MECHANISMS OF THE EFFECT OF ZINC IONS IN
HEPATIC LIPID PEROXIDATION

by

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ABSTRACT

Rat liver microsomes were incubated in the presence of zinc ions and the rate of NADPH oxidation and related metabolism of aniline and ethylmorphine by appropriate oxidases were studied. A competitive mechanism of the inhibition of NADPH oxidation by zinc ion was found, with $V_{\text{max}} = 10.3$ nmoles NADPH/min/mg protein and $K_i = 7.22$ μM zinc. Although spectral analysis of NADPH in the range of 220-400 nm did not show any effect of zinc, gel filtration indicated binding of Zn$^{2+}$ to NADPH in the molar ratio 2:1. The overall formation constant of Zn$_2$-NADPH complex is $10^{6.75}$.

Zinc ion also inhibited the activity of two other microsomal drug oxidases, aniline hydroxylase and ethylmorphine-N-demethylase; 50% inhibition reached at 60 and 55 μM Zn$^{2+}$, respectively. Another microsomal enzyme, glucose-6-phosphatase, independent of NADPH, was not affected by zinc ion. The content and spectral characteristics of cytochrome P$_{450}$ was not affected by zinc ions.

It is concluded that Zn$^{2+}$, by direct binding to the phosphate moiety of NADPH, inactivates this pyridine nucleotide and prevents it from functioning in the microsomal electron transport system. The possibility that Zn$^{2+}$ may interfere with other ions or enzymes involved in microsomal electron transport cannot be excluded.
CHAPTER 1

INTRODUCTION

Unsaturated lipids in the cell membrane are a potential threat to the integrity of the cell because of lability of lipids in the presence of oxygen. Free radicals may be formed in lipids in the presence of oxygen which results in peroxidation of the lipids and the original lipid is destroyed along with the loss of the integrity of the membrane. Factors controlling lipid peroxidation are vitamin E, glutathion, and selenium, which are naturally occurring antioxidants, as well as zinc ion. Other means of preventing lipid peroxidation are spatial separation of the components undergoing lipid peroxidation, low oxygen tension, and metal chelation. This study is concerned with the effect of zinc ions in hepatic lipid peroxidation.

Lipid peroxidation is an irreversible process associated with pathological processes. Lipid peroxidation has been identified as a basic deteriorative reaction in cellular mechanisms of aging processes (1,2), carbon tetrachloride poisoning (3-6), in air pollution oxidant damage to cells (7), ethanol induced liver injury (8), oxygen toxicity (9), and radiation damage (10).

The mechanism of lipid peroxidation has not been fully understood since there are a variety of catalysts and inhibitors present. Also, the profile of unsaturated lipids, catalysts, and antioxidants is

1
not the same in all tissues and subcellular fractions of the tissue.

The proposed reaction scheme for the peroxidation of unsaturated lipids is (11):

1. Unsaturated lipid ($R_1H$)

2. Organic free radical formed by hydrogen abstraction ($R_1^\cdot$)

3. Diene conjugation

4. Peroxide radical ($R_1OO^\cdot$)

Hydroperoxide ($R_1OOH$)

$+R_2^\cdot$ which can attack another unsaturated lipid

The free radicals shown in steps 2 and 4 can react very energetically. They can initiate nonspecific hydrogen abstraction and chemical addition reactions because of their unpaired electrons. The initiation reaction of the above process is the variable step in tissues. Causes of the abstraction of hydrogen from the unsaturated lipid have been shown in vitro to be related to some heavy metals and the microsomal electron transport chain.

The research reported here is concerned with the mechanism of controlling lipid peroxidation particularly by zinc ions, but before
this can be understood the processes of lipid peroxidation must be elucidated.

**Metal Catalyzed Peroxidation of Pure Lipids**

Metal catalyzed lipid peroxidation has primarily been considered in the degradation of hydroperoxides, but recently Heaton and Uri (12) have shown metals to be associated with the chain initiation step. Evidence for this observation was shown when metal chelators were able to stop the autoxidation of tissue homogenates (13). A general scheme for the metal catalyzed propagation step is proposed by Ingold (14) as:

\[ R\,O-O\,H + M^{N+} \rightarrow R\,O^+ + O\,H^- + M^{(N+1)+} \]

where \( M^{N+} \) represents the metal. The \( R\,O^+ \) can then act as an initiator of the oxidation of lipids. Some metals that have been shown to have this catalytic effect are iron, copper, cobalt, and manganese (14-17). Therefore, metals catalyze lipid peroxidation in two ways. They can initiate the first oxidative attack and promote the degradation of hydroperoxides.

Copper ions were shown to enhance the autoxidation of phospholipid emulsions (18). Jarvi (19,20) has shown that a cobaltous chelate has a similar effect by increasing lipid peroxidation. The addition of ferric ions to irradiated suspensions of mitochondria increased the production of hydrogen peroxide (21). Lipid peroxidation has also been associated with other metals such as lead (22), mercury (23), silver (24), and gold (25).
Enzymatic Lipid Peroxidation

Free radical intermediates have been shown to be formed in liver microsomes by means of NADPH oxidation (26). Pfeifer and McCay (26) have evidence that the free radical is responsible for the peroxidative chain scission of unsaturated lipids in the microsomal membrane. Another study by Hochstein and Ernster (27) indicates an NADPH-induced peroxidation of lipids to be present in rat liver microsomes. The reaction requires ADP, NADPH, or other pyrophosphates and is coupled to the NADPH oxidase system of the microsomes. Several authors (28,29) have described an NADPH oxidase system of liver microsomes related to the drug metabolizing system.

Gillette's work (29) indicates that the drug metabolism of liver microsomes is associated with the electron transport system. He has evidence that the drug substrate reacts with the oxidized form of cytochrome P-450 to form a complex by NADPH cytochrome c reductase or indirectly by an unidentified electron carrier. The reduced cytochrome P-450 complex then reacts with oxygen and decomposes to the oxidized drug and oxidized cytochrome P-450. During this process, a free radical intermediate is formed which can attack the unsaturated lipids of the microsomal membrane and cause peroxidative damage characteristic of pathological processes.

Toxicities of Lipid Peroxides

Autoxidized fats which contain peroxides are toxic (30,31). Pure lipid hydroperoxides are not well absorbed from the gastrointestinal tract. Intraperitoneal injections of methyl oleate and methyl linoleate
peroxides (32) have an LD$_{50}$ of 6 and 12 mg/kg body weight, respectively, while 200 mg/kg of the peroxides were not lethal if fed to the animal.

*In vitro* studies of peroxides show that peroxides react with proteins, such as cytochrome c, to make them less soluble, and cause a depletion of or damage to amino acids such as histidine, serine, proline, and valine (33). Peroxides were shown by Wills (34) and Lewis and Wills (35) to oxidize sulfhydryl compounds to sulfonic acids and inactivate sulfhydryl dependent enzymes. Lipid peroxides were added to low density lipoproteins (36,37) which resulted in proteins being denatured. Similarly, denatured proteins may be present in atherosclerotic lesions. Lipid peroxidation products appear to interfere with the normal processes of the cell.

The amount of lipid peroxidation formed in mitochondria exposed to ultraviolet irradiation is correlated to the inhibition of oxidative enzymes (38). O'Malley et al. (39) have shown that lipid peroxides inhibit erythrocyte acetylcholinesterase. The inhibition of synthesis of ascorbic acid in rat liver microsomes incubated aerobically has been associated with the lipid peroxidation reaction (40).

The presence of lipid peroxides in certain tissues has been concurrent with pathological conditions. Lipid peroxides have been demonstrated in patients with thrombosed veins (41) and atherosclerotic aortas (1). High serum values of lipid peroxides have been found in patients with hepatitis and cirrhosis (42,43). In vitamin E deficiencies, Damm and Granados (44) found peroxides in the brown depot fat. Lipid
peroxides appear in skin coincident with the inflammation caused by chemicals or irradiation (45,46).

Radiation

Results of several studies have shown that lipid peroxides are present in the initial phases of radiation damage (47-50). The initial reaction brought about by the ionizing agents is presumably the production of the free radicals which begins the chain of events in lipid peroxidation.

Aging

Barber and Bernheim (1) have identified lipid peroxidation as a deteriorating effect associated with aging. This is based on two ideas. One is that chromolipids (lipofuchsins, aging pigments, ceroids) that accumulate linearly with age result from polymerization of oxidized unsaturated lipids (51,52). The other theory is that environmental factors lead to the production of free radicals which will cause oxidative reactions along with irreversible and damaging effects on the lipids (53).

