THE EFFECT OF SMALL QUANTITIES OF CADMIUM ON ANIMAL METABOLISM

By

Lucile E. Decker

A THESIS

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

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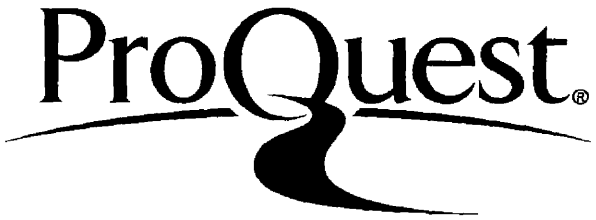

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VITA

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THE EFFECT OF SMALL QUANTITIES OF CADMIUM ON ANIMAL METABOLISM

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ABSTRACT

Cadmium is considered to be a trace element. It is never found free but is usually found combined with zinc ores. Recently, cadmium has been found to occur in small concentrations in some ground waters; however, little experimental evidence is available concerning the effects of prolonged ingestion, by animals or man, of water containing trace quantities of this metal.

There is considerable evidence concerning the toxicity of cadmium in higher concentrations. Inhalation of cadmium oxide or cadmium sulfide fumes or dust has caused practically all of the industrial deaths which have been attributed to cadmium. Less severe poisoning has resulted in symptoms of weakness, drowsiness, loss of weight, vomiting, nausea, proteinuria and kidney damage. In addition, a yellow ring was found on teeth of men who had a history of long exposure to cadmium.

Several cases of acute cadmium poisoning from food contaminated by contact with cadmium plated utensils have been reported. In these cases, vomiting is the usual symptom.

Studies of experimental cadmium poisoning have shown that the concentration of cadmium which causes acute poisoning or death depends on the pathway of administration. The type of experimental diet used influences the severity of toxicity symptoms shown, and, in addition, the toxicity of a given concentration of cadmium is greater in liquid food than in solid food. The amount of cadmium retained by the tissues

varies with the route of administration, but usually the kidney and liver retain the largest amount of the element. The cadmium which does get into the tissues, however, stays a long time and is not easily released.

Cadmium ion has been shown to be active as a cofactor in several enzyme systems, depending on the system, either as an activator or an inhibitor.

Currently, there is considerable interest in the possibility of using cadmium to plate water pipes used for drinking water. Also it would be desirable to have experimental evidence upon which the Public Health Service could base an allowable cadmium standard for drinking water.

The aim of the present study was to determine, by means of animal experiments, the chronic toxicity in drinking water of cadmium in concentrations up to 10 parts per million. Rats were used as experimental animals and measurements were made of growth rate, and pathological changes in the blood and various tissues. Kidney and liver tissues were analyzed for cadmium content after six months and twelve months of exposure of these animals to known levels of cadmium in the drinking water.

In rats it was found that there was no apparent effect on blood hemoglobin or growth rate with amounts of cadmium in drinking water up to ten parts per million. No evidence of pathological changes was noted in any tissues from rats receiving cadmium at these levels. Food and water intake of all groups were essentially the same.

When the results of the cadmium analyses of tissues were calculated as ug. cadmium per gram wet weight tissue, the kidneys were found to have retained two to three times the amount of cadmium as was retained by the liver. However, the total amount of cadmium in the liver was usually equal to or greater than the cadmium content of kidney because of the larger weight of the liver. The cadmium content in ug. per gram tissue of either liver or kidney increased with the cadmium intake, usually in direct proportion to the cadmium intake. In addition, values for tissue content obtained at the end of a year's exposure to cadmium, were roughly double those obtained at the end of the six month's period, which suggests that the amount of cadmium retained depends on the length of time that cadmium-containing water is ingested.

The enzyme formyl-L-glutamic deformylase was isolated from guinea pig liver. The specific activity of the enzyme was increased 11.2 times during purification from the crude extract. As judged by data obtained from electrophoresis of the purified enzyme in both phosphate and veronal buffer, there was one component present in the purified preparation. This component was probably identical with the active enzyme. The activity of the purified enzyme was enhanced by cobalt(ous) ion, whereas cadmium ion and ferrous ion depressed the activity slightly. Which of these metal ions, if any, is concerned with the "natural enzyme" can not be said at this time.

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Cadmium has recently been found to occur in small concentrations in some ground waters; however little experimental evidence is available concerning the effects of prolonged ingestion, by animals or man, of water containing trace quantities of this metal.

In cases of industrial cadmium poisoning, both acute and chronic, and from experiments carried out with animals, it has been found that cadmium in relatively small doses is lethal for humans and animals. The metal induces vomiting in certain species, when given orally, and when introduced into the general circulation, produces a general toxic effect. It also has a tendency to be stored, mainly in the kidney and liver, and elimination of cadmium from the organism is slow.

The aim of the present study was to determine, by means of animal experiments, the chronic toxicity in drinking water of cadmium in concentrations up to ten parts per million. Measurements were made of growth rate, and pathological changes in the blood and various tissues were studied. Kidney and liver tissues were analyzed for cadmium content after six months and twelve months of exposure of animals to known levels of cadmium in drinking water.

In rats it was found that there was no apparent effect on blood hemoglobin or growth rate with amounts of cadmium in drinking water up to ten parts per million. No evidence of pathological changes was noted in any tissues from rats receiving cadmium at these levels.

Food and water intake of all groups were essentially the same. There was a definite storage of cadmium in the liver and kidneys, which increased as the amount of cadmium in the drinking water increased.

It is conceivable that cadmium in very small amounts may have a beneficial physiological action in animals. This has been suggested by enzyme studies in which cadmium was found to activate some enzymatic reactions. One of these enzymes is formyl-L-glutamic acid deformylase, which is involved in the metabolism of L-histidine. After isolation of this enzyme from guinea pig liver, enzymatic activity and the effect of metal ions on the activity were studied. It was also found that the purified enzyme preparation contained only one component as judged from an electrophoretic study. Whether cadmium and the apoenzyme are associated either as a metalloenzyme or as a metal-enzyme complex can not be stated with certainty at this time.

HISTORICAL

Occurrence of Cadmium

Cadmium is generally classed as a trace element or a minor element. Cadmium, like mercury, is found in concentrations of only 0.5 gram per ton of the earth's crust. Cadmium is never found free, but is usually found in zinc ores from which it is separated by it's greater volatility (1).

The soils of the steppes of Russia are said to contain from 1.7×10^{-4} to 4.5×10^{-3} percent cadmium, whereas in Russian natural surface waters, cadmium was found only in one place, the Urov River (2). A Russian review on the occurrence and biological role of cadmium (3) gives data on the content of human embryos and the distribution in four humans from 4 to 64 years of age. The liver and kidney contained the most cadmium (expressed as milligrams per 100 grams of ash) and the amount of cadmium increased as the age increased. No case histories were given; it is not known if these were presumed to be "normal" humans.

In the United States, water in the Long Island area (4) was found to contain 0.05 to 3.2 parts per million (ppm) cadmium. However, this was not water used for drinking purposes. In this case the cadmium was thought to come from pollution by industrial wastes.

In a report on the analysis of various foods (5), canned grapefruit juice, fresh spinach and beef kidney were found to contain,

respectively, 0.019, 0.04, 0.66 ppm cadmium. No cadmium was found in any liver sample. A possible explanation for these amounts of cadmium in the grapefruit juice would be contamination from solder in the can, but the authors have no explanation for the presence of cadmium in the other foods.

Cadmium Poisoning in Man

The first known cases of cadmium poisoning in man were reported by Sovet in 1858 (6) in three servants who became ill after polishing silverware with cadmium carbonate powder. Stephens (7) gave the case history of a 67 year old man who worked in the zinc smelting industry. At autopsy, chemical analyses of the liver showed no lead, traces of copper and 59 mg. cadmium and 50 mg. zinc per pound of liver. During a six year period Stephens examined eight similar cases. From chemical analyses of the liver, he decided that the poisoning was due to zinc and cadmium. There have been many reports of industrial cadmium poisoning, usually as a result of inhaling cadmium oxide fumes or dusts. Several interesting cases have been reviewed by Prodan (8). This article also lists industrial practices where the most hazardous exposures have been encountered. The usual symptoms from inhalation of cadmium dusts are weakness, drowziness, loss of weight, vomiting and nausea. Proteinuria and kidney injury have also been reported (9). On autopsy acute cases showed congestion of the larynx, trachea, bronchi and acute inflammation of the kidneys (10). Cotter and Cotter (11) presented evidence to show that injury to the liver, kidney and

bone marrow occurred in cadmium poisoning although other clinical symptoms were absent. Princi (12) found a characteristic yellow to golden brown ring on teeth of men who had a history of long exposure to cadmium. This ring usually appeared after the second year of exposure and took as long to leave as it did to develop. The color seemed to have been incorporated into the tooth enamel and could not be scraped off.

Other than industrial poisoning, there have been cases of acute cadmium poisoning caused by contamination of food. The poisoning usually resulted from using utensils, plated with cadmium, which contained foods such as fruit juices, jellies, or even coffee (13,14,15). Fortunately, the victim usually loses most of the cadmium by continued vomiting. Two outbreaks of acute cadmium poisoning were traced to drinks contaminated by ice cubes exposed to drippings and scalings from cadmium plated refrigerator evaporators (16). Frant and Kleeman (17) reported that 29 school children were affected by eating popsicles containing from 13 to 15 ppm of cadmium. (This would correspond to ingestion of about 1 mg of cadmium per popsicle.) Monier-Williams (18) in commenting on these cases states that "as with other toxic metals the effect is probably far more pronounced with liquid than with solid food."

