# Effects of increased microsomal oxygen radicals on the function and stability of cytochrome P450 in dietary zinc deficient rats

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*It has been proposed that a biochemical function of Zn is maintenance of the membrane structure and function. Previous research has shown that dietary Zn deficiency causes a leakage of $H_2O_2$ from the NADPH-dependent P450 enzyme system. Microsomal membranes isolated from Zn-deficient rats are more susceptible to lipid peroxidation. The objectives of this study were to investigate the effects of dietary Zn deficiency on the function and stability of liver microsomal P450 in rats and to study the possible mechanisms involved. In rats fed the Zn-deficient diet, the concentration of total liver microsomal protein was unchanged, but P450 concentration and aminopyrine demethylase activity were significantly decreased compared with rats fed the Zn-adequate diet pair-fed or* ad libitum. *The decrease in P450 concentration was not accompanied by the appearance of P420. The decrease in enzyme activity was also reflected by impaired drug metabolism* in vivo *as indicated by increased pentobarbital-induced sleeping time, but a statistically significant difference was found only between the ZnDF and the ZnAL groups. However, membrane lipid peroxidation, as indicated by MDA concentration, was not affected by the dietary treatments. To understand the mechanism by which Zn deficiency causes reduced function and stability of P450, the results obtained from the microsomal membranes isolated from Zn-deficient rats were compared with the results obtained from the microsomal membranes treated with protein denaturants or free radical generating systems* in vitro, *or microsomal membranes isolated from rats challenged with $CCl_4$ or FeNTA* in vivo. *It is concluded that severe dietary Zn deficiency in rats caused a functional and structural impairment of liver microsomal P450 via a free radical medicated-like mechanism.*

**Keywords:** Zn deficiency; cytochrome P450 and P420; aminopyrine demethylase; MDA; free radical generating system/agent

## Introduction

It has been proposed that a biochemical function of Zn is maintenance of the membrane structure and function.[1] Dietary Zn deficiency has been shown to increase the susceptibility of rat hepatic microsomal membranes to lipid peroxidation both in vitro[2,3] and in vivo.[2] Bray et al.[4] reported that dietary Zn deficiency stimulates the production of carbon-centered free radicals in lung microsomes by an NADPH- and cytochrome P450-dependent system. Hammermueller et al.[5] demonstrated that Zn deficiency causes a leakage of $H_2O_2$ from the NADPH-dependent P450 enzyme system. In addition, dietary Zn deficiency causes a significant accumulation of Fe in the microsomes.[5] The consequences of increases $H_2O_2$ production from the P450 catalytic cycle and the accumulation of Fe in the microsomal membranes in the Zn deficient rats are still not clear. This reactive oxygen species may react with the Fe in the microsomal membranes to induce a free radical-mediated attack to either the microsomal proteins, such as cytochrome P450, or the membrane lipids that surround them, or both.

Cytochrome P450 (P450), a hemeprotein, is an essential component of the drug metabolizing enzyme system that is embedded in the endoplasmic reticulum. Both the integrity of P450 structure and of the mem-

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brane lipids surrounding P450 are crucial for the function of P450. Under the presence of a variety of chemical agents, P450 can be denatured to cytochrome P420 (P420).[6] This denaturation results in a loss of the catalytical property and a loss of the characteristic spectral absorption pattern of P450.[7]

The objectives of this study were to investigate the effects of dietary Zn deficiency on the function and stability of liver microsomal P450 in rats and to study the possible mechanisms involved. In this study, the "function" of P450 refers to the enzymatic capacity of microsomal P450 and in vivo drug metabolism that were measured by aminopyrine demethylase activity and pentobarbital-induced sleeping time, respectively. In contrast, the "stability" of P450 mainly implies the structural aspect that is indicated by total P450 concentration, P420 concentration, and total microsomal protein concentration. Malondialdehyde (MDA) concentration was used to indicate the role of membrane lipid peroxidation on the effects of dietary Zn deficiency on the function and stability of microsomal P450.

## Materials and methods

### *Animals and diets*

Severe dietary Zn deficiency was produced by the procedures reported previously.[5] Three-week-old male weanling Wistar rats with a body weight of 40–50 g (Charles River, St. Constant, Quebec, Canada) were housed individually in stainless cages in a temperature- and humidity-regulated room with a 12-hr light:dark cycle. The rats were fed a Zn-deficient diet (< 1.1 mg Zn/kg) (ZnDF) or a Zn adequate diet (100 mg Zn/kg) (ZnAL) ad libitum for 3 weeks. The dietary compositions were reported previously.[5] The severely reduced voluntary feed consumption by ZnDF rats made it necessary to include a second control group, the pair-fed group (ZnPF). The ZnPF rats were fed the Zn-adequate diet in the same amount that the ZnDF rats consumed the day before. Double deionized water was used as the source of drinking water for the rats.