The association of lipid peroxidation to aging is supported by Harman's study (54) in which the addition of antioxidants to the diet of mice prolonged the normal life span. The evidence is indirect, but as Barber and Bernheim pointed out (1, p. 395), "... it is generally not clear whether peroxidation is the cause or effect of these (pathological) changes. However, since lipid peroxidation is autocatalytic, once started it can compound the injuries initiated by other factors and thus
contribute to the overall pathology. Such may be the role of lipid peroxidation in aging."

Carbon Tetrachloride Toxicity

The toxic effect of carbon tetrachloride on rat liver, which is seen as necrosis, is dependent on the metabolism of carbon tetrachloride (55,56). The metabolism of CCl₄ results in free radical products (4,55, 57-59). The free radical products attack unsaturated lipids in intracellular membranes which results in lipid peroxidation. The lipid peroxidation damage is believed to be the necrogenic action of CCl₄ (6,55,59) which is seen in the liver.

The formation of the free radicals from the metabolism of CCl₄ is enzyme dependent and located in the endoplasmic reticulum of the liver (55,56). In vitro studies have shown that a source of NADPH is necessary for the formation of lipid peroxides in the microsomal fraction of liver homogenates (27,60). The enzymatic pathway is involved in the NADPH-cytochrome P-450 electron transport chain or drug oxidizing system. Once the free radical intermediate, CCl₃⁺, is formed lipid peroxidation can occur by way of the enzymatic process as described earlier in this paper, or by a nonenzymatic route which requires ADP and Fe²⁺.

Factors Controlling Lipid Peroxidation

Since lipid peroxidation is considered to be a deteriorating event in biological systems, it would be advantageous to control or limit the process. Extensive studies have been made on dietary
antioxidants which are suggested in controlling lipid peroxidation. Some of these are vitamin E, selenium, glutathion, selenium containing compounds, and zinc ions.

Vitamin E

Chronic avitaminosis E in many animal species is manifested as a degenerative process of the muscles. Vitamine E deficiencies of pregnant mice results in 20% of the newborn possessing necrotic muscles (61). Rats on vitamin E deficient diets develop degenerative lesions of the myocardium and fibrosis develops (62). Dogs which have deranged absorption of lipid soluble vitamins as a result of biliary fistula experience chromolipid pigmentation of muscles (63). Also, dogs on a high polyunsaturated fatty acid diet low in vitamin E show lipid pigmentation (lipofuschins) in the small intestines (64).

These studies indicate that the function of vitamin E is to limit lipid peroxidation. Gram and Fouts (65) incubated rat liver microsomes with an NADPH generating system and observed considerable lipid peroxidation. When they added vitamin E to the incubation medium, lipid peroxidation was abolished. This study, along with numerous reports by Tappel (66-69), indicates that vitamin E inhibits fatty acid oxidation stimulated by different compounds.

Selenium and Glutathion

Selenium has been associated with protecting animals against lipid peroxidative damage induced by diets high in unsaturated fats (70, 71). Studies by Reddy and Tappel (72) indicate that the detoxification
of dietary peroxides by way of the glutathion peroxidase system is higher in rats supplemented with selenium. Flohé, Günsler, and Schock (73) have shown that glutathion peroxidase contains selenium. This supports Reddy and Tappel's (72) work that the detoxification of peroxides is dependent on sufficient amounts of selenium and glutathion to activate the glutathion peroxidase system.

The swelling and lysis of mitochondria has been correlated to lipid peroxides being formed (74). Hunter et al. (74) have shown that glutathion significantly reduced the swelling, lysis, and disintegration of isolated mitochondria.

These in vitro studies indicate that selenium and glutathion reduce lipid peroxidation damage by means of the glutathion peroxidase system. The lipid peroxidation rate is probably not being decreased; instead, the products of lipid peroxidation are being detoxified more rapidly in the presence of the glutathion peroxidase system and therefore tissue damage does not occur.

Zinc Ions

Carbon tetrachloride hepatotoxicity has been related to the enhancement of lipid peroxidation. Several laboratories have shown that the damaging effects of \( \text{CCl}_4 \) are ameliorated by zinc ion supplementation in exposed animals (75-77).

In Chvapil's study (75), rats were dosed with carbon tetrachloride so that half were fed normal levels of zinc and the other half were on a high zinc diet. Results significantly showed that a product of lipid peroxidation, malonaldehyde, was significantly decreased in the
higher zinc rats. Lysosomal stability was significantly increased in the high zinc rats as measured by the amount of β-glucuronidase released from the lysosomes. Collagen accumulation and synthesis in the liver, which is indicative of the repair process, was significantly decreased in rats on high zinc. This study showed that lipid peroxidation damage induced by carbon tetrachloride could be reduced when zinc was administered in conjunction with the noxious agent.

Mechanism of Zinc Ions Controlling Lipid Peroxidation

Zinc ions have been shown to prevent lipid peroxidation in vivo and in vitro (78). The research presented in this paper is concerned with the mechanism of zinc ions inhibiting lipid peroxidation. This has been approached by three different means. First, since an enzymatic lipid peroxidation process involving the NADPH oxidase system exists, the effect of zinc on this was investigated. The oxidation of drugs by liver microsomes in the presence of zinc was studied. Thirdly, because the coenzyme NADPH is involved as a substrate for the above reactions, the effect of zinc ions of NADPH was studied.

These studies do not hope to answer all the questions presented by this process. Another means by which zinc may be inhibiting lipid peroxidation that has not been studied here is that zinc may be interfering with metals which can catalyze lipid peroxidation or metals that are necessary for the electron transport chain. Zinc ions may also be binding directly with enzymes of the electron transport system. More studies are necessary to further explain the mechanism of the effect of zinc ions in hepatic lipid peroxidation.
CHAPTER 2

INHIBITION OF NADPH OXIDATION AND OXIDATIVE METABOLISM
OF DRUGS IN LIVER MICROSONES BY ZINC

Recent observations that zinc inhibits in vitro-induced lipid peroxidation in liver microsomes (78,79) and protects the liver against the toxic effects of CCl₄ (75) pointed to a possible effect of this metal on the activity of mixed-function oxygenases in the endoplasmic reticulum of the liver. Several authors have stressed the essential role of NADPH as a source of electrons for the microsomal cytochrome P-450 drug-metabolizing enzymes (28,80). An NADPH oxidation-linked lipid peroxidation system has also been identified in liver microsomes and first described by Hochstein and Ernster (27). It was also suggested that both the processes of lipid peroxidation and of drug oxidation depend on the same electron transport chain (81). Since CCl₄-induced lipid peroxidation is generally assumed to be initiated by the •CCl₃ free radical produced by the NADPH-dependent, cytochrome P-450 enzyme system (55,56,82), then inhibition of NADPH oxidation may explain the decreased lipid peroxidation and CCl₄-induced hepatotoxicity produced by zinc. In this research, evidence will be presented that NADPH oxidation is inhibited by zinc and that this reaction results in the inhibition of drug metabolism by liver microsomes.
Rat liver microsomes were prepared by a procedure described in previous papers (78,79) and incubated in a medium enriched in NADPH or in NADPH-generating systems, as given in the legend to Fig. 1. The rate of NADPH oxidation was recorded at 340 nm on a Beckman Acta III spectrophotometer at 37° under continuous slow magnetic stirring. Repetitive addition of zinc into the reaction mixture not only slowed down the rate of NADPH oxidation but, at a certain concentration of zinc in the medium, reversed the reaction in the direction of NADP reduction (Fig. 1). The fact that an excess of zinc increases the final content of NADPH above the initial level of NADPH indicates the additional reduction of endogenously present NADP in the microsomal fraction. We have two reasons to believe that the observed inhibition of NADPH oxidation by zinc is related to the effect of this ion on some enzymes present in the microsomal fraction of the liver rather than to a direct interaction of zinc with pyridinenucleotides: 1) heating the reaction mixture at 60° for 10 min abolishes the changes in NADPH content, and 2) there is no spectroscopic evidence on the interaction of Zn²⁺ with NADPH (83).