At the present time the United States Public Health Service Ordinance and Code Regulating Eating and Drinking Establishments (19) forbids the use of utensils "containing or plated with cadmium" and a similar ordinance (20) forbids the use of cadmium in the construction of milk utensils.

Experimental Cadmium Poisoning

The first historical work on the toxic effect of cadmium was that of Marme (21) in 1867. He used dogs, cats, rabbits and pigeons as experimental animals and administered about fourteen cadmium compounds intravenously, subcutaneously, by feeding and by direct application on the skin. He concluded that cadmium compounds which were soluble in water or dilute acids at body temperature were analogous in their poisonous action. He also found that toxic doses of cadmium, regardless of route of administration caused vomiting, diarrhea, loss of energy, loss of appetite and death. Small doses given over a longer period of time caused chronic intoxication as evidenced by gastro-enteritis and continued loss of weight.

In 1896, Severi (22) reported lesions in dog and rabbit kidneys were caused by the subcutaneous injection of ten mg. per kilogram of cadmium (in the form of cadmium chloride). These lesions were similar to those caused by mercuric chloride poisoning. Macroscopically, the kidneys were greater in volume and of paler color than normal kidneys, while microscopically they showed intense necrosis in the convoluted tubules and frequent dilatation of the tubules. The glomeruli showed no apparent alteration.

Schwartze and Alsberg in 1923 (23) published an extensive study on the comparative pharmacology of cadmium and zinc in four species with regard to lethal doses, sublethal doses and emetic properties. Cats and dogs seemed to be more resistant to cadmium than rats and rabbits, as judged by the amount of cadmium needed for a lethal

intravenous dose. Over the period of a year, cats were offered 5 to 50 mg. cadmium per day in their meat (not necessarily eaten nor retained). Tissue analyses showed that the main storage of cadmium was in the kidneys and the liver with the kidneys storing relatively higher amounts. The progressive tendency for storing cadmium when fed in larger doses over longer periods of time proved that cadmium is absorbed more quickly than it is excreted.

Johns, Finks and Alsberg (24) found that rats on diets containing 250, 500 or 1000 ppm cadmium grew little or none and soon died. The initial growth of rats fed 125 ppm cadmium was normal but the male rats all died within 50 days, the majority of the females survived much longer although only one was alive at the end of 280 days. There was no effect on growth rate or food intake of rats eating 62.5 ppm cadmium in the diet. The concentrations of cadmium consumed (calculated from the food intake) were 3 mg., 2.2 mg., 1 mg., 0.8 mg. and 0.56 mg. per rat per day for rats receiving diets containing 1000, 500, 250, 125 and 62.5 ppm, respectively.

The year 1925 marked the beginning of the use of cadmium compounds as therapeutic agents. Kochmann (25) reported qualitative experiments on the pharmacological action of the element. Using cadmium chloride as a typical compound he found that it precipitated serum albumin; when the element was applied on living tissue, it had an astringent action in medium concentrations, and an irritant, caustic action in larger concentrations. Yeast fermentation was inhibited and protozoa were killed in a solution of 1:300,000 of cadmium chloride.

Grouven (25) reported on the clinical treatment of syphilis with cadmium compounds. Experiments with tubercular guinea pigs (26) suggested a bactericidal action of cadmium compounds and led to the clinical application in human tuberculosis patients (27).

Hessel (28), reporting experiments on the therapeutic use of cadmium preparations, showed that cadmium injected intramuscularly or intravenously in rabbits, dogs and men tended to be stored up and excreted very slowly. To illustrate the extremely slow excretion of cadmium he observed a person who was treated for syphilis with cadmium. The urine and feces were collected and analysed. During a 39 day period only two per cent of the total injected quantity (900 mg.) was excreted. It is now considered that the questionable benefits to be derived from medication with cadmium are far outweighed by the disadvantages of toxicity and retention in the tissues.

Classical studies, from the industrial hygienist's standpoint, were Prodan's (29) experiments on cats poisoned with cadmium oxide fumes, cadmium oxide dust and cadmium sulfide dust. Gross macroscopic and microscopic changes were described in detail and cadmium analyses on the cat tissues reported. He found that exposure to cadmium oxide fumes and dust, in general, resulted in cadmium deposition in the lungs, liver and kidneys shortly after the exposure; later the element became stored in the liver, kidney and bones. Cadmium sulfide exposure results in cadmium deposition mainly in the lungs with a small per cent present in the kidneys. The author explained the differences found

between cadmium oxide and cadmium sulfide as due to the greater insolubility of the latter compound.

When Wilson and De Eds (30) fed cadmium chloride to young rats, animals receiving as little as 31 ppm (0.0031 per cent) cadmium in the diet had a lowered growth rate and those receiving 62 ppm were definitely stunted. Severe anemia also occurred in the rats receiving 62 ppm; within two to three months the hemoglobin concentration had decreased from 16.5 grams to 3 to 4 grams hemoglobin per 100 ml. blood; the number of red blood corpuscles had decreased but not proportionally, whereas white blood counts and differential white counts remained unchanged. Incisor teeth were bleached in rats receiving as little as 16 ppm of cadmium in the food. More severely poisoned rats had hearts weighing as much as two times the normal weight. From these results the authors concluded that cadmium probably interfered with iron metabolism.

In the same year, Fitzhugh and Meiller (31) reported that the toxicity of cadmium was increased by low protein diets. Ginn and Volker in 1944 (32), studied the effect of cadmium and fluorine on rat dentition. Rats were divided into four groups; Group I, controls; Group II, 50 ppm cadmium (as cadmium chloride) in the food; Group III, 50 ppm cadmium (as cadmium chloride) in drinking water; and Group IV, 50 ppm fluoride (as sodium fluoride) in drinking water. After eight weeks treatment, Groups I and II showed normal gains in weight, Group III was stunted in growth and exhibited a progressive decrease in hemoglobin. At the end of 86 days, values for hemoglobin were

(I) 15.8, (II) 12.6, (III) 7.7 and (IV) 11.1 grams per 100 ml. blood. These findings are proof of the fact that cadmium ingested in liquids is much more toxic than the same amount obtained in the food. Diminished pigmentation of incisor enamel was noted both from fluoride and cadmium in the water. Unlike fluoride, cadmium did not inhibit experimental caries. In fact, if anything, 50 ppm cadmium in the water apparently increased caries formation. The authors suggest that the pigmentation changes in enamel and the decrease in hemoglobin are related to the ability of fluorine and cadmium to interact with iron-containing proteins. This interaction has also been suggested from experiments with sugar beets grown in sand culture with the addition of equivalent concentrations of heavy metal ions. Cu^{++}, Co^{++} and Cd^{++} were unusually highly active in causing chlorosis, a condition usually associated with an iron deficiency (33).

Leicester (34) studied the effects of cadmium on caries production in rat teeth. One group of mothers was placed on 0.004 per cent cadmium water from two to six days before birth of the young. The litters and mothers were maintained on this intake until the young were 23 days old, when calcification of the molars was complete (35). This group was removed from cadmium and like the controls given tap water while fed a Hoppert-Webber-Caniff caries diet for 100 days. Another group of mothers and young were treated in the same manner except that they were maintained on cadmium water also during the 100 days period on the caries producing diet. Teeth of the control rats drinking tap water and of those drinking cadmium containing water only during the

calcification period did not give even a qualitative test for cadmium, whereas, teeth of rats receiving cadmium for 123 days contained an average of 0.003 per cent cadmium. The conclusions from this experiment were that cadmium did not increase the number of caries produced by the Hoppert-Webber-Caniff caries diet, but did appear to increase the rate at which caries appeared, and that cadmium deposition occurred in rat teeth only after calcification was complete.

The effect of the diet in studies of chronic toxity was observed using two diets, diet A, a "natural" food ration and diet B, purina dog chow (36).

TABLE I

COMPOSITION OF DIET A

Constituent	Per Cent by Weight
Yellow corn meal	73
Linseed oil cake meal	10
Alfalfa meal	2
Casein	10
Cod liver oil	3
Bone ash	1.5
NaCl	0.5

Diet A (Table I) contained 16 per cent crude protein and diet B contained 21 per cent. For comparative purposes, 125 ppm cadmium were added to each diet and rats were fed for 175 days. Animals receiving diet B (with cadmium) showed bleaching of the teeth and hemoglobin values ranged between 8 to 12 grams per 100 cc. blood, and body weights

were almost identical with the controls. Rats receiving diet A (with cadmium) exhibited bleaching of teeth, decreased hemoglobin (five grams per 100 ml. blood) and decreased growth rate. At the end of the feeding period, the average weight of the rats receiving diet A was 175 grams compared to 276 for controls. Therefore, diet A, as compared to diet B, greatly increased the toxicity of cadmium. The authors routinely use diet A for toxicity studies since they feel that the constituents do not vary as much as a commercial diet (such as B) in which the per cent of protein, fat, etc., are kept constant, but the constituents which furnish these nutrients are varied.

