THE EFFECT OF DIETARY ZINC ON INDUCTION OF CYTOCHROME P-450 BY PHENOBARBITAL OR 3-METHYLCHOLANTHRENE IN MALE SPRAGUE-DAWLEY RATS

by

Laurel E. O'Connor

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F.W. Thye, Chairman

M.E. Failla R.E. Ebel

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ABSTRACT

The effects of dietary (oral) zinc, as zinc chloride, on the hepatic microsomal monooxygenase system in Sprague-Dawley rats was investigated in the present study. Three routes of zinc administration - oral gavage, intraperitoneal injection and dietary supplement - were compared for accumulation of zinc in the liver and intestine, and induction of hepatic metallothionein (MT). Ip injection was the most effective method for the elevation of tissue zinc levels. Tissue metal and hepatic MT levels did not differ with dietary Zn supplementations The oral gavage was effective in elevating tissue zinc and hepatic MT levels. Since dietary zinc did not elevate tissue metal content, the oral gavage was used as "dietary" zinc treatment.

When zinc was administered orally in a linear fashion (0-200mg/kg), hepatic MT was elevated in relation to the dose size with 200mg/kg Zn^{+2} resulting in a 7-fold increase. There was an increase in hepatic microsomal enzymes at 25mg and 50mg Zn^{+2} then a decrease with the 100mg and 200mg doses. This could indicate a stabilization-destabilization of the cell membrane.

A one day oral dosing scheme was as effective as a 2 or 3 day schedule in the elevation of tissue zinc and hepatic MT levels, but

with a decrease in hepatic MT levels at day 3. Oral zinc at 40mg/kg had no effect on level of induction of cytochromes P-450 or b_5 by phenobarbital or 3-methylcholanthrene.

Hepatic microsomes isolated from rats that received 40mg Zn^{+2} were as effective in the biotransformation of aniline, aminopyrine, or p-nitroanisole as hepatic microsomes isolated from untreated animals. The K_m of aniline of the zinc treated rats was slightly higher than the control.

The *in vitro* addition of $ZnCl_2$ (0,25,50,100,1000μM) to a microsomal reaction mixture resulted in a decrease in the metabolism/biotransformation of aniline, aminopyrine and p-nitroanisole, with p-nitroanisole being the most sensitive.

This series of experiments indicated that if zinc was given as an oral supplement, the body is protected from a toxic effect by hepatic and probably intestinal MT. MT binds the excess zinc, and then either delivers it as necessary or holds for elimination from the body. If the protective function of MT is bypassed by intraperitoneal injection or intravenous infusion, zinc could possibly have metabolic consequences. Further studies investigating IV zinc on hepatic drug metabolism are indicated.

DEDICATION

If it had not been for the support and love of my brother John and his wife Karen, I would not be where I am now. The completion of this degree also belongs to you. Thank you.

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To Dr. Mark Failla, thank you for your expert guidance. I appreciate the support of Dr. Forrest Thye as my major professor from 1973 through the completion of this paper. But most of all, to Dr. Richard Ebel, who had confidence in me when I didn't think I would ever understand; who guided me expertly through all my experiments, and was there with moral support when I needed it; I sincerely thank you.

I also wish to thank Lon Fanning for all his guidance with the millions of Lowry protein determinations I agonized through.

Finally, to all my fellow poker players; I put my ante in.

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INTRODUCTION

Marginal zinc deficiency has been detected across age, sex, and socio-economic groups in the United States (Sandstead, 1973, Wolvarens *et al.*, 1976 and Hambridge *et al.*, 1972) as indicated by the occurrence of three zinc responsive disorders viz., growth failure, hypogeusia, and impaired wound healing. Nutrient analysis of institutional diets (Sandstead, 1973) and infant formulas (Wolvarens *et al.*, 1976) revealed levels of dietary zinc below RDA. Low income groups are particularly affected due to the expense of animal products which are the recommended zinc sources (Sandstead 1973). Ten percent of children between the ages of 0-4 showed low hair zinc (Denver Children's Study, Hambridge *et al.*, 1972), with associated poor appetite and slow growth. As a result of the attention that has been given to widespread marginal zinc deficiency, many physicians are now aggressively prescribing zinc supplementation of diets, intravenous fluids, and total parenteral nutrition solutions. While much is known about the cause, reversal, and consequences of zinc deficiency, there has been less concern for the metabolic consequences of excessive zinc intake.

Zinc has been reported by several authors to have an important role in membrane structure and function (Betteger and O'Dell 1981, Chvapil 1978). If excess zinc is accumulated within cells, the metal may influence the activity of the monooxygenase system.

The monooxygenase system is located in the endoplasmic reticulum membrane and metabolizes lipophilic foreign compounds and endogenous

substrates (e.g., steroids). It is possible that dietary zinc status might affect the content and/or activity of the monooxygenase system. If excess dietary zinc does affect the activity of the monooxygenase system, the ability to respond to foreign or endogenous compounds (e.g., steroids) could be hindered.

The purpose of this research was to study the effect of oral excesses of zinc on the content and activity of the monooxygenase system in rat liver and to determine if excess oral zinc affects the ability of the monooxygenase system to be induced by phenobarbital (PB) or 3-methylcholanthrene (3-MC).

LITERATURE REVIEW

Zinc as an Essential Element

Zinc was first established as an essential element for *Aspergillis niger* by Raulin in 1869. Todd *et al.*, (1934) suggested the essentiality of zinc for mammals. Because of the ubiquity of this metal in the environment, a deficiency was considered unlikely. However, in 1960 Prasad (1961) identified a pathological condition in Iranian males indicative of zinc deficiency. The symptoms exhibited were growth retardation, emaciation, emesis, conjunctivitis, keratitis, general disability and skin lesions on the abdomen and extremities. All symptoms abated upon initiation of oral zinc therapy.

Zinc is present in all human tissue with the highest concentrations occurring in the prostate, muscle, liver, and kidney. In the liver, zinc is present in the nuclear, mitochondrial, microsomal, and cytosol fractions, with the highest levels per unit of protein in the cytosol and microsomes (Underwood, 1977). Zinc is required for catalytic and/or structural properties of over 300 enzymes, including carbonic anhydrase, carboxypeptidase and alchohol dehydrogenase. The micronutrient is also involved in the stabilization of nucleic acids, proteins, ribosomes, and cell membranes. Zinc has a role in the growth of hair, skin, and bone, and vitamin A metabolism (Nriagu, 1977). Rough skin, susceptibility to infection and impaired wound healing are correlated with zinc deficiency in humans.

Zinc Deficiency

There are two classes of zinc deficiency: 1) that due to less than adequate levels of the metal in the diet, and 2) conditional zinc deficiency. The former state results when zinc is either absent from the diet, present at too low a concentration, or bound with dietary chelators, e.g., phytic acid that reduce its bioavailability. Physiological factors such as stress, inborn errors in zinc metabolism, cirrhosis, infection, and surgical trauma are all known to contribute to conditional zinc deficiency. There are several disease states linked to a zinc deficiency including acrodermatitis enteropathica, sickle cell anemia and arthritis. When zinc is administered as an oral therapeutic agent, the symptoms associated with these states are reversed (Brewer et al., 1979, Campo and McDonald 1976, and Simpkin, 1976). Hallbrook and Lanner (1972) demonstrated that upon initiation of zinc therapy (15 to 30mg orally), there was a significant increase in the rate of wound healing in man. More detailed studies have shown that this effect occurs only in patients with marginal zinc status (Prasad 1979).