More rigorous treatment of the effect of various zinc concentrations on NADPH oxidation was carried out under the conditions recommended for the assay of NADPH oxidase (84) and is presented in Fig. 2. The activity of NADPH oxidase, studied at two different concentrations of the substrate and measured as initial velocities during the first minute of the reaction, was inhibited by zinc. Only a 10 μM concentration of zinc inhibited 50 percent of the enzyme activity. Such a low
Fig. 1. Effect of zinc on the oxidation of NADPH in rat liver microsomes. -- A microsomal fraction was prepared and diluted in Tris-KCl buffer (0.05 M, pH 7.4) to obtain 1.5 mg protein/ml. To samples containing 2 ml suspension, 1 ml of either 0.2 mM NADPH or 2 mM of glucose 6-phosphate, 2 units of glucose 6-phosphate dehydrogenase, 5 mM MgSO$_4$ and 20 mM nicotinamide were added and measured at 340 nm at 37° under stirring. Given amounts of zinc in Tris-KCl buffer were added as indicated.
Fig. 2. Effect of zinc on the activity of NADPH oxidase in liver microsomes at low and high substrate levels. -- Enzyme activity in the microsomal fraction was assayed at pH 5.5 in a system containing 8 μmoles Na₂HPO₄, 54 μmoles KH₂PO₄, 1 μmole MnCl₂, 340 μmoles sucrose and 21.6 or 86.4 μmoles NADPH in 3 ml final volume. Control samples did not contain a subcellular fraction. The rate of oxidation of the reduced coenzyme was scanned at 340 nm.
concentration of zinc suggests a possible effective and specific role of zinc in the control of NADPH oxidation within biological systems, even in vivo.

Additional evidence indicating that zinc ions at relatively low concentrations interfere with NADPH oxidation-linked reactions was determined by studying the microsomal metabolism of ethylmorphine. The incubation procedure has previously been described by Sipes et al. (85). The only modification was a final protein concentration of 2 mg/ml. The data in Fig. 3 summarize the inhibitory effect of zinc on microsomal ethylmorphine N-demethylase activity as related to time. Zinc inhibited the formaldehyde produced by the N-demethylation of ethylmorphine throughout an incubation period of 90 min. Since the reaction was still linear at 10 min, this time point was chosen to determine the effect of various concentrations of zinc ions on the metabolism of ethylmorphine. The results are summarized in Fig. 4. It is evident that the activity of the N-demethylase, as judged from the first 10 min of the reaction, is inhibited by zinc in a concentration-related manner. Similar experiments carried out with various zinc salts (gluconate, SO\textsubscript{4}\textsuperscript{2-}, Cl\textsuperscript{-}, CO\textsubscript{3}\textsuperscript{2-}, and CH\textsubscript{3}CO\textsuperscript{-}) indicate that the anion has no effect on the magnitude of the inhibition (data not presented).

The experimental evidence presented in this study clearly indicates that, in system in vitro at relatively low concentrations, zinc ions inhibit the oxidation of NADPH and the related metabolism of drugs, as represented in this study by ethylmorphine. The mechanism of zinc interactions with NADPH oxidase is under investigation. It is noteworthy,
Fig. 3. Effect of zinc on the liver microsomal N-demethylation of ethylmorphine in vitro. -- A final volume of 3 ml incubation mixture contained 2 mg/ml of microsomal protein, 10 mM ethylmorphine, and NADPH-generating system (NADPH 0.22 mM, glucose 6-phosphate 3.3 mM, nicotinamide 2.0 mM, and glucose 6-phosphate dehydrogenase 1 unit/3 ml). The concentration of zinc sulfate was 70 μM. Incubation was carried out at 37° under slight shaking.
Fig. 4. Effect of zinc concentration on the liver microsomal N-demethylase activity. -- For methodological details, see Fig. 3. Formation of formaldehyde (HCHO) in control samples during a 10-min incubation was 42.3 nmoles/mg of protein.
however, that this enzyme requires Mn$^{2+}$ (84). There is a possibility that zinc may displace this cation in a manner similar to that suggested by Brunel and Cathala (86) for alkaline phosphatase from bovine brain. In any case, the findings indicating that the activity of NADPH oxidase is stimulated by Mn$^{2+}$ and inhibited by zinc differ from the observation made by May and McCay (87), who showed that peroxidation of microsomal phospholipids dependent on NADPH oxidation was inhibited by Mn$^{2+}$. We assume that the described inhibition of NADPH oxidation by zinc may explain our observation of the inhibition of lipid peroxidation in the liver by zinc (78,79) and the protection afforded by zinc against CCl$_4$-induced hepatotoxicity.

Incubation studies in vitro with $^{14}$CCl$_4$ and liver microsomes have implicated NADPH oxidation in the mechanism of CCl$_4$ activation to an active intermediate, probably $^{•}$CCl$_3$. By following the covalent binding of $^{14}$C to $^{14}$CCl$_4$ to microsomal protein as an index of the conversion of $^{14}$CCl$_4$ to $^{•}$CCl$_3$, it was reported that covalent binding was prevented by elimination of NADPH from the incubation mixture (88,89) or by addition of the specific antibody of NADPH-cytochrome c reductase (89) (NADPH oxidase) to the incubation medium. If $^{•}$CCl$_3$ is responsible for the CCl$_4$-induced lipid peroxidation, then decreasing the formation of it may reduce the peroxidation which is destructive to the liver cells. Therefore, the finding that zinc inhibits NADPH oxidase may be the mechanism by which zinc inhibits CCl$_4$-induced lipid peroxidation and the subsequent liver damage.
While studying the mechanism of Zn$^{2+}$ inhibition on NADPH oxidation, the concentrations of all reactants were decreased as compared to the conditions of the experiment in Fig. 1. (Only the initial rate of the reaction was used so that Michaelis-Menton mechanisms could be applied.) Under these conditions, Zn$^{2+}$ inhibited NADPH oxidation as is shown in Fig. 1, but the increase in absorption at 340 nm was not evident. Repeating the conditions of these previous experiments and scanning the reaction at 340 nm and 600 nm showed an increase in absorption at both these wavelengths when 60 µmoles of Zn$^{2+}$ were added to the reaction vessel. Increasing the temperature showed that this increase in absorption occurred at still lower levels of Zn$^{2+}$. From these new findings, it appears that the increase in absorption at 340 nm in Fig. 1 may be due to microprecipitation at these high levels of NADPH and Zn$^{2+}$. Further study is continuing in this area into the possibility of NADPH and Zn$^{2+}$ interactions occurring even though the u.v. spectral analysis which is mentioned in this chapter shows no perturbations.
CHAPTER 3

INHIBITION OF NADPH OXIDATION AND RELATED DRUG OXIDATION IN LIVER MICROSONES BY ZINC

In a recent study, it was shown that the administration of Zn\(^{2+}\) to rats protected the liver from CCl\(_4\)-induced hepatotoxicity (75). The data indicated a decrease in the peroxidation of unsaturated fatty acids, since the rate of malondialdehyde formation was significantly slower in liver microsomes from Zn\(^{2+}\)-treated animals. It is generally assumed that CCl\(_4\) initiates lipid peroxidation after it is converted to a trichloromethyl free radical (•CCl\(_3\)) by microsomal drug-metabolizing enzymes and that •CCl\(_3\) is the actual hepatotoxic species (5,90). Two possible mechanisms could explain these findings: 1) that Zn\(^{2+}\) was preventing the microsomal conversion of CCl\(_4\) to •CCl\(_3\), and/or 2) that Zn\(^{2+}\) was interacting with the polyunsaturated fatty acids of the biomembranes, thus rendering them resistant to peroxidative deterioration.

In this chapter we explore the first hypothesis, namely, that zinc interferes with mixed-function oxidases residing in smooth endoplasmic reticulum of liver microsomes. It has been well established that the mixed-function oxidation consumes equal amounts of NADPH, oxygen, and drug as a substrate. Thus, the initial step in liver microsomal electron transport involves transfer of electrons from NADPH to reduce the oxidized heme P-450-substrate complex by way of NADPH-cytochrome-c
reductase. The reduced cytochrome P-450 substrate complex then reacts with molecular oxygen to form the oxygenated complex (29). The oxidation of NADPH in the initiation of microsomal electron transport resulting in oxidation of a drug was assumed to be the likely target for zinc effect. The preliminary report indicating such an effect has already been published (91).

The microsomes will also catalyze an NADPH-dependent peroxidation of endogenous lipid (26). This reaction involves the transient formation of lipid peroxides, leading to deterioration of polyunsaturated fatty acids and producing a variety of degradation products, including malondialdehyde. Assuming that zinc inhibits microsomal NADPH oxidation (91), we suggest that by this mechanism both oxidation of drugs and NADPH-oxidation dependent lipid peroxidation in the endoplasmic reticulum should be inhibited. The experimental data supporting the view that zinc ions inhibit enzymatic lipid peroxidation were published elsewhere (79).

Methods

All experiments reported in this study were performed with microsomes isolated from adult Sprague-Dawley rats of both sexes. After exsanguination of the animal, the liver was thoroughly perfused with at least 20 ml of ice-cold saline through either the portal vein or the inferior vena cava.