In an attempt to determine the toxicity of household enamel, pigments which contained either cadmium sulfide, zinc sulfide and barium sulfate, or cadmium sulfide, cadmium selenide and barium sulfate were fed in the food to rats (37), at levels of 0.1, 0.25, 0.5 or 1 per cent. No differences in growth rate were noted except that selenium containing pigments seemed to diminish the appetite and therefore the growth gain. The author concluded that cadmium-containing pigments were not harmful even to children. This conclusion seems hard to believe in view of the known toxic effects of cadmium salts, in general, and the effects of selenium on men and animals (38).

In a comprehensive study on prolonged inhalation of cadmium(39), Princi and Geever exposed dogs to definite concentrations of cadmium oxide and cadmium sulfide dusts over the period of one year. Most of the dogs showed some weight gains. The results of various blood tests and liver function tests were reported also. Cadmium analyses of bone,

feces, bile, kidney, and liver were presented. Most inhaled cadmium dust was stored in lungs, liver and kidney, with lesser amounts in the bones and teeth. Cadmium levels in blood and urine of dogs exposed to cadmium sulfide usually were significantly lower than those found in dogs exposed to cadmium oxide. This is consistent with the fact that cadmium oxide is more readily soluble in body fluids than cadmium sulfide and therefore much more easily absorbed.

It has been reported (40) that when rats were injected with Cd^{115} intra-peritonealy and fed diets with protein concentration varying from 16 to 60 per cent that an increased retention of cadmium was found in the liver and spleen with an increase in the protein content of the diet.

In this laboratory (41), 300 gm albino rats were given, by stomach tube, a single 10 microcurie dose of Cd^{115} (as cadmium nitrate). The total weight of cadmium was two mg. which is about 1/30 the oral LD_{50} dose for the rat (28). Approximately 80 per cent of the cadmium was excreted by way of the feces, whereas kidney and liver tissues each showed a maximal uptake of a little over one per cent of the injected dose. At the end of 15 days, however, the kidney and liver concentrations were the same as at the end of eight hours. In another experiment 400 gram rats were injected intravenously with 2 microcuries of Cd^{115} (as cadmium nitrate). The total weight of cadmium administered was 0.25 mg which is about 0.1 of the intravenous LD_{50} for the rat (23). At the end of 4 hours, about 63 per cent of the injected dose was found in the liver and about 1.6 per cent in the kidney. At the end of a five weeks period the liver and kidney contained as much cadmium as

they did at the end of the four hours. The total excretion of cadmium via the feces at the end of five weeks was 22 per cent of the injected dose. Both of these experiments demonstrate that whatever cadmium does get into the liver and the kidneys is very tenaciously held and is not released for some time, if at all.

In summary, previous experimental studies have shown that the concentration of cadmium which causes acute poisoning or death depends on the pathway of administration. In addition, the toxicity of any given concentration of cadmium seems to be much greater in liquid than in solid food. The type of diet used also influences the toxicity. The amount of cadmium retained by the tissues varies with the route of administration, but usually the kidney and liver retain the most cadmium. The cadmium which does get into the tissues, however, stays a long time and is not easily released.

Effect on Enzymes

There have been reports that cadmium, in common with most "heavy metals," inhibits or retards enzymatic action. In 1896, Athanasiu and Langlois (42) published a comparative study of the effects of zinc sulfate and cadmium sulfate on lactic fermentation of colon bacilli. Cadmium sulfate concentrations between 0.18 and 0.2 parts per thousand completely inhibited fermentation; zinc sulfate was effective in concentrations between 1.5 and 1.6 parts per thousand.

Gerber (43) showed that cadmium retarded the action of various rennins and that the activity of amylases (44) may be retarded or

completely checked depending on the cadmium concentration. Inhibition of catalase and peroxidase (45) and distinct retardation of sucrose inversion have been shown to occur in the presence of low concentrations of cadmium. Krebs (46) showed that 1.3×10^{-5} moles of cadmium per liter caused 50 per cent inhibition of the proteolytic action of papain. The enzyme phospholipase C which catalyzes the hydrolysis of lecithin to a diglyceride and phosphoryl choline was inhibited by cadmium (47). This inhibition may be due to the formation of a cadmium-lecithin complex (48). Aminotripeptidase, obtained from thymus, which hydrolyzes a variety of tripeptides at the N-terminal peptide bond, was inhibited 95 per cent by 0.001 M Cd^{++} and 80 per cent by 0.001 M Hg^{++} (49). Aspartase from propionic acid bacteria which catalyzes the formation of fumaric acid and ammonia from L-aspartic acid was strongly inhibited by certain heavy metals including cadmium (50).

One hypothesis is that heavy metals are toxic to biological systems because of their reversible mercaptide-formation with the -SH groups of the protein moiety of cellular enzymes. For this reason, several experimentors have tried to reverse the effects of cadmium poisoning, in vivo and in vitro, with 2-3 dimercaptopropanol (BAL)(51,52,53,54). They explain that dithiols form mercaptides of sufficiently low dissociability to reverse effectively the combination of heavy metals with sensitive cellular enzyme systems. These authors found that administration of BAL to rabbits before lethal intravenous doses of cadmium chloride resulted in amelioration of symptoms of acute intoxication but death usually followed due to fatal renal insufficiency,

even though life was prolonged (51). From the results of these studies it was concluded that "BAL interacts with Cd^{++} *in vivo* to form a mercaptide of low dissociation which is susceptible to intracellular oxidation, but which in presence of excess BAL directs the metal to the kidney for excretion and thereby prevents poisoning of sensitive extra renal loci; the mercaptide, however by glomerular filtration and tubular reabsorption is concentrated in the epithelium of the renal tubule where intracellular oxidation results in the release of toxic amounts of Cd^{++}."

On the other hand, Cd^{++} is only somewhat less active than Mn^{++} or Mg^{++} in satisfying the metal requirements of the α-carboxylases of yeast (55,56). Purified oxalacetic decarboxylase of the bacterium *Micrococcus lysodeikticus* requires a divalent ion. Cd^{++} or Mn^{++} were the most effective coenzymes of various cations tried (57). In crude kidney extracts of prolinase (imino-dipeptidase), the hydrolysis of specific substrates was activated by Mn^{++} or Cd^{++} (58). Suda, *et al.*, have presented evidence to indicate that cadmium activates two enzymes concerned in the metabolism of L-histidine, L-histidine deaminase (59) and formyl-L-glutamic deformylase (60).

The major pathways of L-histidine utilization are (1) incorporation into proteins, (2) excretion into the urine and, (3) degradation. Of these the last pathway is quantitatively the most significant. This can be clearly seen from the experiments of Borsook *et al.* (61) and of Novak (62) in which one hour after intravenous injection of isotopic

L-histidine into mice, about one-third of the radioactivity of the injected amino acid was found in the expired carbon dioxide.

Recent experiments have shown that the degradation of histidine involves, as the first step, the non-oxidative deamination of the α-amino group; the product of this reaction is urocanic acid. The enzyme catalyzing this reaction has been referred to as "histidase," "histidine-deaminase" or "histidine-α-desaminase" by various investigators. Enzymatic conversion of L-histidine to urocanic acid with liver preparations were described as early as 1939 by several investigators in Japan (63,64). In animal tissues the *in vitro* degradation of L-histidine through urocanic acid has been reported in preparations from rat and guinea pig liver (65,66), and from the liver of several other species. When urocanic acid is degraded by liver homogenates or *Pseudomonas fluorescens* extracts, a product is obtained which contains bound forms of ammonia, L-glutamic acid and formic acid. The latter acids can be released by alkaline hydrolysis. The product of urocanic acid degradation has been identified as formamido-L-glutamic acid by Tabor and Mehler (67) and Borek and Waelsch (68,69). The further degradation of histidine beyond formamide-L-glutamic acid varies in different preparations. In liver homogenates (69,70) for example, there is very little degradation beyond this stage, even though the whole animal rapidly degrades histidine to carbon dioxide. Recently however, Knudson (71) has demonstrated the conversion of formamido-L-glutamic acid to glutamic acid in rat liver extracts.

In contrast to the results with liver homogenates, Pseudomonas extracts rapidly convert histidine, urocanic acid, and formamidoglutamic acid (65,67) to stoichiometric quantities of L-glutamic, formic acid, and ammonia. Recently, evidence has been presented (67) that formyl-L-glutamic acid is the immediate precursor of the glutamic acid in this degradation by Pseudomonas extracts. In addition, Suda et al. (60), have reported the isolation of the enzyme which hydrolyzes formyl-L-glutamic from guinea pig liver. (This source is much less active than the Pseudomonas extracts.) They have named this enzyme formyl-L-glutamic deformylase. The enzyme from Pseudomonas extracts which performs the same function has been named glutamic formylase by Tabor (67). Other pathways of histidine degradation and the enzymes concerned, have been adequately reviewed (72,73).

Glutamic formylase (67) is greatly activated by the presence of ferrous (Fe^{++}) ions. On the other hand, it is reported that after formyl-L-glutamic deformylase from guinea pig liver (60) is dialyzed overnight the inactivated enzyme is re-activated best by cadmium ions. These same authors have also reported that L-histidine deaminase from guinea pig liver is an SH-protein which requires cadmium ion as a co-factor (59).