Therapeutic Zinc

Zinc is primarily administered orally as a sulfate, acetate, carbonate, or gluconate salt because of ease of administration. Oral zinc is relatively nontoxic and fairly well tolerated except for a slight gastric irritation when given on an empty stomach (Oelshlegel

and Brewer 1971). The average dose is 220mg zinc sulfate/day (55mg elemental zinc) per individual (Baker 1982). Oral zinc is given after an overnight fast to avoid the binding that can occur due to dietary factors, such as phytate, fiber, arginine, phosphates, other chelating agents or dilution by the food itself (Oberleas and Harland 1977). The percentage of the dose absorbed ranges from 27% to 43% and is inversely related to the zinc status and the amount administered. Yamagichi *et al*. (1980) found that orally administered zinc initially appears in the liver and spleen. Following treatment of rats with 5mg zinc sulfate/100g body wt., hepatic zinc content increased significantly. However, doses of 10, 20, and 40mg Zn^{+2}/100g body wt. only slightly elevated zinc levels in liver above that achieved by the dosage with 5mg Zn^{+2}/100g body wt.

Zinc and Membranes

In addition to the enzymatic functions of zinc, non-enzymatic roles in membrane structure have been suggested. Zinc probably influences membrane structure and function by forming mercaptides with thiol groups of proteins and possibly binding to the phosphate moiety of phospholipids and carboxyl groups of sialic acid on glycoproteins on the plasma membrane (Chvapil 1976). Lysosomal membranes are stabilized by added zinc, preventing rupture of these organelles and resultant peroxidation of unsaturated fatty acids (Sullivan *et al*., 1980).

Chvapil (1973) has suggested that the observed effect of zinc may be due to its existence in a single oxidation state.

Zinc inhibits several membrane bound enzymes; such inhibition may result in the cessation of the energy dependent activity of plasma membrane or improved integrity of membrane structure (Chvapil 1976). Phagocytosis by leukocytes (ATPase dependent) is inhibited by zinc. Addition of the metal to erythrocytes of zinc deficient rats decreases their osmotic fragility. Zinc may also have a role in prostaglandin metabolism as indicated by its inhibition of platelet aggregation (Betteger and O'Dell 1981). The actual mechanism for the zinc effect is unknown.

It has been hypothesized that zinc deficiency disease states do not necessarily result from an effect on a prosthetic group of a metalloenzyme, but rather from an impaired integrity of the cell membrane. This suggestion is supported by the reversal of erythocyte sickling by oral zinc therapy in sickle cell anemia patients (Betteger and O'Dell 1981). Ciejan *et al*. (1981) postulates that zinc deficiency alters membrane fluidity via altered lipid composition. They found an absolute decrease in phospholipid content and a relative increase in the cholesterol to phospholipid ratio.

Zinc and the Monooxygenase System

The role of the diet in the body's ability to respond to xenobiotics (foreign compounds) has become an area of concern.

Xenobiotic metabolism has been reviewed by Clayson (1975), Wattenburg (1975), and Campbell (1979). Alteration in the metabolism of xenobiotics can lead to a grossly exaggerated toxic response and, possibly, to an increased rate of chemically induced pathogenesis or carcinogenesis.

The monooxygenase system is responsible for various activities including steroid metabolism and detoxification and/or activation of exogenous chemicals. This system is composed of three components, viz; NADPH cytochrome P-450 reductase, cytochrome P-450 and a lipid component. NADPH cytochrome P-450 reductase supplies electrons to the heme protein cytochrome P-450. Cytochrome P-450 activates molecular oxygen and inserts one atom of molecular oxygen into a substrate while the second oxygen atom is reduced to water. The functions of cytochrome P-450 have been reviewed by Sato et al. (1978). Hepatic cytochrome P-450 is primarily associated with the endoplasmic reticulum (ER). It is usually isolated as a "microsomal" fraction, obtained as a 105,000xg pellet of the 9000xg post-mitochondrial supernatant of tissue homogenate.

Becking (1977) has reviewed possible effects of dietary metals on drug metabolism. These effects include: 1) alteration of the substrate binding site; 2) alteration of the synthesis or degradation of components of the monooxygenase system; 3) alteration of the ER membrane itself. The above effects could be mediated by 1) a deficiency of the metal; 2) an interaction of two or more metals; or 3)

an interaction of the metal with another dietary component. Dietary metals that have been found to affect the monooxygenase system include Mg, Zn, Cu, Cd, Hg, Fe, Se, and Pb. Both excesses and/or relative deficiencies of these metals have been shown to cause such changes.

Hepatic microsomal cytochrome P-450 content has been found to be decreased by treatment with Cd, Se, Ag, Mg, Pb, and Cr and was correlated with an induction of heme oxygenase, the enzyme which catalyzes the initial step in heme degradation (Eaton et al., 1980). Zinc had no effect on either parameter. Mg^{+2} deficiency reduced the in vivo and in vitro metabolism of aniline and aminopyrine and cytochrome P-450 content. Reduced content of NADPH cytochrome c-reductase was also observed in Mg^{+2} deficient rats (Becking and Morrison 1970). Alverez et al. (1976) reported no effect of high chronic doses of dietary lead on drug metabolism in rats. Adult humans exposed to excessively high environmental lead levels displayed no significant changes in microsomal enzyme activities (Alverez 1975 and 1976). Lead (Pb) inhibited erythocyte aminolevuleni c acid dehydratase, a non-rate limiting enzyme of heme biosynthesis. Copper deficient rats exhibit decreased activity of aniline hydroxylase, increased activity of benzo(a)pyrene hydroxylase (Moffit and Murphy 1973; Teston and Jenner 1981). Cadmium treatment (200mg/kg body wt.) reduced cytochrome P-450 content by 40% and induced hepatic microsomal heme oxygenase in rats (Eaton et al., 1980 and Drummand and Kappas 1980).

Becking and Morrison (1970) reported no effect of zinc deficiency on aromatic hydrocarbon hydroxylase activity. However, oxidation of pentobarbital and the N-demethylation of aminopyrine were decreased in zinc deficient animals. Guzelian *et al*. (1982) reported a significant loss in aminolevulenic acid dehydratase activity in zinc deficient cultured rat hepatocytes. In tandem there is a significant decrease in cytochrome P-450 content. Guzelian has suggested a possible correlation between the decreased aminolevulenic acid dehydratase activity, which is involved in heme biosynthesis and the reduction in cytochrome P-450.

Clejan *et al*. (1981) reported that zinc deficiency in rats resulted in a modification of the lipid composition of liver microsomes. Zinc deficiency may modify hepatic metabolism in a way that could lead to harmful effects on the membrane. Betteger and O'Dell (1980) suggested that zinc may have a unique affinity for, and special function in, lipoproteins and proteins associated with biomembranes. They postulated that the absence of this metal causes alterations in the membrane structure. A high percentage of liver zinc exists in the microsomal fraction, but its functions there are unknown, since there are few known zinc metallo-enzymes located within this organelle (Betteger and O'Dell 1981).

Metallothionein and Zinc Homeostasis

Metallothioneins (MT) are low molecular weight, soluble, proteins. There are responsible, in part, for chelating excess zinc in the body. MT are rich in cysteine. MT are isolated from the cytosolic fraction of tissue homogenate and present in the highest concentration in liver, intestinal mucosa, and kidney. Their postulated functions include intracellular storage of heavy metals until they are either required or excreted. MT may also serve a protective effect by binding potentially toxic metals like cadmium and mercury (Cherian 1978). MT has also been implicated in protection from carbontetrachloride hepatotoxicity (Cagen 1979).

Intestinal absorption of zinc occurs by means of a saturable mechanism that may be carrier mediated (Davies 1980). The zinc passes either to the serosal side to be carried by the portal circulation bound to albumin or remains within the mucosal cell, where it is found associated with metalloproteins and/or MT (Richards and Cousins 1975).

Dietary and parenternally administered zinc induce MT synthesis in intestinal mucosa and hepatocytes. The synthesis of high molecular weight (75,000 daltons) proteins are not affected (Richards and Cousins 1976, Cousins 1979). With an increase in body zinc status, the metal is bound by the intestinal MT, thereby preventing its entry into the body (Richards and Cousins 1975). The intestinal cell apparently monitors zinc status and coordinates the flux of zinc entering the lumen with that which enters the cells from the plasma. Thus, MT's are

an integral part of the homeostatic mechanism that controls zinc absorption. This homeostatic mechanism is found in the plasma, intestinal mucosa and liver, and provides adequate zinc for bodily functions, but prevents uncontrolled, non-specific and potentially deleterious zinc-ligand associations (Cousins 1979).