Liver was homogenized in phosphate buffer (0.01 M, pH 7.0) or Tris-KCl buffer (0.05 M Tris HCl and 0.153 M KCl buffer, pH 7.4) and is described for each study. Tris-KCl buffer had a tendency to decrease
enzyme activity as compared to phosphate buffer but phosphate buffer precipitated Zn$^{2+}$ at higher levels so that the buffer was chosen according to the parameter being studied. Homogenization was performed in an all-glass homogenizer and microsomes were isolated from the supernatant of 15,000 g centrifuged for 20 min as described in a previous paper (92).

In experiments with dialyzed microsomes, an aliquot of microsomal fraction homogenized in 0.01 M phosphate buffer, pH 7.0, was dialyzed for 2 hr at 4° against 5 mM EDTA, 1 mM 1,10-phenanthroline, pH 7.0, and then against 0.01 M phosphate buffer for 24 hr. The pH of the microsomal fraction after dialysis was adjusted to pH 7.0.

NADPH Oxidation

The rate of NADPH oxidation was recorded at 340 nm at 32° on a Beckman Acta III spectrophotometer in quartz cuvettes. The reaction vessel contained 141.7 mM sucrose and 96 μM NADPH (Sigma) in either Tris-KCl or phosphate buffer. Enzyme activity was initiated by adding 1-6 mg of microsomal protein in a final volume of 3 ml. MnCl$_2$, MgCl$_2$, or ZnCl$_2$ was added as indicated in Results of this chapter. The initial velocity of the oxidation was obtained in min 1 of the reaction, since the rate tended to decrease with time.

Microsomal Drug Oxidases

The microsomal fraction for the determination of microsomal drug-metabolizing activity and glucose 6-phosphatase activity was prepared as follows: livers were perfused in vitro as described,
homogenized in 1:3 (w/v) of Tris-KCl buffer (50 mM Tris·HCl-154 mM KCl, pH 7.4), and the homogenate was centrifuged at 15,000 g for 20 min. The resulting supernatant was then centrifuged at 105,000 g for 60 min to obtain the microsomal pellet. This pellet was gently rehomogenized in 1.5 vol of the same buffer. All the above operations were carried out at 2°. The protein content of the microsomal suspension was determined by the method of Lowry et al. (93).

The microsomal metabolism of ethylmorphine and aniline was determined in 3.0-ml mixture consisting of: 5 mM MgCl₂, 12 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase (Sigma), 0.33 mM NADP, 50 mM Tris-KCl buffer (pH 7.4), 6 mg of microsomal protein, and 10 mM ethylmorphine or 1 mM aniline. The mixture was incubated at 37° for 10 min with shaking, after which the reactions were terminated with 1 ml of 15% ZnSO₄ (ethylmorphine) or with 0.8 g NaCl and 25 ml ether (aniline). The degree of N-demethylation was estimated by measuring the amount of formaldehyde formed according to the method of Nash (94). Aniline hydroxylase was assayed according to Smuckler, Arrhenius, and Hultin (95).

In order to insure that Zn²⁺ was not selectively precipitating the microsomal enzymes, the activity of microsomal glucose 6-phosphatase was determined by incubating 8 μmoles glucose 6-phosphate with 1 mg of microsomal protein in 0.2 ml of 50 ml Tris-KCl buffer (pH 7.4). The procedure employed was essentially that described by Harper (96) with a microsomal suspension being substituted for filtered liver homogenate.
The phosphate content was determined by the colorimetric method of Fiske and Subbarow (97) utilizing the Fisher Gram-Pac (A0974).

Cytochrome P-450

Cytochrome P-450 was determined spectrophotometrically (95) and calculated using the extinction coefficient of 91 mM$^{-1}$ cm$^{-1}$ (98).

Metal Analysis

This analysis was done on an atomic absorption spectrophotometer, Perkin Elmer model 305, after digesting the liver sample initially with concentrated nitric acid followed by digestion in equal parts of nitric acid and 30% hydrogen peroxide. The digest was evaporated to dryness, the residue was diluted in deionized distilled water, and appropriate aliquots were taken for the analysis of zinc, iron, manganese, and magnesium.

Results

A linear relation was found between the rate of formation of NADP and the protein content of liver microsomes within the concentration of 1-6 mg proteins in 3 ml medium. Further work reported was carried on within this concentration range of microsomal fraction.

Effect of Zinc on NADPH Oxidation

This effect was studied in a microsomal fraction of the liver at various concentrations of zinc and at 7.2, 14.4, and 28.8 mM NADPH added to the microsomes, suspended in 0.04 M phosphate buffer. The rate of NADP formation in the system was related to the amount of NADPH added.
At any concentration of NADPH its oxidation was inhibited by zinc in a concentration-related manner. The $K_s$ value of this reaction was 6.45 mM NADPH. These data presented in Lineweaver-Burk double-reciprocal plots (Fig. 5) show a competitive mechanism for Zn$^{2+}$ inhibition. With a $1/V$ intercept of 0.097, the $V_{\text{max}}$ for the reaction was 10.3 nmoles NADP/min/mg of proteins. The $K_i$ was calculated to be 7.22 μM Zn$^{2+}$.

There are several possible mechanisms by which Zn$^{2+}$ could inhibit NADPH oxidation. Direct interaction with NADPH may render this pyridine nucleotide more resistant to oxidation. Another possibility is the interference of Zn$^{2+}$ with certain metals involved in the microsomal electron transport system. Finally, the inhibition of NADPH oxidation by Zn$^{2+}$ may reflect the interference of this metal with enzymes at any step of the microsomal electron transport chain. In the next section, the results referring to the last two mentioned hypotheses will be presented. The first hypothesis is under investigation.

Interaction of Zn$^{2+}$ with Other Metals

We tested the possibility that zinc displaces some metals involved in the microsomal electron transport system. It was shown that the granular fraction of polymorphonuclear leucocytes (84,99) and macrophages (100,101) contains an NADPH oxidase which is strongly activated by Mn$^{2+}$ ions (99,101). In a recent paper, it was reported that Zn$^{2+}$ inhibited NADPH oxidase from pulmonary alveolar macrophages in a competitive manner (101). In liver microsomes, it is the activity of cytochrome c reductase which functions as NADPH oxidase.
Fig. 5. Lineweaver-Burk curve of 1/substrate vs 1/velocity based on the initial rate of enzyme activity at four different Zn$^{2+}$ concentrations. -- NADPH oxidation was measured at 340 nm in a cuvette containing 22.5 mM KH$_2$PO$_4$, 13.3 mM Na$_2$HPO$_4$, 141.7 mM sucrose, and the amounts of NADPH and Zn$^{2+}$ given in the figure; 2.0 mg of microsomal proteins were added to initiate the reaction at 32°. The 1/V$_{\text{max}}$ value is 0.097 (nmole NADPH/min/mg of protein)$^{-1}$. The 1/K$_S$ value is 0.155 (mM NADPH)$^{-1}$. 
The studies on the role of Mn\(^{2+}\) in microsomal NADPH oxidation were done with a microsomal fraction dialyzed for 2 hr against 1 mM, 1,10-phenanthroline and 5 mM EDTA at pH 7.0 and then for another 24 hr at 4\(^\circ\) against 0.01 mM phosphate buffer, pH 7.0. The change in the content of zinc, iron, magnesium, and manganese in microsomal fraction due to dialysis is shown in Table 1. Only a minimal amount of iron (less than 9 percent of total iron) was removed by dialysis. After the second dialysis, the content of Mn\(^{2+}\) decreased from the original 3.2 to 1.6 \(\mu\)g/g of microsomal protein. Thus, only 50 percent of manganese was removed by dialysis. Further addition of Mn\(^{2+}\) up to 0.43 mM final concentration resulted in a linear increase of the rate of NADPH oxidation (Table 2). No effect of added Mg\(^{2+}\) within the same concentration range was observed. Surprisingly, addition of small amounts of Zn\(^{2+}\) (up to 5 \(\mu\)M final concentration) to the EDTA-treated and extensively dialyzed microsomal fraction stimulated the oxidation of NADPH (Fig. 6). Within a range of concentrations of 5-30 \(\mu\)M Zn\(^{2+}\), an inhibition of NADPH oxidation was found in two independent experiments. The pertinent data from our experiments are shown in Fig. 6.