Cadmium in Drinking Water

Currently, there is considerable interest on the effects of small amounts of cadmium in the drinking water. According to Garrity (74), the electro-deposition of cadmium has developed rapidly in the past

decade because, in contact with steel, it forms a protective galvanic couple superior to that provided by zinc. He also stated that because cadmium gives better protection than zinc any finding that cadmium is safe in water works would be "welcome news."

To our knowledge the only reported experience of long duration of exposure of humans to cadmium in drinking water is that reported by Princi (75). He states, "Several individuals have been studied who show evidence of long-continued cadmium absorption...In the drinking water of these persons there has been found a cadmium content which averaged 0.047 mg per liter. Both blood and urine levels in this group have been found to exceed 0.10 mg." No adverse effects of cadmium ingestion, other than the yellow cadmium pigmentation of the teeth, were noted by Princi.

Authorities in some cities have considered the use of cadmium of enough danger to regulate its use. In Detroit the local plumbing code prohibits the use of cadmium-plated pipes or fittings, while New York City prohibits the use of cadmium in food utensils and equipment (76). There is no standard based upon experimental facts, and, as Muhlberger (77) has pointed out, the adoption of a standard for drinking water should await evidence concerning the toxicity of small amounts. For consumption of cadmium over the period of many years, the allowable quantity ingested may have to be less than that amount which causes disfiguring effects on the teeth. (This is the basis of the existing Public Health Service fluoride standard.)

Although there has been much work done concerning the effect on animals of intake of high levels of cadmium, both in food and water, to our knowledge, no studies have been carried out on levels of cadmium which might reasonably be expected in drinking water. Neither has there been any study of the effects of long term ingestion of low concentrations of cadmium, nor has there been a systematic analysis of the concentration of cadmium retained in tissues of animals on such a regimen. For this purpose, the present study was begun under the support of a grant from the Public Health Service.

It was decided to use amounts of cadmium less than those which produce clinical symptoms of toxicity, in order to determine the effects, if any, of ingesting small concentrations of cadmium for extended periods. It has been suggested that 15 parts per million or more of cadmium would produce nausea and vomiting in humans, and therefore the top level of cadmium chosen was 10 ppm. Since the highest concentration of cadmium regularly found in water (not used for drinking) was 0.5 to 3.2 parts per million (4), this was used as a guide for determining the concentrations of the lower levels.

It was not expected that a direct correlation between the effects on rats and the effects to be expected in humans could be made, but it was hoped that information could be obtained which would be helpful in the determination of an allowable cadmium concentration in drinking water.

The isolation of the enzyme formyl-L-glutamic deformylase was also undertaken in an effort to determine whether cadmium was really involved in the action of this enzyme. In addition, further characterization of this enzyme was made by means of the electrophoresis apparatus.

EXPERIMENTAL

Part I. Chronic Toxicity Experiments

Rat Care and Diets

Albino rats of the Sprague-Dawley strain were 34 days old when selected for these experiments. With the exception of the control group, the animals were divided into groups of sixteen rats with each group having an equal number of males and females. The control group contained ten males and ten females. At a later time, five males and three females were repeated on group II (0.1 part per million cadmium) since a large number of animals in the original group died. The average initial group weights are given in Table II.

TABLE II

INITIAL AVERAGE GROUP WEIGHTS

Group Number	Body Weight	
	Males g.	Females g.
I	98	102
II	100	92
	100*	99*
III	103	96
IV	97	95
V	98	97
VI	90	95
Average of all groups	97.7	96.7

* Repeats of Group II.

The animals were housed in individual raised cages with the room temperature maintained between 75 to 78°C. All animals received ad libitum the Hoppert-Hunt stock diet shown in Table III.

TABLE III

COMPOSITION OF THE HOPPERT-HUNT DIET

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

The control group (Group I) was given distilled water ad libitum. The other groups were given water containing different quantities of cadmium ad libitum, as shown in Table IV, prepared by diluting a stock solution of cadmium chloride with distilled water. Weekly records were kept of body weight and food and water consumption.

At the end of the first six months period one male and one female from each group, I through VI, were sacrificed. Tissues were taken for pathological studies and for cadmium analyses. The rest of the animals in each group were sacrificed at the end of a year's time. Animals which died during the experimental period were examined for gross pathological changes, and, in some cases, tissue sections were prepared.

TABLE IV

CADMIUM CONTENT OF WATER

Group Number	Cadmium Concentration ppm Cadmium*
I	0.0
II	0.1
III	0.5
IV	2.5
V	5.0
VI	10.0

* Cadmium chloride in distilled water calculated as parts per million (ppm) cadmium ion.

A group of rats (Group VII) consisting of eight males and eight females were given water containing 50 parts per million (ppm) cadmium; all other treatment of these animals was the same as above, with the exception that the rats were sacrificed at the end of three months. Initial and final body weights of these animals are given in Table V.

TABLE V

GROWTH OF RATS RECEIVING 50 PPM CADMIUM--GROUP VII

Initial Weight		Average Body Weight					
		21*		50*		84*	
Males (g.)	Females (g.)	Males (g.)	Females (g.)	Males (g.)	Females (g.)	Males (g.)	Females (g.)
73	89	137	147	211	193	246	215
		Average Weight Gain g./rat/day					
		3.1	2.7	2.5	1.6	1.0	0.6

* Days after experiment began.

Blood Studies

Blood studies included determinations of the total red and white cell counts, differential white cell count and the determination of the hemoglobin concentration by a modified Sanford method (78). Blood analyses were made at monthly intervals on four males and four females in each experimental group and on five males and five females in the control group.

Pathology[1]

Rats at the age of six months and one year were killed with one-quarter ml. of Halatal (sodium-ethyl (1-methyl-butyl) barbiturate) in the thoracic cavity. Samples of the following tissues were taken: kidneys, adrenal glands, liver, spleen, heart, brain, stomach, duodenum, ileum, colon, and cross-section of bone marrow of sternum and femur. Fixatives were 10% saline-formalin and Carnay's fluid for glycogen.

All of the tissues were stained with hematoxylin and eosin. Small portions of the liver, kidney and adrenal gland were stained with Best's Carmine for glycogen and Sudan IV for fat.

Weights of the liver, spleen and kidneys were recorded.

Cadmium Analyses

Liver, kidney and bone samples were frozen on solid carbon dioxide and kept in the deep freeze at -15°C until analyses were performed. Whole liver and kidney samples were wet ashed in Phillips beakers.

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

It was found most satisfactory to add fifteen ml. concentrated sulfuric acid and fifteen ml. concentrated nitric acid to the tissue, then cover the beaker, and allow it to stand overnight. The next day the beaker was heated on a hot plate; ten ml. portions of nitric acid were added until no more charring occurred, and the sample was almost colorless. The volume was reduced to about ten ml. by strong heating and after cooling the beaker, the samples were titrated with sodium hydroxide to a yellow color using thymol blue indicator (pH 2.8). (This step is critical to obtaining low blanks and accurate results.) The sample was then diluted to 25 ml. with distilled water, and, depending on the expected cadmium concentration, either whole samples were analyzed or aliquots were taken. Saltzman's micromethod for cadmium (79) was used. This method has the advantages that it can be applied to samples containing as much as 5 to 10 mg. of common interferring metals and that no additional purification of reagents is necessary.

The procedure consists of extracting the cadmium with diphenylthiocarbazone (dithizone) in chloroform, removing the cadmium ion from the cadmium-dithizone complex by complexing the cadmium with tartaric acid, and then by making the solution basic, re-extracting the cadmium with dithizone. The optical density of the pink solution of cadmium dithizonate in chloroform was determined at 515 mμ in the Beckman Model B, equipped with a special cell holder to accommodate Coleman matched tubes 15 mm. in diameter. A standard curve was obtained by carrying known concentrations of cadmium nitrate solutions through the whole

procedure. Recoveries were run on tissues with known concentrations of cadmium nitrate added before the ashing procedure. Recoveries averaged 95 to 98 per cent. Blanks were also run on the acids used in ashing the tissues. Low values obtained for control tissue blanks were subtracted from concentrations of cadmium found in experimental tissues. The reagents used were C. P., and were not further purified. Ordinary distilled water was used and no interferences from contaminating ions were noted. Under our conditions the range of the working curve was from 0.2 microgram to 10 micrograms of cadmium in the final solution of 15 ml. chloroform. Over this range Beer's law was followed; in fact there was only a small deviation for a 20 microgram standard cadmium solution. The sensitivity of the method was 0.05 micrograms in a volume of 15 ml. Contrary to the findings of the published method, it was noted that the final chloroform solution lost color upon standing as little as three hours at room temperature. Readings were therefore made immediately after each sample was carried through the extraction procedure.

Bone samples may be analyzed with the following modifications. Ten ml. of concentrated sulfuric acid and ten ml. of concentrated nitric acid were added to a bone weighing approximately one gram. This mixture was heated on the hot plate, no longer than an hour and a half. At this time the solution was colorless, but there was a large amount of precipitate in the flask. After cooling the flask, 10 ml. of water were added and the sample was titrated with concentrated ammonium hydroxide to a yellow color with phenol red (pH 8.3). Solid sodium citrate was

then added with constant swirling, until all the precipitate was in solution. (This usually took about 18 grams of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) for a one gram bone.) The clear solution was transferred to a separatory funnel, and 10 ml. of dithizone in chloroform (the same concentration as was used in Saltzman's procedure) was added. After shaking the funnel, the chloroform layer was drawn into another separatory funnel containing 30 ml. of 1 N HCl. The solution in the first separatory funnel was rinsed with 10 ml. of chloroform and after shaking the funnel, the chloroform layer was again drawn into the second funnel. After shaking the second funnel, the chloroform layer was discarded, and the solution was washed with 10 ml. of chloroform, which was also discarded. The solution of cadmium chloride was then neutralized with NaOH to the thymol blue endpoint (pH 2.8) as described previously, and the solution carried through the usual Saltzman procedure.