Albumin bound zinc is transported to the liver where it becomes part of the hepatic zinc pool. Hepatic zinc is bound to zinc containing proteins and MT. When liver zinc levels increase above normal, much of the additional metal is bound to MT (Richards and Cousins 1976). Increased serum zinc is correlated with appearance of hepatic MT. Hepatic MT are also thought to block toxic effects by binding the excess metal and preventing it from binding to various enzymes or proteins (Cherian 1978).

In summary, the use of zinc salts as therapeutic agents and in oral dietary supplement has increased markedly. Toxic episodes as a result of zinc ingestion are rare. However, because high-dosage zinc administration for extended periods has increased, it is important to understand the metabolic consequences of added zinc. Zinc has been reported to have an important function in membrane function and integrity. Cytochrome P-450, a membrane bound enzyme, is responsible for the metabolism of endogenous compounds and external agents. Therefore, it is possible that, if excess zinc alters the integrity of cell membranes, changes in monooxygenase content and activity might be expected. The purpose of this work was to: 1) administer zinc by

three routes (gavage, intraperitoneal injection, and dietary supplementation) and compare zinc and copper levels in intestine and hepatic cytosol; 2) determine the maximum level of zinc that could be administered by oral gavage; 3) compare the effect of various levels of oral zinc (25, 50, 100, 200mg Zn^{+2}/kg body wt.) on hepatic MT levels and the monooxygenase system; 4) assess whether excess dietary zinc affects cell membranes by investigating induction of cytochrome P-450 by phenobarbital or 3-methylcholanthrene; and 5) assess whether excess dietary zinc affects cell membrane integrity.

MATERIALS AND METHODS

Male Sprague Dawley rats (Harlan Industries, Inc., Indianapolis, Indiana) weighing 175-200 grams were used. Animals were housed in pairs in hanging stainless steel cages in a temperature (68-72°F) and light/dark (12:12) controlled room. A minimum three day acclimation period was employed prior to experimentation. Rats were given free access to commercial chow (Purina 5001) and deionized water, except where indicated. All chemicals used were reagent grade.

Treatment

Zinc (as $ZnCl_2$) was dissolved in either 0.10M (pH6.0) or 0.5M (pH4.0) citrate buffer. Phenobarbital (PB), dissolved in 0.2M NaOH to a concentration of 40mg/ml was injected intraperitoneally (i.p.) at a dose of 80mg/kg body wt. 3-Methylcholanthrene (3-MC) was dissolved in corn oil (10mg/ml) and injected i.p. at a dose of 20mg/kg body wt. Control animals for the 3-MC group received the same amount of vehicle alone while the PB control animals received no treatment.

Sample Preparation

Rats were sacrificed by decapitation and blood was collected and incubated at 4°C in tubes containing lithium heparin. The liver was perfused via the hepatic portal vein with cold saline (0.9% NaCl). About 20cm of small intestine (duodenum) was removed below the pyloric sphincter and luminal contents flushed with 50ml ice cold saline. All

tissues were held on ice until processed. Blood was centrifuged at 5,000 rpm in a table top clinical centrifuge. Plasma was carefully decanted and stored frozen (-20°C).

For the isolation of microsomes and cytosol, whole livers were homogenized in 4 volumes (4ml/gram wet liver wt.) of ice cold 0.25M sucrose -0.5M Hepes (pH7.4) buffer using a motor driven, Potter-Elvejham homogenizer with a Teflon pestle. All isolation procedures were carried out at 4°C. After the total volume of homogenate was measured, 7 milliliters were removed and stored frozen for measurement of liver zinc and copper. The homogenate was centrifuged at 8700xg (maximum) for 10 minutes. The resultant supernate was centrifuged at 18,000xg (maximum) for 15 minutes. The 18,000xg supernate was centrifuged at 105,000xg (maximum) in a Type 30 rotor (Beckman) for 45 minutes. The final supernate (cytosol) was stored at -20°C for metallothionein determination. The pellet was resuspended in 20ml ice cold 0.15M KCl -0.05M Hepes (pH7.4), homogenized as before, and centrifuged at 105,000xg for 60 minutes. The microsomal pellet was resuspended in 0.15M KCl -0.05M Hepes (pH7.4) and protein concentration was adjusted to 10mg microsomal protein/ml. Intestine was ashed in an electric muffle furnace for 24 hours at 550°C.

Enzyme Assays

Microsomal content of cytochromes P-450 and b_5 was analyzed by the method of Omura and Sato (1964). NADH and NADPH cytochrome *c* reductase activities were measured at 25°C by monitoring the reduction of cytochrome *c* at 550nm. Activities were reported as nmoles cytochrome *c* reduced/min/mg protein.

Drug biotransformation assays were used to measure the activity of the monooxygenase system. Comparison of endogenous zinc (oral) treatment to *in vitro* addition of zinc to hepatic microsomes was made using p-nitroanisole, aniline or aminopyrine as substrates. Incubations were carried out at 37°C with 1mg of microsomal protein/ml in mixtures containing 0.05 Hepes and 0.15M KCl at pH 7.4 for aniline and aminopyrine and at pH 8.0 for p-nitroanisole. A NADPH generating system, 0.01M $MgCl_2$, 0.01M D,L-isocitrate, 0.4 unit/ml of isocitrate dehydrogenase and 0.2mM NADPH was used. p-Nitroanisole - o - demethylase activity was determined by measuring the formation of p-nitrophenolate according to the procedure of Netter *et al*. (1964). Aniline hydroxylase was determined by measuring the formation of p-aminophenolate according to Imai *et al*. (1966). Aminopyrine-N-demethylase was determined by measuring the formation of formaldehyde according to Nash (1953). Enzyme activities were expressed as nmoles of product formed/min/mg microsomal protein.

Protein and Metal Assays

Metallothionein was measured on pooled cytosol fractions. A 4.0ml aliquot of cytosol was fractionated on Sephadedx G-75 using a column (2.1 x 60cm) equilibrated with 0.01M Tris acetate buffer (pH 8.6) at a flow rate of 20ml/hr. Column fractions were collected (4.0ml each) and the absorbance at 280nm recorded. Copper and zinc levels in each fraction were determined by atomic absorption spectrophotometry. MT content was quantified as the amount of metal (Zn+Cu) that eluted at v_e = 1.8 to 2.0 x void volume per mg cytosol protein.

Tissue, plasma, and MT zinc and copper were determined by air/acetylene flame atomic absorption spectrophotometry (Perkin Elmer Model 560). Zinc (1ppm) and copper (5ppm) standards were prepared from a concentrated stock solution. Intestine and liver homogenate (2.5ml) were dried in preweighed porcelain crucibles at 100°C for 24 hours. Samples were then ashed in an electric muffle furnace for 24 hours at 550°C. Ashed samples were dissolved in 5ml of 0.12N ultrapure HCL. Plasma was diluted 1:4 with distilled water. Column eluents were read directly. Samples containing high concentrations of metals were diluted with ultrapure HCL (0.12N) to bring them into the range of the metal standard. Liver and intestinal metal levels were expressed as μg metal/total liver or intestine. Plasma metal was reported as μg metal/ml.

Microsomal protein was determined by the method of Lowry et al., (1951). Cytosolic protein was determined by the microbiuret method

(Baily, 1962). Bovine serum albumin was used as the standard for both methods.