At 6 weeks of age, the rats were anesthetized and their livers were perfused with ice-cold saline and immediately frozen in liquid nitrogen before storing at −80° C. Microsomes were isolated by differential centrifugation[8] in a Tris-HCl buffer (50 mmol/L Tris, 154 mmol/L KCl, 5 mmol/L EDTA, and 25% glycerol, pH 7.4) and were bubbled with He for 10 minutes prior to use.

### *Biochemical assays*

Liver microsomal P450 and P420 concentrations were determined by difference spectrophotometry[7] with some modifications.[9,10] The extinction coefficient of 91 $cm^2$/mmol (450–490 nm) and 111 $cm^2$/mmol (420–490 nm)[10] were used to calculate P450 and P420 concentrations, respectively.

Microsomal aminopyrine demethylase activity was determined by measuring the formation of formaldehyde[8] using the Nash reagent.[11] The concentration of formaldehyde formed was determined against a standard curve prepared from concentrated formaldehyde (75%, Fisher Scientific Company, Fair Lawn, NJ, USA).

Total microsomal MDA concentration was determined on the basis of its reaction with thiobarbituric acid (TBA), which forms a pink TBA-MDA complex that can be quantified spectrophotometrically.[12] Microsomal TBA-MDA complex was quantitated using a standard curve prepared from synthesized MDA (kindly donated by Dr. H.H. Draper, University of Guelph, Ontario, Canada). Microsomal protein was measured by Lowry's method[13] with bovine serum albumin as the standard.

### *Pentobarbital-induced sleeping time*

To determine if the changes in P450 concentration caused by dietary Zn deficiency would effect in vivo drug metabolism, pentobarbital was injected intraperitoneally at a dose of 28.6 mg pentobarbital/kg body weight to ZnDF, ZnPF, and ZnAL rats and sleeping time was recorded.

### *Other in vitro and in vivo studies*

To investigate the possible mechanisms by which dietary Zn deficiency causes an impaired function and a decreased stability of microsomal P450, some in vitro and in vivo studies were carried out. In the in vitro studies, the liver microsomes isolated from ZnAL rats were suspended in the same buffer described above. Each microsomal preparation was a pooled sample of three rat livers with three replications for each treatment. The incubation mixtures contained approximately 2 mg microsomal protein /mL. Additions to these complete incubation mixtures and incubation conditions are described in the figure legends. In the in vivo studies, $CCl_4$ or ferrous nitrilotriacetate (FeNTA) was used. ZnAL rats ($n=6$) were intubated with $CCl_4$ in corn oil at a dose of 0, 1, or 2 mL of $CCl_4$/kg body weight following a 24-hr fasting period. Freshly prepared FeNTA (pH 7.4) was injected intraperiotoneally to ZnAL rats ($n=6$) at a dose of 0, 9, or 18 mg Fe/kg body weight. Two hr after $CCl_4$ intubation and 24 hr after FeNTA injection, livers were removed using the procedure described above.

### *Mineral analyses*

Liver (1 g) was dried to a constant weight at 100° C and wet-ashed with redistilled nitric acid.[14] Plasma and the digested liver samples were diluted with 0.1 N nitric acid to an appropriate concentration to determine the plasma Fe and Zn and liver Fe concentrations by flame atomic absorption spectroscopy.

### *Statistical analysis*

Significant differences ($P < 0.05$) between the treatment means were determined using Tukey's honestly significant difference procedure.[15]

## Results

Typical signs of severe dietary Zn deficiency in rats, such as severe reduction of feed intake, growth retardation, decreased feed efficiency, and dermal lesions around the mouth and the paws were observed in the rats fed the Zn-deficient diet for 3 weeks. The body weight of ZnDF rats was significantly lower than the body weight of ZnPF and ZnAL controls *(Table 1)*. The growth retardation observed in ZnDF rats seems to be attributed largely to reduced energy intake caused by the severely reduced feed intake in dietary Zn deficiency. ZnDF and ZnPF rats had lower liver weight when compared with ZnAL rats *(Table 1)*. Hematocrit

**Table 1** The effect of dietary Zn deficiency on body weight, liver weight, haematocrit, and plasma Zn concentration*

| Treatment† | Body weight (g) | Liver weight (g) | Hematocrit (%) | Plasma Zn (μg/ml) |
|---|---|---|---|---|
| ZnDF | 49 ± 1[c] | 2.0 ± 0.1[b] | 48 ± 2[a] | 0.33 ± 0.04[b] |
| ZnPF | 73 ± 1[b] | 2.5 ± 0.1[b] | 45 ± 2[a] | 1.81 ± 0.19[a] |
| ZnAL | 193 ± 3[a] | 8.5 ± 0.4[a] | 37 ± 1[b] | 1.81 ± 0.06[a] |

*Values are means ± SEM, $n$ = 6 rats. Means within the same column having different superscript letters are significantly different ($P < 0.05$).
†ZnDF, Zn deficient group: ZnPF, Zn adequate pair-fed group: ZnAl, Zn adequate ad libitum control.

level was significantly elevated by dietary Zn deficiency and reduced energy intake. The severity of the dietary Zn deficiency status was indicated by the significantly lower plasma Zn concentration in ZnDF rats compared with ZnPF and ZnAL rats *(Table 1)*.