Part II. Enzyme Studies

Preparation of Formyl-L-glutamic Acid

Formyl-L-glutamic acid was prepared from formic acid and L-glutamic acid according to Tabor and Mehler's procedure (67). It was found that the ethanol-benzene solution of formyl-L-glutamic acid described in the procedure must be left in the cold room at 5°C. several days in order for crystals to form, and that the beaker must be scratched occasionally during this time. Difficulty was experienced in trying to remove the benzene completely from the acid. The benzene was finally removed by

lyophilization of the compound for several hours. The melting point was 112°C.; (m. pt. found by Tabor was 112°C.) and when mixed with known formyl-L-glutamic acid[1] there was no depression of the melting point.

Ascending chromatograms were run in the following solvents and the following R_f values were found, which agree well with values given in the literature (67,80).

TABLE VI

CHROMATOGRAPHY OF L-GLUTAMIC ACID AND FORMYL-L-GLUTAMIC ACID

Compound	Solvent		
	t-Butanol: 90% formic acid: H_2O		Phenol: H_2O
	R_f Value	70:15:15	75:25
Formyl-L-glutamic acid	0.74		0.61
L-glutamic acid	0.44		0.31

Detection of L-glutamic acid and formyl-L-glutamic acid on the paper was accomplished as follows. After air drying the chromatogram to remove the solvent, the paper was placed in a jar with a small amount of concentrated HCl. After two hours the paper was removed and allowed to remain overnight in air to remove the HCl fumes. Both glutamic acid and formyl glutamic (hydrolyzed to glutamic acid by the HCl) were visualized by spraying with ninhydrin.

[1] Obtained through the kindness of Dr. Herbert Tabor, National Institutes of Health, Bethesda, Md.

Isolation of Formyl-L-Glutamic Acid Deformylase

Albino guinea pigs[1] weighing from 400 to 700 grams were injected intramuscularly with a suspension of L-histidine monohydrochloride[2] in Mazola (corn) oil. One hundred mg. of histidine were injected per 100 grams of body weight. The animals were sacrificed 18 hours later by a blow on the head. The liver was removed and an acetone powder prepared immediately by homogenizing the fresh liver for one to two minutes in a Waring Blendor with five volumes of cold acetone (cooled to -30° to -40°C. with solid carbon dioxide). The temperature of the preparation stayed between 2 to 5°C. although the entire operation was done at room temperature. The suspension was rapidly filtered with suction using a Buchner funnel and Whatman no. 1 filter paper, and the precipitate was washed with two volumes of cold acetone. The solid was not allowed to dry on the funnel, but was rehomogenized with five more volumes of cold acetone, filtered and washed on the filter with two volumes of cold acetone. The filter cake was allowed to stand on the funnel until it was nearly dry and was then removed to a large sheet of Whatman no. 1 filter paper and air dried after crumbling with the hands. The powder was then kept in the deep freeze at -20°C. until needed. The yield of acetone powder was 31.6% (18 grams of acetone powder from 57 grams wet weight of liver).

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[1] The guinea pigs were obtained through the kindness of Dr. L. P. Hedeman of the Michigan State Health Department, Lansing, Michigan.

[2] L (+) histidine-monohydrochloride, C. P. (monohydrate). Pfanstiehl Chemical Company, Waukegan, Illinois.

An initial attempt to isolate formyl-L-glutamic deformylase by the procedure of Suda et al. (60) resulted in a preparation with no activity. Therefore, various modifications of the preparation were tried. The procedure outlined below resulted in an acetone powder of the highest activity. All solutions were made up in glass redistilled water. The acetone powder was extracted with 20 volumes of 5×10^{-2} M phosphate buffer, pH 5.6. The powder and buffer were rubbed together in a mortar and pestle. The mixture was transferred to a beaker and stirred slowly with a mechanical stirrer for two hours at room temperature. This mixture was then centrifuged. A two per cent solution of protamine sulfate[1] was added with continuous stirring until the ratio of the optical density of the supernatant at 280 to 260 mμ was about 0.8. The volume of protamine sulfate required was about 0.1 the volume of the crude protein solution. After centrifuging, the supernatant was heated in a beaker for ten minutes at 50°C. The solution was brought to 50° by rapidly heating the beaker in a water bath, with continuous stirring, during the ten minutes time. The mixture was then cooled quickly to 20°C. (in an ice-salt bath) and centrifuged. The above operations except where stated otherwise were carried out at room temperature.

The following acetone fractionations of the protein solution were carried out in the cold room at 5°C. One-half volume of cold acetone

[1]Protamine sulfate, Nutritional Biochemicals, Inc. Two grams of protamine sulfate were dissolved in a small amount of water with the addition of two drops of 10 N NaOH, the solution neutralized to pH 5.0 with acetic acid and made up to 100 ml.

(-40°C.) was added to the supernatant (the heat-treated enzyme). A precipitate formed which was centrifuged and discarded. A volume of cold acetone was then added to the supernatant, equal to the volume of the supernatant and the mixture was again centrifuged. The supernatant was discarded, and the precipitate obtained from this second acetone precipitation was dissolved in a volume of redistilled water equal to that of the original extract. The mixture was centrifuged and the precipitate discarded. To one volume of the supernatant containing the acetone-fractionated enzyme, one-half volume of saturated ammonium sulfate was added; the pH was adjusted to 5.0 with 25 per cent acetic acid.[1] The beaker containing the solution was covered and left in the cold room overnight. The next day the mixture was centrifuged and the supernatant discarded. The precipitate was put into water solution, in 0.1 the volume of the original crude extract, and the pH adjusted to 7.0 with 1 N NaOH. The solution was dialyzed[2] against redistilled water in the cold room for two hours. During this time the water was changed once. The material in the dialysis bag was centrifuged and the supernatant designated as the two-hours-dialyzed enzyme.

Determination of Enzymatic Activity

One ml. aliquots of the enzyme solution at various states of purification were incubated with 0.5 ml. phosphate buffer

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[1] All pH determinations were made with the Beckman Model H-2 pH Meter, with the glass electrode.

[2] The dialysis was carried out on the rotating external dialyzer. Visking tubing was used as the dialysis membrane.

(2×10^{-1} M, pH 5.6), 0.2 ml. of 10^{-1} M formyl-L-glutamic acid (neutralized to pH 6.0 with NaOH), 0.2 ml. of metal ion solution (if added) and enough redistilled water to make a total volume of two ml. The concentrations of metal ions used were 10^{-2} M cadmium sulfate solution, 2×10^{-2} M cobalt(ous) chloride solution and 10^{-2} M ferrous sulfate solution. The mixture was incubated in a 25 ml. Erlenmeyer flask, which had a ground glass joint. The flask was attached to a Warburg manometer with rubber bands by means of a metal collar attached to the outside of the neck of the flask and the vessels were shaken in the constant temperature bath of the Warburg apparatus for one hour at 37°C. Ninhydrin positive substances were determined at the beginning and end of the incubation period and the increase, glutamic acid formed from the hydrolysis of formyl-L-glutamic acid, was calculated. Enzyme with no substrate present, enzyme and metal ions with no substrate present, and substrate with no enzyme added were run as controls.

Determination of Glutamic Acid

Glutamic acid was determined by the ninhydrin method of Troll and Cannan (81). One-tenth ml. of the sample, containing from 0.05 to 0.8 u mole of amino acid, one ml. potassium cyanide-pyridine reagent and one ml. of 80 per cent phenol reagent were added to Folin-Wu sugar tubes. Two-tenths ml. of the ninhydrin reagent was added to each tube and the rack containing the tubes was immediately placed in a boiling water bath for four minutes. The tubes were cooled in cold running water and made up to 12.5 or 25 ml. with 60 per cent ethyl alcohol.

Under these conditions formyl-L-glutamic acid alone gave no more color than the reagent blank. Aliquots of the incubation mixture were analyzed in triplicate, and solutions in which protein was precipitated (crude enzyme extracts) were centrifuged after dilution with the alcohol. The optical density of the solutions was determined in the Beckman spectrophotometer Model B at 570 mμ, using the cell holder adapter with 15 mm. diameter Coleman cells or the regular carriage with one cm. corex cells. The concentration of glutamic acid in the unknown was read from a standard curve, prepared from ninhydrin determination of known concentrations of L-glutamic acid.

Formic Acid Determination

Formic acid was determined by the method of Pickett (82). Formic acid was oxidized using 0.5 M mercuric acetate in 0.1 N hydrochloric acid. The carbon dioxide formed was determined manometrically with a standard Warburg apparatus. For the reaction to go to completion, the time required was six to eight hours at 37°C. in the Warburg apparatus. Due to the difficulty of running large numbers of samples and the time required for each sample, formic acid was not determined routinely.