Experiments

Experiment #1

The effect of parenteral zinc administration was investigated in eight (8) Sprague Dawley rats (175-200g), four (4) per group, by injection (ip) with either 3mg Zn^{+2}/200g body wt. (as $ZnCl_2$) in 0.1M citrate buffer (pH6.0) or an equivalent amount of citrate buffer alone (control). Rats were fasted overnight. Animals were sacrificed 18-20 hours after injection. Hepatic microsomal and cytosolic fractions were prepared. Hepatic MT was determined on pooled cytosols. Tissue zinc and copper were measured by the method described above. Hepatic cytochromes P-450 and b_5 were measured. NADH and NADPH cytochrome c reductase activities were determined as described in earlier sections of Materials and Methods.

Experiment #2

The effects of route and duration of zinc administration on tissue zinc levels was determined on male Sprague Dawley rats (175-200g). Animals were divided into the four (4) treatment groups as follows: 1) four (4) animals received no treatment (control); 2) eight (8) animals were injected (i.p.) with 5mg Zn^{+2}/kg in 0.10M citrate (pH6.0); 3) eight (8) animals were oral dosed by gavage with 5mg Zn^{+2}/kg; and 4)

eight (8) animals were fed a purified diet with the zinc level at 200ppm. This zinc level had no effect on food consumption. Four (4) animals per group (except control) received treatment one day and the remaining animals for three days. All animals, throughout the study, were maintained on a purified diet of the following composition: sprayed egg white albumin (20%), glucose monohydrate (33%), cornstarch (33%), Alphacel (3.2%), corn oil (5%), vitamin mix (5%). Biotin supplementation was at 4mg per kg. Detailed composition of the diet is described elsewhere (Failla and Kiser 1981). The zinc level in the control diet was 20ppm. Animals were sacrificed at 8:00 a.m. without an overnight fast, and tissues and MT were analyzed as above.

Experiment #3

The effect of increasing levels of zinc on hepatic metals and microsomal membrane enzymes was determined. Male rats were orally dosed with 0(4), 25(1), 50(1), 100(4) or 200(4)mg zinc. Numbers in parentheses indicate size of the treatment group. Food was removed six hours prior to oral dose. Animals were dosed at noon. Food was not returned, but animals were allowed access to water. Rats were sacrificed 18-24 hours after dosing. Tissues and MT were analyzed as before. Hepatic microsomal cytochromes P-450 and b_5 content and NADH and NADPH cytochrome c reductase activities were determined as described above.

Experiment #4

It was necessary to determine whether treatment for one day was adequate to elevate liver zinc content. In this experiment, four (4) male rats (175-220g) were subjected to a multiple oral dosing scheme. Animals were dosed with 200mg Zn^{+2}/kg in 0.5M citrate buffer (pH4.0) for either 1, 2, or 3 days (N=1 per group). The control received 0.5M citrate alone. Food was removed at 6:00 a.m. Animals were anesthetized and dosed at noon. Food was not returned. Animals were sacrificed at noon the following day. Tissues and MT were analyzed. Hepatic microsomal cytochromes P-450 and b_5 and NADH and NADPH cytochrome c reductase activities were measured as described above.

Experiment #5

The effect of zinc on the inducibility of cytochrome P-450 by phenobarbital and 3-methylcholanthrene was determined. Male Sprague Dawley rats (175-200g) were divided into five groups: 1) Zn^{+2} orally dosed at 200mg/kg in 0.5M citrate buffer; 2) phenobarbital (PB) (80mg/kg in 0.2M NaOH); 3) PB + Zn; 4) 3-methylcholanthrene (3MC) (20mg/kg in corn oil); and 5) 3MC + Zn. Food was removed six hours prior to oral dosing with the metal. Animals were orally dosed at noon. PB and 3MC were injected (i.p.) at 4:00 p.m. Food was not returned. Animals were sacrificed 24 hours after injection. Tissues and MT were analyzed as before. Hepatic microsomal cytochromes P-450 and b_5 levels were measured as described above.

Experiment #6

Male rats (175-200g) were divided into two groups of four rats each. One group was orally dosed with 200mg Zn^{+2}/kg (0.5M citrate pH4.0) and one group was orally dosed with 0.5M citrate alone. Dosing followed a six hour fast. Food was not returned. Access to water was not limited. Rats were sacrificed 18-20 hours after treatment. The effect of orally dosed Zn^{+2} on drug biotransformation by the hepatic microsomal monooxygenase system was investigated using p-nitroanisole, aniline and aminopyrine as substrates. Hepatic microsomes were prepared as described above.

Experiment #7

Hepatic microsomes were prepared from four fasted male rats as previously described. Tissues were pooled prior to centrifugation. *In vitro* drug biotransformation of aniline, p-nitroanisole or aminopyrine in the presence of zinc was followed. Zinc (0, 10, 25, 50, 100, 250, or 1000μM) was added and biotransformation assays were performed as described in *Materials and Methods*. This study was necessary to investigate potential direct effects of zinc on cytochrome P-450 activity.

RESULTS

Intraperitoneal injection of Zn^{+2}(30mg/kg) elevated hepatic zinc and hepatic metallothionein (MT) levels about three fold (Table 1) over control animals. Zinc treatment depressed cytochrome P-450 levels 32%, but had no effect on cytochrome b_5. NADPH-cytochrome c reductase activity was also reduced 32% following zinc treatment. The observed effects of administered zinc on tissue zinc levels and hepatic MT agree with previous results from this laboratory (Table 1). The primary discrepancy between the studies was that in the 1981 study, control hepatic MT was four fold higher than the value obtained in 1980. Possibly, animals in the present study were stressed more during treatment due to inexperienced handling. The smaller change in hepatic zinc levels would account for the smaller change in hepatic MT content. Cytochromes P-450 and b_5 levels were almost identical between the two experiments. The influence of zinc treatment on NADPH-cytochrome c reductase activity was similar in both studies.

Before investigating the effect of zinc on the induction of cytochrome P-450 by phenobarbital or 3-methylcholanthrene, it was necessary to determine the optimal condition for elevating liver zinc by oral administration of the metal. Oral administration was preferable to intraperitoneal injection since the former mimics the usual means for nutrient supplementation in humans. Table 2 presents the comparison of three routes of zinc administration on tissue distribution of zinc and hepatic MT levels. Intraperitoneal injection

Table 1. The Effect of Zinc Intraperitoneal Injection (Experiment 1) on Hepatic Zinc Metallothionein and Enzyme Levels in Two Different Trials with Rats*

	1980-1981**		1981-1982	
	Control	Zn	Control	Zn
Hepatic Zinc Status				
Zinc concentration (μg/g dry wt.)	194 ± 8	707 ± 26	139 ± 19	441 ± 88
Metallothionein (μg metal/mg cystosol protein)	0.044	0.416	0.189	0.512
Microsomal Membrane Enzymes				
Cytochrome P-450 (nmol/mg)	0.92 ± 0.03	0.69 ± 0.01	0.92 ± 0.11	0.62 ± 0.04
Cytochrome b_5 (nmol/mg)	0.36 ± 0.01	0.32 ± 0.01	0.32 ± 0.02	0.28 ± 0.01
NADPH-cytochrome-*c*-reductase (nmol/min/mg)	120 ± 3	88 ± 3	89 ± 10	60 ± 11

*Control animals received 0.1M citrate injected by intraperitoneal injection. The zinc-treated group received 30mg Zn^{+2}/kg as $AnCl_2$ in 0.1M citrate buffer. There were four animals per group. Rats weighed approximately 200 grams.