The differing effect of increasing concentrations of zinc ions on NADPH oxidation in nondialyzed and dialyzed microsomes (Fig. 6) was suggestive of possible interaction between Zn\(^{2+}\) and Mn\(^{2+}\). Further analysis of this effect indicated rather complex relations between both metals. Table 3 represents the results of one typical experiment, which has been reproduced in three similar experiments. The data show that increasing Zn\(^{2+}\) concentration in a dialyzed sample from 3.3 to 13.3 \(\mu\)M
Table 1. Content of some metals in intact and CaNa₂EDTA dialyzed rat liver microsomes.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe (µg/g protein)</th>
<th>Zn (µg/g protein)</th>
<th>Mn (µg/g protein)</th>
<th>Mg (µg/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondialyzed</td>
<td>878</td>
<td>95</td>
<td>3.2</td>
<td>452</td>
</tr>
<tr>
<td>Dialyzed</td>
<td>800</td>
<td>63</td>
<td>1.6</td>
<td>167</td>
</tr>
<tr>
<td>% Dialyzed out</td>
<td>9</td>
<td>30</td>
<td>50</td>
<td>63</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Liver microsomes (250 mg protein) were dialyzed for 2 hr against 5 mM EDTA and 1 mM phenanthroline, pH 7.0, at 4° and then 0.01 M PO₄ buffer, pH 7.0, for 24 hr at 4°. Metal content was determined by atomic absorption. Data are presented/g protein of microsomal fraction.

Table 2. Rate of oxidation of NADPH in EDTA-treated and dialyzed liver microsomes in the presence of various divalent cations.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Mn\textsuperscript{2+} or Mg\textsuperscript{2+} concn (mM)</th>
<th>Zn\textsuperscript{2+} concn (µM)</th>
<th>Mn\textsuperscript{2+} (nmole/min/mg protein)</th>
<th>Mg\textsuperscript{2+}</th>
<th>Zn\textsuperscript{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0</td>
<td>29</td>
<td>29</td>
<td>19.7</td>
</tr>
<tr>
<td>0.10</td>
<td>3.33</td>
<td>50</td>
<td>29</td>
<td>37.5</td>
</tr>
<tr>
<td>0.33</td>
<td>10.00</td>
<td>157</td>
<td>30</td>
<td>24.0</td>
</tr>
<tr>
<td>0.43</td>
<td>13.33</td>
<td>169</td>
<td>29</td>
<td>20.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Liver microsomes were dialyzed as is described in Methods. NADPH oxidation was initiated by 2.0 mg of microsomal proteins being added to a cuvette containing 22.5 mM KH₂PO₄, 13.3 mM Na₂HPO₄, 141.7 mM sucrose, 96 µM NADPH and the above metals at 32°. The pH value of the incubation medium was 7.4. Indicated metal concentration refers to exogenous amount of single metal added to the microsomal sample.
Fig. 6. Effect of Zn$^{2+}$ additions on dialyzed and nondialyzed liver microsomal fractions in phosphate buffer. -- NADPH oxidation was initiated by 1.5 mg protein of microsomal preparation being added to a cuvette containing 22.5 mM KH$_2$PO$_4$, 13.3 mM Na$_2$HPO$_4$, 141.7 mM sucrose, 96 µM NADPH, and the given concentrations of Zn$^{2+}$. The nondialyzed liver microsomes contained 3.5 µg Zn$^{2+}$/g protein and the dialyzed sample contained 2.5 µg Zn$^{2+}$/g protein as determined by atomic absorption.
without exogenous Mn$^{2+}$ resulted in inhibition of NADPH oxidation. When 0.1 mM Mn$^{2+}$ was added, the effect was just the opposite; nevertheless, at 0.3 mM Mn$^{2+}$, the oxidation of NADPH was again inhibited with increasing Zn$^{2+}$. The analysis of the kinetic data suggests that the mechanism involving various metal ions in NADPH oxidation is too complex for rigorous treatment at the present time.

Table 3. Rate of oxidation of NADPH in dialyzed microsomes at various concentrations of Zn$^{2+}$ or Mn$^{2+}$.a

<table>
<thead>
<tr>
<th>Zn$^{2+}$ (μM)</th>
<th>Mn$^{2+}$ (mM)</th>
<th>NADP (nmoles/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.1</td>
<td>20.8</td>
</tr>
<tr>
<td>3.33</td>
<td></td>
<td>17.5</td>
</tr>
<tr>
<td>10.00</td>
<td>28.5</td>
<td>35.1</td>
</tr>
<tr>
<td>13.33</td>
<td>42.8</td>
<td>25.6</td>
</tr>
</tbody>
</table>

aMicrosomal fraction was dialyzed as described in Methods. NADPH oxidation was measured in a cuvette containing 22.5 mM KH$_2$PO$_4$, 13.3 mM Na$_2$HPO$_4$, 141.7 mM sucrose, 96 μM NADPH, the above amounts of metals and 1-6 mg of microsomal proteins, initiating the reaction at 32°. The pH value of the medium was 7.4. The indicated concentrations of individual metals refer to amounts added to the sample in excess of their content left after dialysis. The oxidation rate was based on min 1 of the reaction.

Effect of Zinc on Some Enzymes of Microsomal Electron Transport

To test the hypothesis that zinc interacts directly with some enzyme components of liver microsomal electron transport, we studied the
Effect of this metal cation on cytochrome P-450. Supplementation of Zn$^{2+}$ at two different concentrations (1 and 100 µM) to isolated microsomal fraction incubated under slight shaking in Tris-KCl buffer for 10 or 40 min at 25° did not result in any change of spectral characteristics of cytochrome P-450. The characteristic maximum at 450 nm as well as the shoulder at 420 nm was identical in all samples analyzed.

Effect of Zinc on the Activity of Some Oxidases in Liver Microsomes

Inhibition of NADPH oxidation in liver microsomes by zinc, as documented above, should result in inhibition of drug oxidation. To experimentally verify this statement, we studied the effect in vitro of Zn$^{2+}$ in a final concentration of 5-150 µM on the NADPH-dependent microsomal oxidation of aniline and N-demethylation of ethylmorphine. The data in Fig. 7 indicate the inhibition of aniline hydroxylase activity by zinc, 50 percent inhibition being obtained at 60-70 µM zinc concentrations. These data are similar to the previously published data for ethylmorphine N-demethylation (91). Under similar experimental conditions, the activity of glucose 6-phosphatase, an enzyme independent of NADPH oxidation, was not affected by the presence of Zn$^{2+}$ (Fig. 7).

Figure 8 presents data on the effect of zinc on the activity of aniline hydroxylase as well as ethylmorphine N-demethylase by plotting the reciprocal rates for velocity of product formation as a function of zinc concentration. The slopes of linearly transformed data clearly indicate the inhibitory effect of zinc on both enzymes dependent on NADPH.
Fig. 7. Effect of Zn\(^{2+}\) on the activity of aniline hydroxylase and glucose 6-phosphatase in liver microsomes. -- Aniline (1 mM) was incubated for 10 min in a 3-ml incubation medium (pH 7.4) consisting of 5 mM MgCl\(_2\), 12 mM glucose 6-phosphate, 1 unit glucose 6-phosphate dehydrogenase, 0.33 mM NADP, 6 mg of microsomal protein, 50 mM Tris, and 154 mM KCl. Glucose 6-phosphatase activity was determined by incubating glucose 6-phosphate with microsomes suspended in Tris-KCl and the Pi determined by the colorimetric procedure of Fiske and Subbarow (97). Each point represents the mean of two incubations.
Fig. 8. Reciprocal plot of specific activity of aniline hydroxylase and ethylmorphine N-demethylase as a function of Zn$^{2+}$ concentration. Aniline (1 mM) or ethylmorphine (10 mM) was incubated for 10 min in 3 ml medium (pH 7.4) consisting of 5 mM MgCl$_2$, 12 mM glucose 6-phosphate dehydrogenase, 0.33 mM NADP, 6 mg of microsomal protein, 50 mM Tris and 154 mM KCl. The rate of reaction was determined by measuring the product of each reaction: HCHO for ethylmorphine N-demethylase and p-aminophenol for aniline hydroxylase. Each point is the mean of two incubations.
To ascertain if various anions of zinc compounds play any role in the inhibitory effect of Zn\(^{2+}\), we studied the effect of four zinc salts at 0.1 mM concentration on the activity of ethylmorphine N-demethylase in liver microsomes. Our results indicate that zinc gluconate was the least inhibitory (50 percent inhibition) and zinc acetate the most inhibitory (63 percent) when initial rates of product formation were followed. Zinc chloride and zinc sulfate inhibited by 50 and 56 percent, respectively.