Protein Determination

Protein was determined either by the method of Warburg and Christian (83), by the biuret method (84), or by both methods. Warburg and Christian's method depends on the determination of the ratio of the optical density of a protein solution at 280 to 260 mμ. For a solution containing only protein, this ratio should be about 1.60 to 1.75.

The optical densities were determined on a solution of 0.5 ml. of the sample and either five or ten ml. of phosphate buffer (5×10^{-2} M, pH 5.6) in the Beckman D. U. using one cm. silica cells. A standard curve was prepared using dilutions of either bovine serum albumin[1] or standard casein solution.[2] One can also calculate by means of Warburg's factor that for a one cm. cell and a 280;260 ratio of 1.75, optical density x 1.10 x dilution factor = mg. protein per ml. solution.

In order to determine protein concentration by the biuret method, 0.5 ml. of the protein solution and either 2.5 or 5.0 ml. of biuret reagent were added to a test tube, the tube was stoppered and mixed gently by inversion several times. The mixture was allowed to stand at room temperature for at least 15 minutes, and the optical density determined at 545 mμ in the Beckman B in one cm. corex cells. Under these conditions, protein concentration up to 15 mg. per ml. may be determined with 2.5 ml. biuret reagent and protein concentration up to 30 mg. per ml. with 5.0 ml. biuret reagent. Very good agreement was obtained with protein concentration determined by all of the described methods.

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[1] Bovine plasma albumin, 35 per cent solution. Armour Laboratories, Armour and Co., Chicago, Illinois.

[2] Twelve grams of air dried casein and 40 ml. of 0.2 M NaOH were added to distilled water. The mixture was shaken mechanically until the casein dissolved and the solution was made up to 200 ml. The per cent protein was corrected for the moisture found by drying another sample in the oven at 100°C.

Electrophoretic Studies[1]

The material designated as two-hour-dialyzed enzyme was dialyzed against redistilled water for three more hours in the cold room. The water was changed twice and after this time, a portion of the solution outside the dialysis bag gave no precipitate with barium chloride (test for sulfate ions). The volume of the solution in the dialysis bag (20 ml.) was reduced by per-evaporation at room temperature to 10 ml. After this solution was dialyzed for 12 hours at 5°C. against phosphate buffer, pH 7.0,[2] the solution was subjected to electrophoresis, and conductivity measurements were made on the buffer and the solution. The remainder of the solution was dialyzed against redistilled water for about three hours until a portion of the solution outside the bag gave no yellow precipitate with ammonium molybdate (test for phosphate ions). The solution in the dialysis bag was then dialyzed against Veronal buffer, pH 8.6[3] for 12 hours, and the dialysate subjected to electrophoresis as before.

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[1]I wish to thank Mr. Harald Nielsen and Mr. Rashid Anwar, who very kindly performed the electrophoresis experiments. Schlieren diagrams and mobility data were obtained by use of a Tiselius electrophoresis instrument, the Perkin-Elmer Model 38. Conductivity measurements were made with a Model RC-1B conductivity bridge manufactured by Industrial Instrument, Inc., Jersey City, New Jersey. The conductivity cell supplied by Perkin-Elmer had a cell constant of 0.4893.

[2]Phosphate buffer pH 7.0 made from 0.925 grams $NaH_2PO_4.H_2O$, 4.77 grams of $Na_2HPO_4.12H_2O$ and 8.78 grams of NaCl made up to one liter. The ionic strength was 0.19.

[3]Veronal buffer pH 8.6 made from 21.20 grams of 5,5 diethylbarbituric acid ($C_8H_{12}N_2O_3$) and 4.0 grams of sodium hydroxide made up to one liter. The ionic strength was 0.1.

RESULTS AND CONCLUSIONS

Chronic Toxicity Study

The group average body weights of rats at various periods are presented in Table VII and the group average weight gains in Table VIII. It can be seen that there were no differences between groups as to the amount of weight gained (Groups I through VI). Values for the average food consumption of rats receiving cadmium in the drinking water are given in Table IX. As would be expected, the male rats ate more food than the females, but there was no difference in food consumed by the same sex in one group as compared with another.

The average water intake for each period is given in Table X. Regardless of the weight difference between males and females, there was no difference in their water intake. Therefore, the water intake is given as the group average, in ml. per day. These values are in the same range as values given for water consumption of controls in an experiment (85) concerning toxicity of germanium.

The total cadmium intake during each period, in Table XI, was calculated from the water intake. The total amount of cadmium ingested during the year's exposure is also given.

An analysis of variance which was carried out on all blood data obtained at the end of one year's administration of different quantities of cadmium showed no significant differences between any of the experimental groups and the control groups. The average value of the hemoglobin was 15.5 grams per 100 ml. of blood.

TABLE VII

AVERAGE BODY WEIGHT OF EACH GROUP

Group Number	28‡ Males g.	28‡ Females g.	52‡ Males g.	52‡ Females g.	91‡ Males g.	91‡ Females g.	181‡ Males g.	181‡ Females g.	271‡ Males g.	271‡ Females g.	362‡ Males g.	362‡ Females g.
I	217	197	296	227	363	257	423	285	455	301	494	311
II	256	188	326	218	400	252	468	283	491	300	505	314
	272*	178*	300*	218*	398*	251*	473*	275*			546*	333*
III	247	186	315	212	395	251	454	268	497	287	532	296
IV	252	188	332	223	414	258	466	278	511	311	566	327
V	242	191	309	220	383	249	443	276	480	293	none	303
VI	235	186	311	215	382	287	459	271	508	293	539	295
Average+	242	189	315	219	390	253	452	277	490	297	527	308

‡Days after experiment began.
*Average weight of rats which were repeated on 0.1 ppm.
+Weight of the rats repeated on Group II were not included in these averages.

TABLE VIII

AVERAGE WEIGHT GAIN OF EACH GROUP

Group Number	28*		52*		91*		181*		271*		362*	
	Males g./day/rat	Females	Males g./day/rat	Females	Males g./day/rat	Females	Males g./day/rat	Females	Males g./day/rat	Females	Males g./day/rat	Females
I	4.2	3.4	3.3	1.2	1.7	0.8	0.6	0.3	0.4	0.2	0.4	0.1
II	5.6	3.4	2.9	1.2	1.9	0.9	0.7	0.3	0.3	0.2	0.2	0.2
III	5.1	3.2	2.8	1.1	2.1	1.0	0.6	0.2	0.5	0.2	0.4	0.1
IV	5.6	3.3	3.3	1.5	2.1	0.9	0.6	0.2	0.5	0.4	0.6	0.2
V	5.2	3.3	2.8	1.2	1.9	0.7	0.6	0.3	0.4	0.2	-	0.1
VI	5.2	3.3	3.2	1.2	1.8	0.8	0.8	0.3	0.5	0.2	0.3	0.0
Average	5.2	3.3	3.1	1.2	1.9	0.8	0.6	0.3	0.4	0.2	0.4	0.1

* Days after experiment began.

TABLE IX

AVERAGE FOOD CONSUMPTION

Group Number	28* Males g./rat/day	28* Females g./rat/day	91* Males g./rat/day	91* Females g./rat/day	181* Males g./rat/day	181* Females g./rat/day	271* Males g./rat/day	271* Females g./rat/day	362* Males g./rat/day	362* Females g./rat/day
I	17.5	14.8	19.0	14.5	18.3	13.7	17.6	12.1	17.6	11.6
II	18.7	15.5	17.8	14.8	17.6	14.3	17.4	12.5	17.1	14.1
III	20.0	14.8	19.1	14.1	17.8	13.5	18.5	13.1	18.9	12.4
IV	18.9	16.4	18.9	14.7	18.4	14.0	19.3	15.3	18.7	15.1
V	18.0	15.7	18.4	14.8	18.7	13.8	17.3	13.3	-	13.1
VI	19.0	15.5	18.7	15.0	18.3	14.5	16.8	14.0	16.6	14.2
Average	18.7	15.4	18.6	14.6	18.2	14.0	17.8	13.3	17.8	13.4

* Days after experiment began.

TABLE X

AVERAGE WATER INTAKE OF EACH GROUP

Group Number	28* ml./day/rat	52* ml./day/rat	89* ml./day/rat	181* ml./day/rat	271* ml./day/rat	362* ml./day/rat
I	28	32	36	30	32	30
II	28	30	37	32	33	28
III	28	34	38	32	32	29
IV	26	33	38	30	36	33
V	27	30	35	30	29	30
VI	24	27	32	29	27	26
Average	27	31	36	31	32	29

* Days after experiment began.

TABLE XI

AVERAGE CADMIUM INTAKE OF EACH GROUP

Group Number	During Experimental Period µg.Cd./rat/day*	Total Intake for One Year mg.Cd./rat*
I	0	0
II	3.1	1.132
III	15.9	5.750
IV	82.4	29.82
V	150.2	54.36
VI	276.2	100.0

*Cadmium (as cadmium chloride) calculated from the water consumption.

Data for rats receiving 50 ppm of cadmium (Group VII) are presented separately in Table V. These rats were not part of the chronic toxicity experiments, but were started in order to compare the results obtained with a cadmium level which was thought to be high enough to enable the rats to show gross signs of toxicity. It can be seen that, compared to the average weights of the first six groups, rats receiving 50 ppm cadmium were stunted in growth.