**For comparative purposes, results obtained in this laboratory by others during 1980-1981 are also listed.

Table 2. The Effect of a One or Three Day Zinc Exposure (Experiment 2) by Three Routes of Zinc Administration (Dietary, Oral Gavage, or Intraperitoneal Injection) on Tissue Metal and Hepatic Metallothionein Levels.*

	Control	Dietary		Orally		Injected	
		Day 1	Day 3	Day 1	Day 3	Day 1	Day 3
Tissue Zn (μg/g dry wt.)							
Liver	81 ± 4	88 ± 4	93 ± 6	128 ± 24	163 ± 43	217 ± 27	297 ± 16
Intestine	136 ± 14	109 ± 43	160 ± 43	146 ± 14	155 ± 24	193 ± 20	303 ± 41
Tissue Cu (μg/g dry wt.)							
Liver	14 ± 2	14 ± 4	12 ± 2	15 ± 2	16 ± 3	17 ± 2	13 ± 3
Intestine	11 ± 3	8 ± 3	10 ± 1	14 ± 4	11 ± 2	11 ± 2	18 ± 2
Hepatic Metallothionein (μg metal/mg cytosol protein)	.022	.035	.048	.151	.238	.372	.508

*Animals were divided into four treatments with seven groups (four animals per group). Rats were treated with zinc for one and three days. Dietary treatment consisted of purified diet supplemented with 200ppm Zn^{+2} as $ZnSO_4$. Food consumption was approximately 20g/d. Orally treated animals received 5mg Zn^{+2}/kg in 0.1M citrate buffer by gavage. Injected animals received 5mg Zn^{+2}/kg by intraperitoneal injection. Control animals received no treatment. All animals, except dietary treatment, received purified diet with a zinc level of 20ppm. Metallothionein determined on pooled tissue.

elevated liver and intestine zinc levels and hepatic MT 2.6, 1.4, and 16-fold, respectively above control levels after the first day.

There was a two fold increase in liver zinc, a seven fold elevation of hepatic MT and a slight increase in duodenal zinc when the metal was given orally. Three days of zinc treatment by injection or gavage elevated zinc levels in liver and intestine to a greater extent than addition of excessive levels to the diet. One day dietary treatment had little or no effect on any parameter. Elevated intestinal zinc, but not hepatic zinc levels, at day 3 of the dietary treatment, illustrated the effective control mechanism of zinc homeostasis. The excess zinc, not necessary for normal body functions, was bound in the intestine, by MT, to be used later, or excreted. Intestinal MT provides short term (24-48 hrs.) storage for excess zinc. There was not enough of a difference between one or three day oral dose to warrant a longer treatment period for the following experiments. Since dietary treatment failed to significantly elevate hepatic zinc content, it was decided that the oral gavage was the method of choice.

Tissue copper was a concern because of its role in iron metabolism. Ceruloplasmin, a copper containing protein, is essential for iron metabolism. In the copper deficient state, iron cannot be released from the liver, causing depressed levels of serum iron and erythrocytes. Therefore, an excess zinc mediated copper deficiency

could lead to anemia. Mode of zinc administration had no effect on tissue copper levels.

Liver and intestinal zinc content and hepatic MT levels increased as the quantity of the metal administered orally was increased (Table 3). The tissue response was not linear. Hepatic MT content responded to the greatest extent. There was a 2, 3, and 7-fold increase in MT following oral doses of 50mg, 100mg, or 200mg Zn^{+2}/kg body wt., respectively, whereas, liver zinc increased 1.8, 2.1, 2.2, and 2.5-fold with oral zinc doses of 25mg, 50mg, 100mg, or 200mg. The maximum dose of Zn^{+2} used was 200mg/kg due to the limited solubility of $ZnCl_2$ in 0.05M citrate buffer and since one milliliter was the maximum volume that was introduced into the rat's stomach. Hepatic cytochrome P-450 content was elevated with the 25mg and 50mg Zn^{+2}/kg body wt., but decreased below control levels following treatment with 100mg and 200mg Zn^{+2}/kg body wt. (Table 3). Due to the small sample number, the results were not significant. NADH and NADPH cytochrome c reductase activities were elevated in all Zn-treated animals. As in the previous experiment, zinc treatment did not alter the tissue copper levels. A dose of 40mg Zn^{+2}/200g body wt. (avg. animal wt.) was chosen for subsequent experiments because it resulted in the highest elevation of hepatic levels of zinc and MT and maximally reduced cytochrome P-450 content (15%) below control values.

It was necessary to determine whether a single oral treatment with zinc was adequate to attain a maximum elevation of liver zinc. The

Table 3. The Effect of Orally Dosed Zinc (Experiment 3) on Tissue Metal Metallothionein and Enzyme Levels.*

	Control (n=7)	Treatment Groups 25mg (n=1)	50mg (n=1)	100mg (n=3)	200mg (n=6)
Tissue Zn					
Liver (μg/g dry wt.)	117 ± 5	213	249	263 ± 20	294 ± 39
Liver (μg/total liver)	231 ± 20	424	454	588 ± 17	575 ± 104
Intestine (μg/g dry st.)	129 ± 15	301	257	596 ± 10	433 ± 64
Tissue Cu					
Liver (μg/g dry wt.)	16 ± 3	13	12	11 ± 2	19 ± 3
Liver (μg/total liver)	35 ± 4	35	28	30 ± 7	38 ± 8
Intestine (μg/g dry wt.)	9 ± 0.6	9	7	9 ± 1	16 ± 3
Hepatic Metallothionein*					
(μg metal/mg cytosol protein)	.213	.435	.71	1.711	1.492
Microsomal Membrane Enzymes					
Cytochrome P-450 (nmol/mg)	.74 ± .04	.84	.84	.69 ± .13	.64 ± .07
Cytochrome b_5 (nmol/mg)	.32 ± .02	.36	.37	.28 ± .02	.30 ± .02
NADPH-cytc-reductase (nmol/min/mg)	55 ± 3	75	87	71 ± 4	ND
NADH-cytc-reductase (nmol/min/mg)	239 ± 14	353	288	379 ± 18	ND

*Animals were divided into five groups and gavaged with zinc after a 6 hr fast. Zinc was administered at a concentration of 25, 50, 100 or 200mg Zn^{+2}/kg body wt. in .05M citrate buffer at pH 4.0. Control animals were oral dosed with .5M citrate alone. Animals were sacrificed 18-20 hours after dosing. Metallothionein determined on pooled tissues.

results are presented in Table 4. These values represent the mean for two animals per group. A one day dose of Zn^{+2} was sufficient to elevate liver zinc and subsequent Zn^{+2} doses were not cumulative Tissue copper levels remained unchanged. Hepatic MT was elevated to the same degree (3-fold) on days one and two. There was an unexpected decrease in the MT levels on day three. Copper levels in liver and intestine remained unchanged. Decreased levels of cytochrome P-450 were present in livers on days 1 and 2, but the quantities were similar to control by day 3. Cytochrome b_5 content and NADH cytochrome c reductase activities were decreased on day 1 then rose on days 2 and 3.

One observation can be made when comparing the control MT levels in Tables 1-4. The MT level in the zinc exposure study (experiment 2) was 0.022μg/mg cytosol protein. The values in the other experiments ranged from 0.171 to 0.213μg/mg cytosol protein. The animals in experiments 1, 3, and 4 were fasted prior to dosing and sacrificing. Bremmer and Davies (1975) reported that an overnight fast induced hepatic MT.

With the completion of experiments 2 through 4, a treatment consisting of a single one ml dose containing 40mg Zn^{+2}/200g animal was chosen to investigate the effects of zinc on hepatic drug metabolism.