**Discussion**

The aim of this study was to explain the possible mechanism(s) by which zinc administered *in vivo* protects the liver against CCl\(_4\) hepatotoxicity (75,76). The results of this study *in vitro* indicate that zinc inhibits the oxidation of NADPH in liver microsomes by a competitive mechanism. A logical consequence of blocking NADPH oxidation in the presence of Zn\(^{2+}\) would be inhibition of all microsomal reactions dependent on the transfer of electrons from NADPH. The activity of two NADPH-dependent microsomal enzymes, i.e., aniline hydroxylase and ethylmorphine N-demethylase, was inhibited by the addition of Zn\(^{2+}\) to the incubation medium. Another enzyme, bound also to endoplasmic reticulum, but independent of NADPH oxidation, i.e., glucose 6-phosphatase, was not affected by zinc ions. This finding indicates that the described effect of Zn\(^{2+}\) on NADPH oxidation or the metabolism of two drugs was not related to eventual microprecipitation of microsomal proteins by Zn\(^{2+}\), but to specific interaction of the metal with some components of the drug-oxidizing system. Thus, the decreased oxidation of some drugs
metabolized to a hepatotoxic product might explain the protective effect of zinc on carbon tetrachloride-induced liver damage (75,76).

Zinc is obviously not the only metal inhibiting microsomal electron flow. Mn$^{2+}$ and Co$^{2+}$ were shown to inhibit peroxidation of phospholipids in liver microsomes (102), possibly by competing with Fe$^{3+}$ for binding sites on the microsomes (81). It has to be stressed, however, that contrary to the Zn$^{2+}$ effect both Mn$^{2+}$ and Co$^{2+}$ were effective at mM concentrations, while Zn$^{2+}$ was inhibitory at µM concentrations. While this study suggests that increase in zinc content in liver microsomes may reduce hepatic drug metabolism, a similar effect was reported by an opposite situation, i.e., by zinc deficiency (103). Furthermore, despite the complexity of Zn$^{2+}$ and Mn$^{2+}$ interaction in the tested system, the data do not suggest an additive or synergistic effect.

It would be premature to speculate on the possible biological implications of this finding. A direct proof should be presented first that such an inhibition of drug metabolism and eventually lipid peroxidative deterioration of polyunsaturated fatty acids by zinc occur also after administration in vivo of this metal. The already mentioned protection of CCl$_4$ hepatotoxicity by zinc in rats supports such an assumption (75,76).
CHAPTER 4

EVIDENCE FOR THE FORMATION OF THE BINUCLEAR COMPLEX, Zn₂-NADPH

We have shown that zinc ions inhibit the enzymatic microsomal electron transport chain essential to the drug oxidizing system of liver cells in vitro (91,104). The mechanism of this inhibition is unknown. Since oxidation of NADPH¹ is the initial reaction of the system, we investigated the possibility that binding of zinc to NADPH could be involved in this inhibition.

Results of other studies have indicated interactions of zinc with related nucleotides. Zinc forms stable complexes with 5'-AMP (105), ADP (106), and ATP (107,108). NADH was found not to bind zinc (109,110). All of these nucleotides contain adenine and phosphate moieties and all except NADH bind with zinc.

Since the relative stability of some zinc-nucleotide complexes appears to increase with increasing numbers of phosphate groups in the nucleotide (110), we hypothesized that while zinc might not bind to NADH it could bind NADPH as the latter compound contains an additional phosphate group. In this study, we demonstrate that zinc does, in fact, bind to NADPH and we suggest a structure for the resulting zinc-NADPH binuclear complex.

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**Materials**

NADPH (tetrasodium salt, 99% pure) and NADH (disodium salt, 98% pure) were obtained from Sigma Chemical Co. ZnCl$_2$ (ultrapure) was packed under argon from Alfa Inorganics. Sephadex G-10 was obtained from Pharmacia and D$_2$O from Bio Rad Laboratories. All experiments were performed in 0.05 M Tris-HCl buffer, 0.154 M KCl, pH 7.4 or 0.01 M Hepes, 0.154 M KCl, pH 7.2 as is indicated in the experiment.

**Methods**

The interaction of zinc ions with NADPH and NADH was studied by four methods.

**Equilibrium Gel Filtration**

Equilibrium gel filtration was used to study the binding of zinc ions to NADPH and NADH as described by Mantoura and Riley (111). This method was chosen instead of a titration technique because: (1) we did not know the number of metal ions bound to each pyridine nucleotide molecule, (2) the nucleotide bound to the metal ion could not be distinguished from free nucleotides, and (3) the limited solubility of zinc hydroxide. Zinc and pyridine nucleotide equilibrium gel filtration was carried out with a Sephadex G-10 column (2x100 cm) in 0.01 M Hepes buffer, 0.154 M KCl, pH 7.2, and flow rate of 0.5 ml per min maintained by a peristaltic pump. After equilibrating a Sephadex G-10 column with a buffered solution containing a known concentration of the zinc ion of interest (see Results), 13.3 μmoles of pyridine nucleotide in 0.2 ml of buffer was added to the column and eluted with the zinc solution used
for equilibrating the column. Fractions of approximately 1 ml were collected and NADPH, NADH, and zinc were measured. NADPH and NADH concentrations were obtained by measurement of their absorbances with a Beckman Acta III spectrophotometer at 340 nm using the experimentally determined extinction coefficient of 5.456 cm$^{-1}$ mM$^{-1}$. This value is approximately equal to that reported by Hrycay and O'Brien (112). Zinc ion content in the eluate was measured by atomic absorption in a Perkin Elmer Model 305A spectrophotometer. Recovery of the pyridine nucleotides from the column was at least 87% in all experiments.

The binding of zinc to NADPH was studied in Tris-HCl and Hepes buffers since Hanlon, Watt, and Westhead (113) have shown an interaction of Tris-HCl buffer with zinc ion. Our studies also suggest an interaction since ZnCl$_2$ was soluble in Tris-HCl buffer beyond that indicated by the solubility product of Zn(OH)$_2$ at pH 7.4. This suggested a zinc-tris(hydroxymethyl)aminomethane complex being present which was soluble. Hepes buffer at pH 7.2 was chosen because of no apparent interaction with the zinc, i.e., ZnCl$_2$ solution in Hepes did not exceed the solubility product of Zn(OH)$_2$.

Ultraviolet Spectrum

The ultraviolet spectrum of a solution of 96 µM NADPH was recorded in the range of 220-400 nm in the presence of 13.3 µM ZnCl$_2$, as in Bruice and Benkovic's study (109) with NADH and zinc. NADPH and ZnCl$_2$ were made up in 0.05 M Tris-HCl buffer, 0.154 M KCl, pH 7.4.
Fluorescence Spectrum

The fluorescence spectrum of NADPH at 1 μM with and without 1.25 μM ZnCl₂ was recorded with excitation of 345 nm and emission at 460 nm. The zinc and NADPH were in 0.05 M Tris-HCl buffer, pH 7.4, and measurements were made on an Aminco-Bowman fluorophotometer.

Nuclear Magnetic Resonance

Nuclear magnetic resonance studies of NADPH and NADH were performed on a Varian CFT-20 spectrometer at 32 MHz for ³¹P. 32 μmoles of the pyridine nucleotides were recorded in 10 ml of 0.05 M Tris-HCl buffer, pH 7.4, 20% D₂O, and zinc additions of 40 μmoles.

Acid Dissociation Constants

The acid dissociation constants of the phosphate groups of NADPH were determined by acid titration of the tetrasodium salt of NADPH. 34 μmoles of the NADPH in 20 ml of 0.157 M NaNO₃ were titrated with 0.1 M HCl. Titrations were performed at 25° under a nitrogen atmosphere. The glass electrode saturated calomel electrode pair was calibrated at pH 6.86 and pH 3.56 with National Bureau Standard buffers.

Results

A characteristic elution pattern of NADPH from the Sephadex G-10 column equilibrated with ZnCl₂ in Hepes buffer is shown in Fig. 9. The elution pattern indicates that zinc binds to NADPH. The elution patterns of NADH under identical conditions indicate that zinc binding does not occur.
Fig. 9. Elution pattern of zinc and NADPH in 0.01 Hepes buffer, 0.154 M KCl, pH 7.2, from Sephadex G-10 column. -- The column was equilibrated with 111 μM ZnCl₂ (●) in Hepes buffer and 13.3 μmoles of NADPH (○) was added and eluted with the zinc solution. Recovery rate of the NADPH was 87%.
In order to determine the number of zinc ion binding sites and the association constants of the zinc-NADPH complex, gel filtration columns were equilibrated at different concentrations of zinc ion. Analysis of the binding data was based on the equilibrium equations:

\[
\text{NADPH} + Zn^{2+} \rightleftharpoons k_{1,\text{app.}} \text{NADPH}\cdot Zn
\]  
[1]

\[
\text{NADPH}\cdot Zn + Zn^{2+} \rightleftharpoons k_{2,\text{app.}} \text{NADPH}\cdot Zn_2
\]  
[2]