Teeth of some of the rats receiving 50 ppm of cadmium showed the typical bleaching response as reported by Ginn and Volker (32). Rats from Groups I through VI were checked periodically for this bleaching, but no evidence of it was found.

The average water intake of the rats receiving 50 ppm of cadmium was only 14 ml. per day. This was roughly one-half of the water consumption of the other groups. The food intake of this group of rats was also less than that of the animals drinking lower concentrations of cadmium.

The blood hemoglobin of rats in Group VII dropped within two weeks to 8.0 grams per 100 ml. of blood and stayed between 7.7 to 9.0 grams for the remainder of the three months period. Microscopic studies showed marked anisocytosis, with many microcytic, hypochromic red blood cells and polychromasia with eight to ten nucleated red blood cells per 100 white blood cells.

The pathological changes described pertain to those of Groups I through VI. Pneumonia was the most important change observed in all the various groups of rats. The disease was occasionally accompanied by a pleuritis and an empyema. The pneumonia appeared in the controls and various levels of cadmium as follows:

Levels of Cadmium	Number of Rats	Number with Pneumonia	Per Cent with Pneumonia
Controls	22	13	59
0.1 ppm	26	18	69
0.25 ppm	16	12	74
0.5 ppm	16	12	74
5.0 ppm	16	11	69
10.0 ppm	16	12	74

In addition to the pneumonias, one rat had a pleural tumor which was diagnosed as a squamous cell carcinoma and another rat had an undetermined nervous disturbance. The various levels of cadmium did

not produce any recognizable microscopic changes in the various organs. Systematic studies of pathology were not performed on tissues from rats receiving 50 ppm cadmium, but several animals were observed for gross pathological changes and none were noted.

Cadmium Analyses of Liver and Kidney

The tissue cadmium content of the kidney is given in Table XII and that for liver in Table XIII. Analytical values for tissue cadmium in animals receiving cadmium for six months are given in terms of µg. cadmium per gram wet weight tissue, since portions of the liver and kidney were analyzed and weights of the whole tissues were not recorded. It will be remembered that the six months values were obtained from only two rats; therefore, averages of these groups do not have as much significance as the values from the twelve months period. The values for tissue cadmium following twelve months exposure to cadmium are presented both as µg. cadmium per gram tissue and µg. cadmium in the whole organ. It can be seen that as the cadmium content of the drinking water increased, the amount of cadmium retained in the kidney increaed. In most cases, tissue cadmium concentration was roughly proportional to the increase in the cadmium content of the water. For example, ingestion of 5.0 ppm resulted in a deposit of 52 µg. of cadmium in the kidney per gram tissue whereas 2.5 ppm resulted in an average cadmium content in kidney of 26 µg. The cadmium concentration per gram wet weight of kidney at the end of a year was about 2.5 times as much as at the end of six months, whereas in liver the

TABLE XII

CADMIUM CONCENTRATION IN RAT KIDNEY

Group Number	ppm Cd. in Water	µg.Cd./g. Wet Tissue Wt. 6 months	12 months*	µg.Cd. Total Organ 12 months
I	0	0	0	0
II	0.1	0	1.68 (1.39-2.10)	3.44
III	0.5	4.50 (1.72-7.35)	5.83 (2.44-8.91)	11.3
IV	2.5	10.1 (7.25-12.9)	25.9 (14.9-40.2)	47.6
V	5.0	17.6 (12.8-22.3)	51.8 (49.4-56.9)	94.6
VI	10.0	30.2 (28.6-31.9	83.6 (57.1-112.8)	174.0

Values in parentheses are the range of values obtained.

* These are the average values from 4, 5 or 6 rats in each group.

cadmium concentration at the end of the 12 months was about double the level at the end of six months.

If one compares the µg. cadmium per gram in the kidney and liver, it will be seen that there was more cadmium retained in the kidney. In fact, there was from two to three times the amount of cadmium retained by the kidney as by the liver. Usually, however, the total cadmium content of the liver was equal to or greater than the cadmium level of the kidney, because of the larger weight of the liver.

TABLE XIII

CADMIUM CONCENTRATION IN RAT LIVER

Group Number	ppm Cd. in Water	µg.Cd./g. Wet Tissue Wt. 6 months	12 months*	µg.Cd. Total Organ 12 months
I	0	0	0	0
II	0.1	0	0.23 (0.16-0.29)	2.32
III	0.5	0.90 (0.16-1.56)	1.09 (0.34-1.22)	9.18
IV	2.5	3.80 (3.67-3.91)	6.11 (3.71-10.3)	49.4
V	5.0	7.50 (7.27-7.72)	16.3 (11.3-21.0)	141.0
VI	10.0	20.2 (13.6-26.8)	39.1 (27.8-58.8)	344.8

Values in parentheses are range of values obtained.

* These are the average values from 4, 5, or 6 rats in each group.

Table XIV compares the total concentration of cadmium present in the liver and kidney and the per cent of the total ingested cadmium that each tissue retained. The per cent of the cadmium ingested which was retained by both the kidney and the liver ranged from 0.3 to 0.5 per cent. The percentage of cadmium retention was also calculated from tissue analyses of the rats which received 50 ppm. These values are not directly comparable with the other groups, since the rats receiving 50 ppm accumulated the element for only three months, whereas the values for the other groups were obtained at the end of a year's

TABLE XIV

CADMIUM RETENTION AFTER ONE YEAR

Group Number	Cd. in Total Liver µg.	Cd. Ingested for One Year Retained in Liver per cent	Cd. in Total Kidney µg.	Cd. Ingested for One Year Retained in Kidney per cent	Per Cent of Cd. Ingested Retained by Liver and Kidney	Total Cd. Ingested for One Year mg. Cd.
I						
II	2.32	0.205	3.44	0.304	0.50	1.132
III	9.18	0.160	11.3	0.197	0.36	5.75
IV	49.4	0.166	47.6	0.159	0.33	29.82
V	141.0	0.260	94.6	0.174	0.43	54.36
VI	344.8	0.345	174.0	0.174	0.51	100.00
VII*	276.5	0.472	93.0	0.158	0.63	58.8

* Values for Group VII (animals which received 50 ppm Cadmium in water) are for three months only.

exposure. However, the data do show that, except after administering 0.1 ppm of cadmium, the kidney retained about the same per cent of the ingested dose regardless of the concentration of cadmium in the water. On the other hand, the per cent of the ingested cadmium stored in the liver seems to increase with an increase in dietary cadmium.

Pooled samples of blood from rats receiving 50 ppm were analyzed for cadmium content. The concentration found was 11.5 µg. per 100 ml. of blood. If one assumes a blood volume of 6.7 ml. per 100 grams of body weight (86), then these rats, when sacrificed, would have had a blood volume of 14 to 16 ml., and the cadmium content in their total blood volume would be only 1.4 to 1.6 µg. per rat. Because of the difficulty of obtaining enough blood from the rats on the chronic toxicity study, no blood cadmium analyses were made on these groups, but it can be assumed from the results given above that the cadmium concentration of the blood would be insignificant, except perhaps in rats receiving the 10 ppm of cadmium in water.

From the results of the chronic toxicity experiment, it can be seen that the presence of cadmium in the drinking water had no effect on growth rate, food consumption, water consumption or blood composition. This finding was not unexpected since the levels of cadmium were deliberately chosen to be those which might be as high as would occur in drinking water, but not high enough to cause any obvious signs of acute toxicity. It is obvious that the maximum allowable concentration of cadmium in drinking water would have to be much below the

level necessary to cause emesis and, also, probably below the level necessary to cause the appearance of a yellow ring on the teeth.

However, from the results of the analyses of tissues for cadmium it can be seen that the element is deposited in liver and kidney, even on the lowest concentration level administered, 0.1 ppm. Since the concentration of cadmium in the tissues at the end of twelve months was roughly twice the amount in the tissue at the end of the six months feeding period, it might be assumed that in cases of continual ingestion of drinking water, that the amount in the tissues would be a reflection of the length of time that the water had been consumed.

The question of whether the amount of cadmium which is stored in the tissues is detrimental to the health of the animal is more difficult to answer. It is true that there were no noticeable effects on animals receiving any of the concentration of cadmium for a year. On the other hand, there could well be harmful physiological effects either to the animals themselves or to their offspring which were not measured by the criteria used in this experiment. The effect of ingested cadmium on enzyme systems present in these animals was not ascertained. There is the possibility that ingestion of cadmium would affect some enzymes which are either activated or inhibited by cadmium.

As was noted in the results, we were troubled by many cases of pneumonia. It was not possible to determine whether the cadmium had anything to do with the incidence of pneumonia. The incidence of the disease in the control group was probably not significantly lower than the incidence in rats which had cadmium in the drinking water.

It can be said that the presence of cadmium in drinking water had no effect on the rats, as measured by the criteria of growth rate, food and water intake or blood studies. This study gives information on the chronic toxicity of cadmium in drinking water in relation to rats, but more work needs to be done before these data can be translated into a standard for drinking water for humans.

Enzyme Studies

Tables XV and XVI present the activity obtained with the most purified preparation of formyl-L-glutamic deformylase. Table XV lists the volume and the protein concentration in each fraction of the purification procedure, while Table XVI presents values for the enzymatic activity of the crude enzyme and the purified enzyme which had been dialyzed for two hours.