The influence of zinc on phenobarbital (PB) and 3-methylcholanthrene (3-MC) mediated induction of cytochrome P-450 was

Table 4. The Effect of Single Versus Multiple Oral Dose (Experiment 4) on Tissue Metal, Hepatic Metallothionein and Enzyme Levels.*

	Control	Day 1	Day 2	Day 3
Tissue Zn				
Liver (μg/g dry wt.)	102	191	156	177
Liver (μg/total liver)	337	606	491	320
Intestine (μg/g dry wt.)	106	400	409	392
Tissue Cu				
Liver (μg/g dry wt.)	10	14	12	12
Liver (μg/total liver)	44	48	40	45
Intestine (μg/g dry wt.)	8	7	7	8
Hepatic Metallothionein (μg metal/mg cytosol protein)	.171	.549	.583	.270
Microsomal Membrane Enzymes				
Cytochrome P-450 (nmol/mg)	.74	.68	.63	.72
Cytochrome b_5 (nmol/mg)	.41	.34	.37	.38
NADPH-cyt <u>c</u> reductase (nmol/min/mg)	145	147	126	140
NADH-cyt <u>c</u> reductase (nmol/min/mg)	513	478	590	680

*Following a 6 hr. fast, one animal was dosed with 200mg Zn^{+2}/kg in .5M citrate buffer, pH4.0, for 1, 2 or 3 days. Control animal was dosed with citrate alone one day. Animals were sacrificed 18-20 hours after dosing. N=1 in all samples.

examined in experiment 5. Zinc treatment elevated hepatic zinc levels 2.6, 2.5, and 3-fold and duodenal zinc levels 2.1, 2.4, and 3.0-fold in +Zn, PB+Zn and 3-MC+Zn groups, respectively (Table 5). Hepatic MT was elevated 5.4-fold in the PB+Zn group, when compared to controls. MT levels in the 3-MC control group were 50% lower than untreated animals, whereas, MT was 50% higher in the PB treated group than the untreated controls. Tissue copper levels were unchanged except in the 3-MC+Zn animals. In PB+Zn animals, 73% of the liver zinc was associated with MT, whereas, only 45% of the zinc was associated with MT in 3-MC+Zn animals. Both the concentration and the total amount of MT-associated copper were elevated two-fold. Administration of zinc alone again reduced cytochrome P-450 content 15% compared to control. However, zinc had no effect on the degree of induction of cytochrome P-450 by PB or 3-MC. Cytochrome b_5 content appeared to increase, but the control value of 0.29nmol/mg protein, are below all the previous values of cytochrome b_5 averaging 0.36nmoles/mg protein. When the average value of 0.35nmoles/mg of cytochrome b_5 is applied as the control level, there was no change in levels of this cytochrome.

In experiments 5 and 6 the influence of *in vitro* zinc addition on the activity of cytochrome P-450 was studied. Activity was measured by following the biotransformation of p-nitroanisole, aniline, and aminopyrine to their respective products by the monooxygenase system. Oral zinc treatment did not affect the maximum velocities for the

Table 5. The Influence of Hepatic Zinc Status (Experiment 5) on Phenobarbital and 3-methylcholanthrene Mediated Induction of the Monooxygenase System.*

	Control	Zn	PB	PB + Zn (40mg)	3MC	3MC + Zn (40mg)
Tissue Zn						
Liver (μg/g dry wt.)	106 ± 6	279 ± 40	109 ± 11	265 ± 4	125 ± 3	318 ± 12
Liver (μg/total liver)	222 ± 35	624 ± 35	274 ± 99	568 ± 83	297 ± 65	505 ± 38
Intestine (μg/g dry wt.)	114 ± 9	247 ± 15	114 ± 16	278 ± 71	101 ± 10	348 ± 39
Tissue Cu						
Liver (μg/g dry wt.)	11 ± 2	11 ± 1	12 ± 3	14 ± 1	15 ± 1	25 ± 7
Liver (μg/total liver)	27 ± 4	27 ± 3	31 ± 5	30 ± 6	32 ± 2	33 ± 5
Intestine (μg/g dry wt.)	6 ± 2	6 ± 1	7 ± 1	7 ± .5	8 ± 1	8 ± 1
Hepatic metallothionein (μg metal/mg cytosol protein)	0.21	---	0.30	1.21	0.11	0.55
Microsomal Membrane Enzymes						
Cytochrome P-450 (nmol/mg)	.74 ± 0.07	.61 ± 0.07	1.6 ± 0.19	1.5 ± 0.13	1.1 ± 0.06	.98 ± 0.03
Cytochrome b_5 (nmol/mg)	.29	---	.38 ± 0.07	.38 ± 0.02	.37 ± 0.05	.35 ± 0.01

*Animals were divided into six treatments (six per group). Following a 6 hr. fast, rats receiving Zn treatment were oral dosed by gavage, 200mg Zn^{+2}/kg. Animals were injected with either PB (80mg/kg) or 3-MC (20mg/kg) four hours after oral dose. Animals were sacrificed 10-20 hours after injections. Control animals received no treatment. Metallothionein determined on pooled tissue.

three compounds (Table 6). The K_m value of aniline was elevated 35% with treatment.

In vitro addition of zinc to microsomes prepared from control animals was used to determine whether there was a direct effect of zinc on cytochrome P-450. As the zinc concentration was increased in the incubation mixture, the percent of compound (aniline, p-nitroanisole, or aminopyrine) converted to its product was decreased (Figure 1). This indicated a loss of cytochrome P-450 activity. Mixture pH was unaffected by the different zinc levels. p-Nitroanisole conversion appeared to be the most sensitive to zinc addition. One substrate concentration was near substrate K_m and the second was ten times higher. The decreased activity was the same for both substrate concentrations.

Table 6. The Effect of Added Zinc on Hepatic Biotransformation of Three Substrates.*

Substrate	Group	Km	Vmax (nmol/min/mg)
Aniline	Control	13 M	5.53
	Zn	20 M	4.50
Aminopyrine	Control	1.25 mM	6.10
	Zn	1.00 mM	5.70
p-Nitroanisole	Control	16 M	1.80
	Zn	15 M	1.40

*Zinc treated animals were oral dosed after a 6 hr fast, with Zn^{+2} at 200mg/kg in 0.5M citrate, pH4.0. Control animals were dosed with citrate buffer alone. Animals were sacrificed 18-20 hours after oral treatment. Assays were performed in duplicate on pooled tissue from four animals per group.

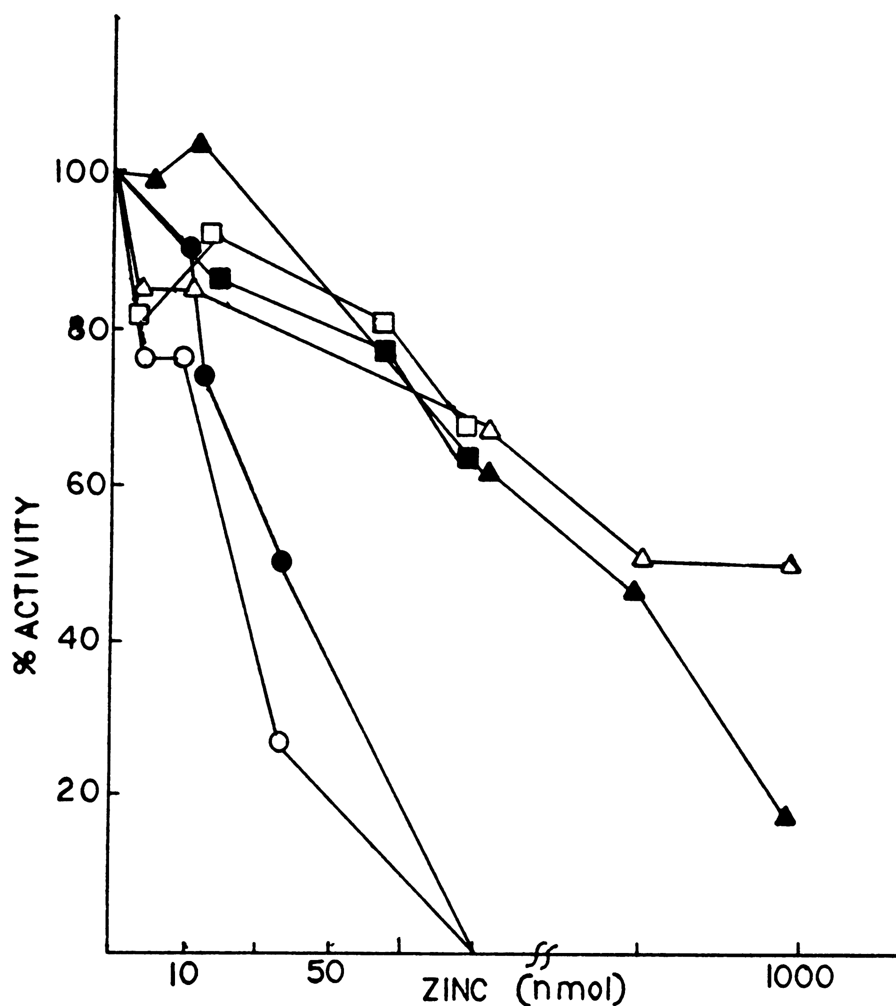

FIGURE 1. Effect of Endogenous Zinc Addition on *In vitro* Biotransformation of Aniline, Aminopyrene or p-Nitroanisole.