The apparent association constants \(k_{1,\text{app.}}\) and \(k_{2,\text{app.}}\) at pH 7.2, and ionic strength 0.157 M, can be written as

\[
k_{1,\text{app.}} = \frac{[\text{NADPH}\cdot Zn]}{[\text{NADPH}][Zn^{2+}]} \quad [3]
\]

\[
k_{2,\text{app.}} = \frac{[\text{NADPH}\cdot Zn_2]}{[\text{NADPH}\cdot Zn][Zn^{2+}]} \quad [4]
\]

where \([\text{NADPH}]\) represents all forms of the uncomplexed ligand in moles/liter as in eq. 5:

\[
[\text{NADPH}] = [H_4L] + [H_3L^-] + [H_2L^{2-}] + [HL^3-] + [L^4-]
\]  
[5]

Equation 5 is pH-dependent and is taken into account later in the calculations. \([Zn^{2+}]\) represents the free molar concentration of \(Zn^{2+}\), and \([\text{NADPH}\cdot Zn]\) and \([\text{NADPH}\cdot Zn_2]\) are the molar concentrations of the complexed species.
A function \( \bar{\nu} \) is defined as the number of moles of zinc bound per mole of total NADPH, \([\text{NADPH}_T]\):

\[
\bar{\nu} = \frac{\text{(moles of Zn}^{2+} \text{ bound)}}{\text{(moles of NADPH}_T)}
\]

where

\[
[\text{NADPH}_T] = [\text{NADPH}] + [\text{NADPH} \cdot \text{Zn}] + [\text{NADPH} \cdot \text{Zn}_2]
\]

Substitution of eqs. 3, 4, and 7 into eq. 6 gives:

\[
\bar{\nu} = \frac{k_1[Zn^{2+}] + 2k_1k_2[Zn^{2+}]^2}{1 + k_1[Zn^{2+}] + k_1k_2[Zn^{2+}]^2}
\]

Rearrangement of this equation gives:

\[
\left[\frac{[Zn^{2+}]^2(\bar{\nu} - 2)}{\bar{\nu}}\right] + \frac{1}{k_2} \left[\frac{[Zn^{2+}]}{\bar{\nu}} (\bar{\nu} - 1)\right] + \frac{1}{k_1k_2} = 0
\]

which is the equation for a straight line with the slope equal to \( \frac{1}{k_2} \) and the y-intercept equal to \( -\frac{1}{k_1k_2} \). When the observed gel filtration data (Table 4) are plotted according to eq. 9, the resulting straight line (Fig. 10) fits the assumption that two moles of zinc are bound per mole of NADPH. The values of \( k_1, \text{app.} \) and \( k_2, \text{app.} \) obtained from a least squares analysis of the data are \( 10^{3.73} \) and \( 10^{2.99} \), respectively.
Table 4. Determination of $\bar{v}$ from Sephadex G-10 columns.$^a$

<table>
<thead>
<tr>
<th>Zn (M)</th>
<th>$\bar{v}$ (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$8.0 \times 10^{-6}$</td>
<td>$0.0212 \pm 0.00025$</td>
</tr>
<tr>
<td>$8.6 \times 10^{-6}$</td>
<td>$0.0310 \pm 0.00035$</td>
</tr>
<tr>
<td>$9.6 \times 10^{-6}$</td>
<td>$0.0433 \pm 0.00025$</td>
</tr>
<tr>
<td>$9.7 \times 10^{-6}$</td>
<td>$0.0410 \pm 0.00030$</td>
</tr>
<tr>
<td>$11.1 \times 10^{-6}$</td>
<td>$0.0648 \pm 0.00028$</td>
</tr>
</tbody>
</table>

$^a$Columns were equilibrated with the above concentrations of zinc ion and NADPH eluted as described in Methods. $\bar{v}$ was determined as described in the text.
Fig. 10. Graph of the experimental data from the Sephadex G-10 column run five times each time in one of five different concentrations of zinc ion in Hepes buffer. -- o = 80 µM, • = 86 µM, ▲ = 97 µM, △ = 96 µM, and □ = 111 µM. Derivation of the straight line equation is presented in the text. Least squares analysis has a correlation coefficient of 0.993 and $k_{1,\text{app.}} = 10^{5.76}$ and $k_{2,\text{app.}} = 10^{2.99}$. 
To determine whether the binding of zinc to NADPH involved the chloride ions in the solution, the column was run under the same conditions except that Zn(NO$_3$)$_2$ replaced ZnCl$_2$ and NaNO$_3$ replaced KCl. Also, the possibility of the Hepes buffer reacting with the zinc was tested by running the column with the buffer concentration increased by a factor of ten. Values for $\bar{v}$ from these columns fit the straight line plot as in Fig. 10. The column with Tris-HCl buffer instead of Hepes buffer at the same zinc concentration also resulted in the same elution pattern as Fig. 9, indicating that the zinc-tris(hydroxymethyl)aminomethane complex did not change the metal-nucleotide complexes.

To determine the zinc binding sites of NADPH, the ultraviolet spectra of the compounds were recorded. Scanning of NADPH and NADH from 220-400 nm with and without the addition of zinc resulted in identical maxima at 260 nm and 340 nm. This agrees with Bruice and Benkovic's (109) observations that zinc caused no shift in the UV spectrum of NADH. Also, the fluorescence spectrum of NADPH with zinc gave maxima identical with those obtained with NADPH alone, at an excitation wavelength of 346 nm and emission at 460 nm. These maxima are similar to those observed by Elevitch (114) with NADH. Thus, our results indicate that zinc does not cause any perturbations in the fluorescence spectrum of NADPH.

The possibility of zinc binding to the phosphate moieties of NADPH was investigated by doing the $^{31}$P NMR spectra of NADPH and NADH with and without zinc additions. The spectra indicate binding of zinc to NADPH but not NADH. As seen in Fig. 11a, the peak at 5 ppm is due to
Fig. 11. $^{31}$P NMR spectrum of 3.2 mM NADPH. -- (a) Tris-HCl buffer, 20% D$_2$O, pH 7.4; 15,242 transients, 0.511 sec acquisition time, 10 µsec pulses with broad band proton decoupling. (b) 4 mM ZnCl$_2$ in Tris-HCl buffer, 20% D$_2$O, pH 7.4; 11,427 transients, 0.511 sec acquisition time, 10 µsec pulses with broad band proton decoupling.
the diphosphates, while the peak at 20 ppm is due to the phosphate on
the 3-carbon of the adenine ribosyl portion of NADPH. The spectrum of
NADPH with zinc (Fig. 11b) indicates that zinc causes a shift of the
phosphate peak at 20 ppm and a slight narrowing of the diphosphate peak
at 5 ppm.

Since the binding sites of zinc to NADPH involve the phosphate
moieties of NADPH, the association constants of the four phosphate groups
were determined. The titration curve of the tetrasodium of NADPH with
HCl is shown in Fig. 12. Also shown in Fig. 12 is the Gran plot
analysis (115) of the pH-volume data obtained from the titration. By
plotting the data as described by Seymour and Fernando (115), the slope
of the Gran plot is \(-\frac{1}{K_4}\) and the interaction with the x-axis is the
volume at the equivalence point. The titration was carried out at the
same ionic strength as the gel filtration (0.157 M) and the value of \(pK_4\)
was found to be 6.01. The first three pK values were found to be
several orders of magnitude less than \(pK_4\) and were not calculated.
Since the binding studies were performed at pH 7.2, the only significant
proton interactions of NADPH will be:

\[
\begin{align*}
\text{adenine} & \quad \text{adenine} \\
\text{O--CH}_2\text{--ribose} & \quad \text{O--CH}_2\text{--ribose} \\
0 = P--O^- & \quad 0 = P--O^- \\
0 & \quad 0 \quad 0 = P--OH \\
0 = P--O^- & \quad 0 = P--O^- \\
0 & \quad 0 \quad 0 = P--OH + H^+ \\
0--CH_2 & \quad 0--CH_2 \\
\text{nicotinamide} & \quad \text{nicotinamide} \\
\text{ribose} & \quad \text{ribose}
\end{align*}
\]
Fig. 12. Acid titration of the tetrasodium salt of NADPH and the Gran plot derived from the acid titration curve. -- o = acid titration and • = Gran plot.
or as shown in eqs. 10 and 11:

$$\text{HL}^3^- \overset{K_4}{\leftrightarrow} \text{L}^4^- + \text{H}^+ \quad [10]$$

$$K_4 = \frac{[\text{H}^+][\text{L}^4^-]}{[\text{HL}^3^-]} \quad [11]$$

The other three protonated species are stronger acids and they will not be present in significant amounts at pH 7.2.

