdCl_2$, what probably happened was that cadmium accelerated the synthesis of Cd-BP, and once this defense mechanism was triggered, the cells developed a tolerance against subsequent larger dose of cadmium. This is in agreement with the postulate of Piscator (1964) that such a defense mechanism is only effective if the organism is exposed to relatively small doses of cadmium before a single toxic load.

In humans cadmium accumulates with age and its concentration in the kidneys is higher than in the liver (Schroeder et al., 1967). Analyses performed on the liver and kidney tissues from an older individual (64 years old) revealed that the concentration of Cd-BP in these tissues also followed the described distribution pattern. The kidney tissue contained more of the Cd-BP component per unit weight than the liver tissue (Figures 31A and B). Results of in vitro experiments suggested that physico-chemically the human kidney Cd-BP and rat liver Cd-BP were very similar molecules. From human kidney tissue a similar protein (metallothionein) has been purified by Pulido et al. (1969).

Cadmium-binding proteins have been isolated from various animal and human tissues. Our present state of knowledge suggests that these intracellular proteins are unique in their amino acid composition, showing an extremely high content of cysteine residues and at the same time a lack of aromatic amino acids. Furthermore, the biological properties of the Cd-BP indicate a perferential binding of ^{109}Cd in vivo. Apart from these ions, the Cd-BP also bind ^{65}Zn as well as ^{203}Hg. Since the biosynthesis of the Cd-BP appears to be regulated in vivo by the body burden of cadmium, the Cd-BP may act as an intracellular protector against toxic Cd^{2+} ions. In addition, the Cd-BP may also function as a protector against other divalent heavy-metal ions in vivo.

5. ARE CADMIUM-BINDING PROTEINS UNIVERSAL?

The low molecular weight cadmium-binding proteins do not seem to be limited to mammalian tissues. Lucis *et al.* (1970) have shown that human fetal skin and muscle fibroblasts cultured *in vitro* also contained Cd-BP. The presence of very similar molecules in a blue-green alga *Anacystis nidulans* has also been indicated by the observations of MacLean *et al.* (1971).

It is tempting to speculate, therefore, that the Cd-BP may have universal importance in plant and animal cells. The present work, which traced the location of ^{109}Cd in a few mammalian tissues, is only a pioneer effort in this direction. Further investigations are needed to characterize the Cd-BP in other forms of life. A comparison of the physicochemical properties and the physiological function of these proteins isolated from different organisms may contribute towards better understanding of biochemical adaptation to the environment.

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