Zinc (0,10,25,50,100,250 or 1000 nmol) was added to incubation mixtures containing control microsomes. Substrate concentrations used Aniline (▲.07nmol or △.43nmol), Aminopyrine (■.05nmol or □.40nmol), or p-nitroanisole ●.17nmol or ○.61nmol). Each value is a mean of three separate measurements.

DISCUSSION

Pharmacologically administered doses of zinc have been found to be beneficial in promoting wound healing (Pories _et al._, 1967), treatment of sickle cell disease (Brewer _et al._, 1977), acrodermatitis enteropathica (Campo 1976, Cartside 1975) and rheumatoid arthritis (Simpkin 1978). Therefore, it appears zinc will be used increasingly for nutritional and/or pharmacologic purposes.

Zinc has also been found to promote cohesiveness and maintain the activity of membrane bound enzymes (Betteger and O'Dell 1981). The mechanisms remain unknown. Zinc stabilizes lysosomal membranes against lipid peroxidation, and affects the activities of membrane enzymes with varying degrees of specificity. Included in this group are guanylate cyclase, adenylate cyclase, $Na^{+}K^{+}$-ATPase, Ca^{+2}-ATPase and microsomal NADH oxidase (Betteger and O'Dell 1981, Chvapil 1979).

Zinc as a therapeutic agent or dietary supplement is easily administered by the oral route. It is nontoxic and fairly well tolerated (Oelshlegel and Brewer 1977). Zinc is also added to IV fluids (1-2mg/kg) for leukemia patients (Green 1977) and as a dietary supplement in parenteral nutrition solutions. Concerns about the metabolic consequences of zinc supplementation and therapy remain.

Data in Tables 1-4 illustrated the homeostatic mechanism controlling zinc metabolism. Comparison of the liver zinc level following parenteral administration of the metal [441μg/g dry wt. (30mg Zn^{+2}/kg)] to liver zinc levels of 213μg/g dry wt. (25mg

Zn^{+2}/kg) or 249µg/g dry wt. (50mg Zn^{+2}/kg) following oral dosing show that the injection of Zn^{+2} at approximately the same level was more effective in elevating liver zinc. This point is also illustrated in Table 2 where three routes of zinc administration are compared. Intraperitoneal injection of the metal bypassed the homeostatic control mechanism at the intestine. Therefore, the liver received a greater level of zinc administered. This bypass occurred because intraperitoneal zinc entered the lymph in the peritoneal cavity, then was transferred to the blood to be carried to the liver. When given orally, an effective mucosal block, probably in the form of intestinal metallothionein, was induced. Also, only 30% of orally or dietary administered zinc is absorbed in the intestine resulting in a maximal exposure of 1500µg or 1200µg respectively. Since intraperitoneal injection bypassed the intestine, these animals were exposed to a larger circulating dose of zinc, 5000µg.

Oral zinc administration (gavage) was better able to elevate liver zinc than dietary zinc (Table 2). First, it is well documented that interfering dietary substances impede absorption of zinc in the duodenum. Dietary fiber and other metal chelators decrease the bioavailability of dietary zinc. There can also be dilution of the metal by other dietary constituents (Oberleas and Prasad 1977). Oral zinc was administered after a 4 to 5 hour fast avoiding any dietary interference with absorption in the intestine. Also, the zinc in the solution (40mg Zn^{+2}/ml precipitated at a pH greater than 4.0. Zinc

was added to solutions containing citrate buffers to facilitate solubility of the metal.

Comparison of the one day and three day zinc exposure in all treatments indicated there was not a continual or linear accumulation of zinc in the liver (Table 2). This same phenomenon is also illustrated in Table 4. Hepatic MT and duodenal zinc levels were elevated in Experiment 4 (Table 4). This suggests that an induction of the mucosal block controls the flux of zinc from the lumen to the liver. The actual liver MT content in Table 2 is lower that Table 4, but the rate of increase is approximately the same. Animals in Experiment 4 were fasted, a stress which is known to elevate MT levels (Bremmer and Davies 1975).

Once oral dosing was demonstrated to be effective in increasing hepatic zinc levels, it was necessary to find the maximum dose that could be administered. A linear dosing scheme was utilized and the effect of these doses on cytochrome P-450 content reported (Table 3).

There was not a linear relationship between hepatic zinc content (Table 3) and the dose of Zn^{+2} administered. On a percentage basis, smaller doses of Zn^{+2} were absorbed better than larger doses (Yamaguchi 1980, Menard _et al._, 1981, and Oelshlegel and Prasad 1977). As the oral zinc levels increased, the hepatic MT (Table 3) levels increased markedly. According to Richards and Cousins (1976), as Zn^{+2} intake increases (0-150mg) the MT levels increase exponentially. While the hepatic MT levels reported in Table 3 do not

increase in an exponential manner, they do increase by a factor of two with the increasing doses. Cytochrome P-450 levels decreased from 50mg to 200mg zinc. Substantial increases in dietary zinc resulted in a transient elevation in plasma zinc, which was accompanied by a reduced absorption of zinc by the small intestine (Menard et al., 1981).

With a functioning homeostatic mechanism, excess dietary zinc is either chelated in the intestine or liver by metallothionein. The zinc is then released, to be excreted from the carcass or incorporated into zinc metalloproteins. Therefore, the hepatic and intestinal MT protects the rat against excess and potentially toxic levels of zinc.

Why would the hepatic monooxygenase system be chosen to illustrate possible effects of excess zinc? First, the liver is the clearing house for virtually all compounds that enter the body and the monooxygenase system is responsible for the detoxification of harmful compounds as well as steroid metabolism. If the interaction of zinc with cell membranes is integral to function, a disruption of zinc metabolism could result in an aberration in the content and activity of cytochrome P-450. It has been shown that zinc deficiency results in membrane changes characterized by increased lipid peroxidation in the lysosomal membrane and increased erythrocyte osmotic fragility (Brewer et al., 1979, Chvapil et al., 1972). Second, cadmium, another transition element of the same group as zinc, elicits deleterious effects on the monooxygenase system (Means et al., 1979).

Oral zinc had an interesting effect on the content of hepatic cytochromes P-450 and b_5. At the 25mg and 50mg zinc doses (Table 3) the content of P-450 and b_5 increased slightly, while they decreased at 100mg and 200mg zinc. The influence of zinc on NADPH -cytochrome c reductase activity followed the same pattern. Zinc levels of 25mg and 50mg could have a stabilizing effect on the membrane leading to increased enzyme levels or activities. The same fluctuation in microsomal membrane enzyme activities is seen in Table 4. In this example content of cytochromes P-450 and b_5 decreased over days one and two, but rose at day 3. The cell membrane could be adjusting over time to the zinc dose. Even though an oral dose of 40mg zinc reduced the content of P-450 (Tables 3, 4, and 5, Control vs Zn) the reduction is probably not considered physiologically significant. Yamaguchi et al. (1980) and Chen et al. (1977) reported that the majority of oral zinc absorbed appears in the cytosolic fraction. There is only a slight elevation in the plasma membrane levels. Possibilities exist for an oral zinc effect, if a dose could be administered that would saturate hepatic and intestine MT and a mechanism found to raise the membrane bound zinc level.