The apparent association constants determined by the gel filtration are conditional constants, dependent on the pH and the ionic strength of the solution (eq. 5). The true formation constants are expressed by:

$$\text{L}^4^- + \text{Zn}^{2+} \overset{k_1}{\leftrightarrow} \text{ZnL}^2^- \quad [12]$$

$$\text{ZnL}^2^- + \text{Zn}^{2+} \overset{k_2}{\leftrightarrow} \text{Zn}_2\text{L} \quad [13]$$

$$k_1 = \frac{[\text{ZnL}^2^-]}{[\text{L}^4^-][\text{Zn}^{2+}]} \quad [14]$$

$$k_2 = \frac{[\text{Zn}_2\text{L}]}{[\text{ZnL}^2^-][\text{Zn}^{2+}]} \quad [15]$$

From eq. 14, $k_1$ is determined by the amount of ligand in the $\text{L}^4^-$ form, and not uncomplexed NADPH as shown in eq. 3. However, $\text{L}^4^-$ is related to the uncomplexed NADPH concentration by the expression:
\[ [L^4^-] = \alpha_4 [\text{NADPH}] \quad [16] \]

where \( \alpha_4 \) is the fraction of uncomplexed NADPH in the \( L^4^- \) form. Recalling that at pH 7.2 the only important ionic species of NADPH are the \( HL^3^- \) and \( L^4^- \), \( \alpha_4 \) may be written as:

\[
\alpha_4 = \frac{[L^4^-]}{[HL^3^-] + [L^4^-]} \quad [17]
\]

Substitution of eq. 11 into eq. 17 gives:

\[
\alpha_4 = \frac{K_4}{[H^+] + K_4} \quad [18]
\]

Since the value of \( K_4 \) and pH are known, the value for \( \alpha_4 \) is 0.93 or \( 10^{-0.033} \). The relationship between the apparent \( k_1 \) and the true \( k_1 \) can be shown by substituting eq. 16 into eq. 14:

\[
k_1 = \frac{[ZnL^2^-]}{[Zn^{2+}] \alpha_4 [\text{NADPH}]} \quad [19]
\]

Rearranging eq. 19 and substitution from eq. 3 gives:

\[
k_1 = \frac{k_{1,\text{app.}}}{\alpha_4} \quad [20]
\]

\( k_{1,\text{app.}} \) of the Zn-NADPH complex from Fig. 10 is \( 10^{3.73} \); therefore, \( k_1 \) from eq. 20 is \( 10^{3.76} \). It is seen that \( k_2 \) is not dependent on the
concentration of $L^4$ so that the value of $k_2$ obtained from the linear least squares analysis is $10^{2.99}$.

**Discussion**

Zinc binds to NADPH to form a mononuclear complex and a binuclear complex. The formation constant of the mononuclear complex is $10^{3.76}$ at an ionic strength of 0.157 M. The formation constant of the binuclear complex is $10^{2.99}$. The zinc binding sites of NADPH were studied by examining the ultraviolet, fluorescence, and $^{31}$P NMR spectra in the presence and absence of zinc ion.

The addition of zinc ion did not change the UV absorption maxima of NADPH at 340 and 260 nm. These maxima have been attributed to the reduced form of the nicotinamide moiety and the adenine portion, respectively. Hence, zinc does not bind at these sites and does not cause any changes in the electron distribution in these groups. This agrees with Iweibo and Weiner (116), who showed that zinc does not bind to the adenine moiety of NADH. The fluorescence spectra of NADPH which arises from the reduced form of the nicotinamide moiety of the molecule is also not influenced by the addition of zinc ion and indicates that zinc is not bound to any of the donor atoms in the nicotinamide group. Both the UV and fluorescence spectra support Takahashi and Harvey's (117) studies which report that neither nicotinamide nor the adenine portions of NADH interact with the zinc atoms in alcohol dehydrogenase.

The $^{31}$P NMR studies show that zinc forms complexes with NADPH and not with NADH. Furthermore, the spectra indicate that zinc interacts with the monophosphate group on the 3'-carbon on the adenine ribosyl
portion of NADPH. Similar zinc-ribosyl phosphate interactions have been reported with AMP (105), RNA (118), and inosine 5'-monophosphate (119). Weitzel and Spehr (110) theorized that the stability of the zinc-nucleotide complex increased as a function of the number of phosphate groups present which is supported by this study. Similar findings were reported by Rifkind and Eichhorn (105) with Zn-5'-AMP in which a phosphate-metal interaction as well as a phosphate-metal-adenine linkage is shown. Our studies with NADPH and NADH did not show adenine interactions with zinc.

The possibility of zinc binding to the chloride ions as indicated by McCall and Taylor (120) in the Zn-(9-methyl-adenine) complex was eliminated since the gel filtration with Zn(NO₃)₂ instead of ZnCl₂ did not change the elution characteristics.

Our results indicate zinc is bound to NADPH as shown in Fig. 13. In this model, the first zinc atom forms a linkage between the monophosphate and diphosphate moieties. The second zinc atom links the remaining oxygen of the monophosphate with the diphosphate to form a ten-membered ring structure. The ring is "puckered" as seen in Fig. 13 so that there is minimal stress. The overall formation constant of the binuclear complex is 10⁶.75. The formation of a ring will restrict the rotation of the adenine and nicotinamide groups which in turn may limit the availability of the reduced nicotinamide ring to enzyme oxidation. A similar effect where zinc stabilizes DNA (121) was reported. The evidence that zinc inhibits NADPH-dependent microsomal reactions (104) may be explained by the binding of zinc to NADPH, thereby making the complex
Fig. 13. Proposed scheme of zinc binding with NADPH based on the experimental results of two zinc moles bound to one NADPH molecule.
more resistant to enzymatic electron loss or hindering the formation of the enzyme-substrate intermediate. Since zinc interacts with the substrate this does not exclude the possibility of zinc ions interfering with some enzymes of the microsomal electron transport chain or other necessary metal ions of this system.
CHAPTER 5

GENERAL CONCLUSIONS AND FURTHER PROJECTIONS

The formation of the binuclear complex, Zn\(^{2+}\)-NADPH strongly indicates that zinc ions inhibit hepatic lipid peroxidation by preventing this pyridine nucleotide from functioning in the microsomal electron transport system. Evidence is presented in this study which shows that NADPH oxidase (cytochrome c reductase), aniline hydroxylase, and ethylmorphine demethylase are inhibited by zinc ions. These enzymes are NADPH-dependent, while glucose-6-phosphase, which is also bound to the endoplasmic reticulum but not dependent on NADPH, was not inhibited by zinc ions. Therefore, zinc ions are inhibiting the production of lipid peroxides.

Lipid peroxidation must first be initiated as in the microsomal electron transport chain, and then it must be perpetuated by producing free radicals as in the unsaturated lipids of the biomembranes. Zinc ions may be inhibiting hepatic lipid peroxidation by interacting with the phospholipids of the membranes, thus making them more resistant to peroxidative damage. The stabilization of lysosomes and erythrocytes by zinc ions from lipid peroxidation has been shown by Chvapil and coworkers (122-124). Results of several studies indicate that zinc ions increase the rigidity of model systems of phospholipids (125).
The research reported here only begins to explain the mechanism of zinc ions inhibiting hepatic lipid peroxidation, namely the microsomal electron transport system. Another aspect of zinc ions in lipid peroxidation which has been documented (122-124) must still be investigated, that is, the stabilization of biomembranes.

To complete the study of the mechanisms of the effect of zinc ions in hepatic lipid peroxidation, membrane stability will be investigated, and then these mechanisms which have been elucidated in the in vitro system will, in conclusion, be tested in the in vivo system.

Biomembrane stability will be studied by measuring the degree of "fluidity" of the membrane as a function of zinc ions. This will be done by measuring phospholipid exchange in the presence and absence of zinc ions. Transport of ions across the membrane will also be examined. The profile of polyunsaturated fatty acids of the membrane may also vary in the presence of zinc ions. Since the binding of zinc ions to the phosphate moieties is well known, the possibility of zinc ions interacting with the phospholipids of the membrane is very real.

The final proof of the mechanisms of zinc ions inhibiting hepatic lipid peroxidation will be in the animal studies in which conditions known to be due to hepatic lipid peroxidation will be tested on high zinc fed rats and normal zinc fed rats. Parameters of lipid peroxidative damage will then be measured. A model system of hepatic lipid peroxidation is carbon tetrachloride poisoning which has already been shown to be ameliorated by zinc administration (75-77). Other systems
to be investigated are ethanol-induced liver injury, oxygen toxicity, and radiation damage.

These studies which are outlined here, it is felt, are necessary to complete the research on the mechanisms of the effect of zinc ions in hepatic lipid peroxidation.
LIST OF REFERENCES