One can calculate that the specific activity increased 11.2 times when the crude extract was purified to the state of the dialyzed enzyme. This is about one-half the increase in specific activity (21.7 times increase) obtained by Suda (60) using a similar purification procedure for the enzyme. It can be seen that Co^{++} activated the enzyme slightly. On the other hand, Cd^{++} and Fe^{++} decreased the activity slightly although the decrease obtained with Cd^{++} may not be significant. Fe^{++} was tried as a co-factor since Tabor has reported that it activated the formyl-L-glutamic deformylase obtained from Pseudomonas fluorescens cells. Suda et al. reported that Fe^{++} inhibited the enzyme from guinea pig liver unless ascorbic acid was

TABLE XV

PURIFICATION OF FORMYL-L-GLUTAMIC DEFORMYLASE

	Volume ml.	Protein Concentration mg./ml.
Crude extract	290	17.5
Protamine ppt.	310	15.0
Heat treated	270	12.5
Acetone fractionated	290	5.7
Ammonium sulfate fractionated	25	
Dialyzed 2 hours	26	1.29

TABLE XVI

ENZYMATIC ACTIVITY

	μ M Glutamic Acid Formed Per Hour	Specific Activity*
Crude enzyme	10.2	0.583
Dialyzed 2 hours enzyme	8.4	6.48
" + Co^{++}	10.0	7.72
" + Cd^{++}	7.8	6.02
" + Fe^{++}	7.0	5.41

The incubation mixture consists of phosphate buffer (pH 5.6, 2 x 10^{-1} M.) 0.5 ml.; formyl-L-glutamic acid (10^{-1} M) 0.2 ml.; metal ions (Co^{++}, 2 x 10^{-2} M; Cd^{++}, 10^{-2} M; Fe^{++}, 10^{-2} M) 0.2 ml., if added; enzyme 1.0 ml.; and redistilled water to make a total volume of 2 ml. The crude enzyme contained 17.5 mg. protein/ml., and the dialyzed 2 hours enzyme contained 1.29 mg. protein/ml.

* Specific activity represents μ moles of formyl-l-glutamic acid decomposed by 1 mg. of protein at 37°C. for one hour.

added with the iron. They explained that perhaps the presence of ascorbic acid prevents oxidation of Fe^{++} to Fe^{+++}. The effect of ascorbic acid was not tried in our determinations of enzymatic activity.

Vallee (87) has classified enzymes which have metal ions as co-factors as metalloenzymes or metal-enzyme complexes. A metalloenzyme is defined as an enzyme with a metal attached firmly and uniquely in such a manner that dialysis or other gentle means will not destroy the binding between the metal and the enzyme. During purification, the ratio of metal to enzyme-protein increases. With complete purification, a protein homogeneous by physical-chemical criteria is obtained, and the ratio of metal to protein becomes constant. The metal, in all instances known thus far, is a reactive group of the enzyme molecule, and removal of the metal results in irreversible inhibition. However, the addition of ions of the bound metal or any other metal to the highly purified metalloenzyme may result in lowered, raised, or identical enzyme activities, but this is not necessarily a guide to the presence or function of the "intrinsic" metal. According to Vallee, metals in metalloenzymes belong to the first and second transition group of the periodic system.

Characteristics of metal-enzyme complexes are that the metal is bound loosely to the protein and dissociates readily. The apoenzyme can be readily obtained metal-free and the binding is therefore much weaker than that in metalloenzymes. Removal of the metal by dialysis may lower the activity and the activity may then be increased by adding back the same metal, or other metal ions.

It can be seen that it is difficult to establish whether or not a given metal may be the "physiologically active" metal in an enzyme reaction, unless complete analyses of several metals are carried out simultaneously as the enzyme preparation is progressively purified. This is the approach taken by Vallee and his co-workers and is usually satisfactory only with the metalloenzymes.

In this experiment an enzyme preparation was obtained which, judging from electrophoretic data, contained one component. It is not possible at this time to say which metal, if any, is concerned with this enzyme in the "natural" state.

ELECTROPHORETIC PATTERNS (TRACED) OF AN ACTIVE PREPARATION OF FORMYL-L-GLUTAMIC DEFORMYLASE

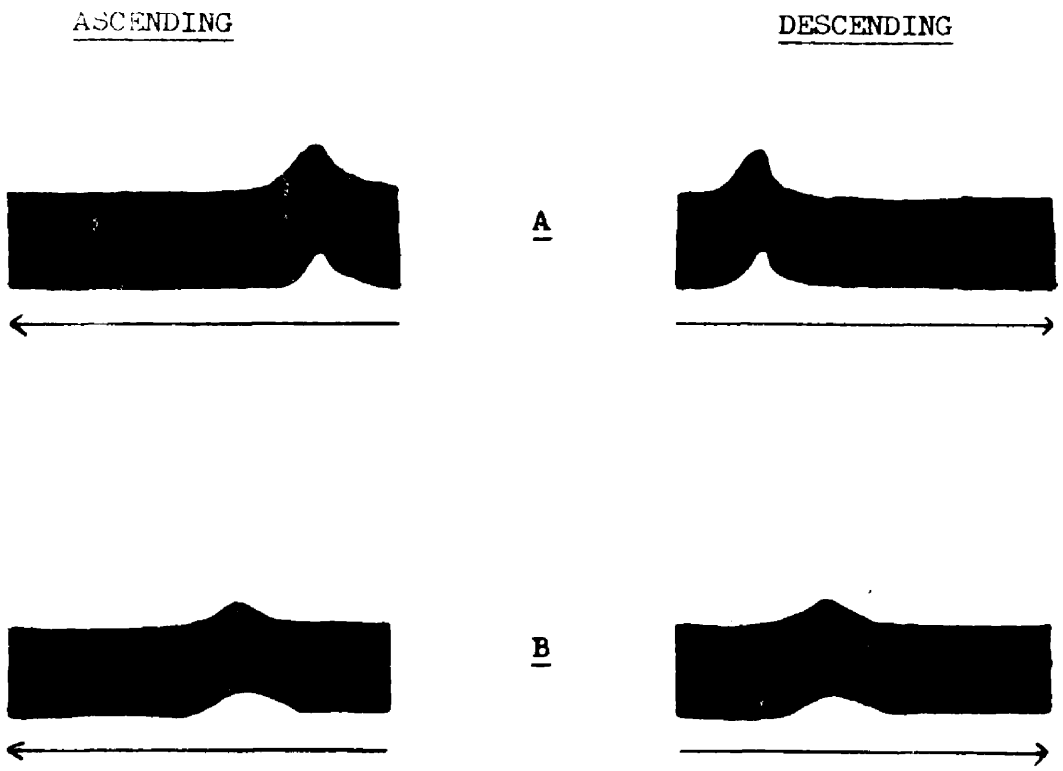

Figure 1.

A. 2 mg. protein per ml. in 0.02 M phosphate buffer pH 7.0 and 0.15 M NaCl, 7200 sec., pot. grad. 5.21 volts per cm., mobility 2.7 x 10^{-5} cm.^{2} per volt per sec.

B. 2 mg. protein per ml. in 0.02 M phosphate buffer pH 7.0 and 0.15 M NaCl, 14400 sec.^{2} pot. grad. 5.21 volts per cm., mobility 2.5 x 10^{-5} cm.^{2} per volt per sec.

SUMMARY

1. Five groups of rats were administered different concentrations of cadmium, between 0.1 to 10.0 parts per million, in the drinking water. During a one year experimental period, there were no differences between these groups or the controls as to the water intake, food consumption, or weight gain.

2. An analysis of variance on the blood composition data obtained at the end of one year's time showed no significant differences between any of the experimental groups and the control group.

3. A group of rats which were given drinking water containing fifty parts per million cadmium for three months drank only one-half of the amount of water consumed by the other groups. In addition, their growth was stunted and bleaching of the teeth was evident.

4. Hemoglobin values for rats receiving 50 parts per million cadmium averaged 8.0 grams per 100 ml. of blood as compared with an average value of 15.5 grams per 100 ml. of blood for controls and for rats receiving 0.1 to 10.0 parts per million cadmium.

5. Liver and kidney samples were analyzed for cadmium content at the end of six months and at the end of a year's consumption of the various concentrations of cadmium. When the results were calculated as µg. cadmium per gram wet weight tissue, the kidneys were shown to have retained two to three times the amount of cadmium as was

retained by the liver. However, the total amount of cadmium in the liver was usually equal to or greater than the cadmium content of kidney because of the larger weight of the liver.

6. The cadmium content in μg. per gram tissue of either liver or kidney increased with the cadmium intake, usually in direct proportion to the cadmium intake. In addition, values for tissue content obtained at the end of a year's exposure to cadmium, were roughly double those obtained at the end of the six month's period, which suggests that the amount of cadmium retained depends on the length of time that cadmium-containing water is ingested.

7. The enzyme formyl-L-glutamic deformylase was isolated from guinea pig liver. The specific activity of the enzyme was increased 11.2 times during purification from the crude extract. As judged by data obtained from electrophoresis of the purified enzyme in both phosphate and veronal buffer, there was one component present in the purified preparation. This component was probably identical with the active enzyme.

8. The activity of the purified enzyme was enhanced by Co^{++}, whereas Cd^{++} and Fe^{++} depressed the activity slightly. Which of these metal ions, if any, is concerned with the "natural enzyme" can not be said at this time.

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