There was also no zinc mediated inhibition of the V_{max} of cytochrome P-450 for aniline, p-nitroanisole or aminopyrine even though in vitro zinc addition inhibited P-450 reactions, nor were there any changes in K_m values of aminopyrine and p-nitrophenol (Table 6). Excessive zinc increased the K_m value of aniline by 35%. While there

is no previous literature concerning effect of zinc on aniline metabolism, Means *et al.* (1979) reported a concentration dependent inhibition of the metabolism of aniline by cadmium.

Oral zinc decreased hepatic microsomal cytochrome P-450 content, but had no effect on the degree of induction of cytochrome P-450 by phenobarbital (PB) or 3-methylcholanthrene (3-MC).

An interesting observation is illustrated in Table 5. Hepatic MT levels in the 3-MC + Zn animals are 50% lower than the PB + Zn animals. There is also only 40% of the hepatic zinc associated with MT in the 3-MC + Zn animals compared to 70% in PB + Zn animals. The latter agrees with Bremmer and Davies (1975), who reported that 70% of administered zinc was associated with MT. There was also an unexplained elevation in liver and intestine copper levels. When this experiment was repeated, the copper levels were comparable to control. The elevated copper may have resulted from contaminated crucibles or an error in calculation.

From the data presented above, one would assume that the induction of MT synthesis facilitates the sequestration of excess orally administered zinc and subsequently protects the monooxygenase system from detrimental effects. But what would be the consequences if this protective mechanism were overwhelmed or bypassed? One major means of bypassing the homestatic mechanism of zinc control is through the use of zinc supplemented IV fluids or subclavical administered total parenteral nutrition. These methods are being used extensively in

hospitals to combat widespread malnutrition in seriously ill patients. We used the *in vitro* addition of zinc to hepatic microsomes to illustrate possible problems.

A description of general toxic effects on the monooxygenase system is needed prior to discussion for the *in vitro* experiment. Normally, when the body is exposed to a foreign compound, the monooxygenase system is induced. Thus the synthesis of monooxygenase polypeptides and heme are increased to provide adequate enzymes (NADPH cytochrome P-450 *c* reductase and cytochrome P-450) to metabolize toxic substrates. If it was possible to get an excess of zinc associated with the cellular membranes and if this excess zinc adversely affected cytochrome P-450, the body's ability to detoxify these foreign compounds would be compromised. This scenerio did not occur with the exogenous zinc administration, since the intestine prevented absorption of excessive amounts of the metal. Since there is very little literature on the effect of excess zinc on the monooxygenase system, previously reported effects of cadmium will be discussed. There will then be a discussion of correlations between zinc and cadmium.

In vitro addition of either cadmium (Schnell 1978) or zinc (Figure 1) to the microsomal monooxygenase system inhibits drug metabolism. According to Jeffrey (1983), zinc either binds to the oxidized cytochrome preventing reduction or inhibits reduced cytochrome:ligand formation by masking the drug binding site. In the present experiment, zinc added *in vitro* inhibited the metabolism of p-nitroanisole,

aminopyrine, and aniline at different substrate concentrations. The metabolism of p-nitroanisole (Figure 1) appeared to be more sensitive to *in vitro* Zn^{+2} administration that that of aniline or aminopyrine. Two classes of substrates metabolized by the monooxygenase system have been characterized on the basis of difference spectra upon interaction with cytochrome P-450. Type I substrates show a peak absorbance at 385-390nm and a trough at 420nm. Type II substrates show a peak at 425 to 430nm. p-Nitroanisole and aminopyrine are Type I substrates, while aniline is a Type II substrate. Cytochrome P-450 occurs in multiple forms and the types of substrate may be specific for a form of cytochrome P-450 (Sato *et al.*, 1978). Therefore, it is possible that *in vitro* zinc has a direct effect on one of the types of cytochrome P-450. Means *et al.* (1979) also reported a concentration dependent inhibition of aniline metabolism by cadmium.

Schnell *et al.* (1978) observed that cadmium accumulation in the liver was correlated with adverse effects on the monooxygenase system. Cadmium injection has been shown to adversely affect several parameters of the monooxygenase system (Stebbins *et al.*, 1980, Schnell *et al.*, 1978, Means *et al.*, 1979). Intraperitoneal injection of Cd^{+2} (2.0mg/kg) reduced the levels of cytochromes P-450 and b_5 by 20% and 30%, respectively. NADPH cytochrome *c* reductase activity was not altered. Exogenous Cd^{+2} also reduced biotransformation of p-nitroanisole and aniline by 29% and 46%, respectively. Zn^{+2}

injection (Table 1) caused a 30% decrease in cytochrome P-450 and NADPH cytochrome *c* reductase, but no effect on cytochrome b_5.

Cadmium injection reduced PB mediated induction of cytochrome P-450 by 38% (Stebbins *et al*., 1980). The metabolism of aniline and p-nitroanisole were also reduced by 53% and 28%, respectively in PB treated animals. The investigators concluded that the effect of cadmium on the monooxygenase system resulted from either a direct effect of the metal on susceptible components of the monooxygenase system leading to inactivation and/or an indirect effect whereby the levels of the necessary components are reduced (Means *et al*., 1979). As illustrated in Table 5, zinc had no effect on the level of induction of cytochrome P-450 by PB or 3-MC.

Comparison of results obtained with cadmium, a toxic metal, are difficult to project to zinc, and essential micronutrients. Cadmium has a higher affinity for sulfhydryl groups than zinc. Therefore, cadmium more readily reacts with essential sulfhydryl groups in protein causing the protein to be non-functional. Cadmium also induces microsomal heme oxygenase, an enzyme responsible for heme degradation, in addition to the inhibitory effect of Cd^{+2} on the microsomal system (Krawsky and Holdbrook 1978).

As stated before, zinc toxicity in humans is rare. Zinc has a stabilizing influence on lysosomal and erythrocyte membranes (Betteger and O'Dell 1981). Chvapil *et al*. (1972) also found that dietary zinc controls or inhibits lipid peroxidation by the liver, which involves

the membrane associated monooxygenase system. Chvapil et al. (1975) suggest that zinc retards the oxidation of NADPH, which in turn retards the transfer of electrons to cytochrome P-450, further inhibiting the oxidation of the substrate. Jeffrey (1983) found that where zinc (200mM) was mixed with NADPH (100mM) a complex forms (zinc:NADPH) preventing the oxidation of NADPH. Also shown was a noncompetitive inhibition of NADPH cytochrome c reductase by zinc (100mM). The inhibition of either component would affect drug metabolism.

Since small concentrations of zinc have been reported to be a membrane stabilizer, the possibility exists that excess zinc could lead to a decrease in membrane fluidity. With a decreased fluidity, or an unavailable protein, appropriate structure of proteins required for specific functions might not be achieved.

The use of zinc as a supplement in IV fluids and total parenteral nutrition (TPN) has increased. Considering the results from the in vitro experiment, this zinc could have adverse metabolic consequence. If parenteral zinc inhibits drug metabolism in the human, then persons on TPN could have an altered response to other administered drugs. Intraperitoneal zinc would also affect the body's ability to handle foreign compounds from the surrounding environment, inhibiting their clearance from the liver and body.

SUMMARY

1) Intraperitoneal zinc administration decreased the content of cytochrome P-450 in hepatic microsomes.

2) With a linear increase in oral zinc, there was a nonlinear increase in hepatic MT.

3) Singular oral dosing with zinc was as effective as a three day dose on elevation of hepatic zinc levels.

4) Oral zinc slightly decreased cytochrome P-450 levels; the degree of reduction would not appear to be physiologically significant.

5) Zinc treatment did not affect the inducibility of cytochrome P-450 by phenobarbitol or 3-methylcholanthrene.

6) Oral zinc treatment had no effect on the biotransformation of aniline, p-nitroanisole or aminopyrine.

7) *In vitro* addition of zinc to hepatic microsomes inhibited the metabolism of aniline, p-nitroanisole or aminopyrine to their respective products.

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