The effects of dietary Zn deficiency on the total microsomal protein, P450 and P420 concentrations, aminopyrine demethylase activity, and MDA concentration in the liver are shown in *Figure 1*. There was no significant difference in the total microsomal protein concentration among all three dietary treatment groups. Zn deficiency significantly lowered P450 concentration to 0.4 nmol/mg microsomal protein in ZnDF rats. This is about 60% of the P450 concentration in ZnPF (0.6 nmol/mg microsomal protein) and ZnAL (0.6 nmol/mg microsomal protein) rats. There was no measurable amount of P420 in all three groups. Along with the decrease in the P450 concentration, aminopyrine demethylase activity was also significantly lower in ZnDF rats (4.6 units/min) when compared with ZnPF rats (6.9 units/min) and ZnAL rats (7.2 units/min). Dietary Zn deficiency had no effect on MDA production.

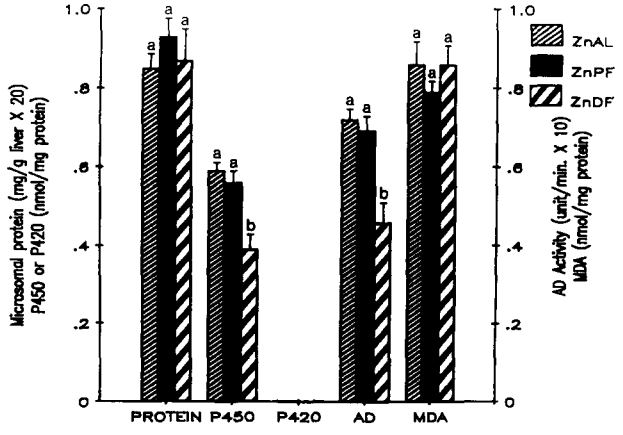


**Figure 1** The effects of dietary Zn deficiency on the function and stability of liver microsomal P450 in rats. Values are means ± SEM, $n$ = 6 rats. Means of the same parameter having different superscript letters are significantly different ($P < 0.05$). The P420 concentration was undetectable in all treatments. ZnAl, Zn-adequate ad libitum control; ZnPF, Zn adequate pair-fed group; ZnDF, Zn-deficient group. Protein, total microsomal protein. P450 or P420, cytochrome P450 or P420. A.D. activity, aminopyrine demethylase activity (1 unit activity = 1 nmol/mg microsomal protein). MDA, malondialdehyde.

*Table 2* shows the effect of dietary Zn deficiency on the in vivo drug metabolism. When injected with pentobarbital, the sleeping time observed in ZnDF rats was about twice that of the controls. However, the only significant difference was between ZnDF and ZnAL rats.

The effect of the protein denaturants, acetone and urea, on the stability of microsomes is shown in *Figure 2*. The P450 concentration was decreased with an increasing concentration of acetone *(Figure 2A)*. There was no measurable amount of P420 in the control nor in the 10% acetone incubation. However, P420 concentration was significantly increased when the acetone concentration in the incubations was increased to 20% and 30% (vol/vol). MDA concentration was unchanged among the treatments. Similarly, increasing the concentration of urea caused a significant decrease in the P450 concentration *(Figure 2B)*. There was no measurable amount of P420 in the control and the 1 M urea incubation. However, P420 concentration was significantly increased with the increasing concentration of urea in the microsomal incubations. The MDA concentration was elevated in the control and 1 M urea incubation when compared to the incubations containing 2 or 4 M urea. This elevation in MDA concentration was probably due to the prolonged incubation period.

The effects of $H_2O_2$ and free radical generating systems on the stability of microsomal membranes is shown in *Figure 3*. When $H_2O_2$ was used to challenge

**Table 2** The effect of dietary Zn deficiency on the pentobarbital-induced sleeping time*

| Dietary treatment† | Sleeping time (min) |
|---|---|
| ZnDF | 151 ± 40[a] |
| ZnPF | 68 ± 8[ab] |
| ZnAL | 52 ± 3[b] |

*Pentobarbital was injected intraperitoneally at a dose of 28.6 mg pentobarbital/kg body weight. Values are means ± SEM, $n$ = 4 rats. Means with different superscript letters are significantly different ($P < 0.05$).
†ZnDF, Zn deficient group: ZnPF, Zn adequate pair-fed group: ZnAl, Zn adequate ad libitum control.

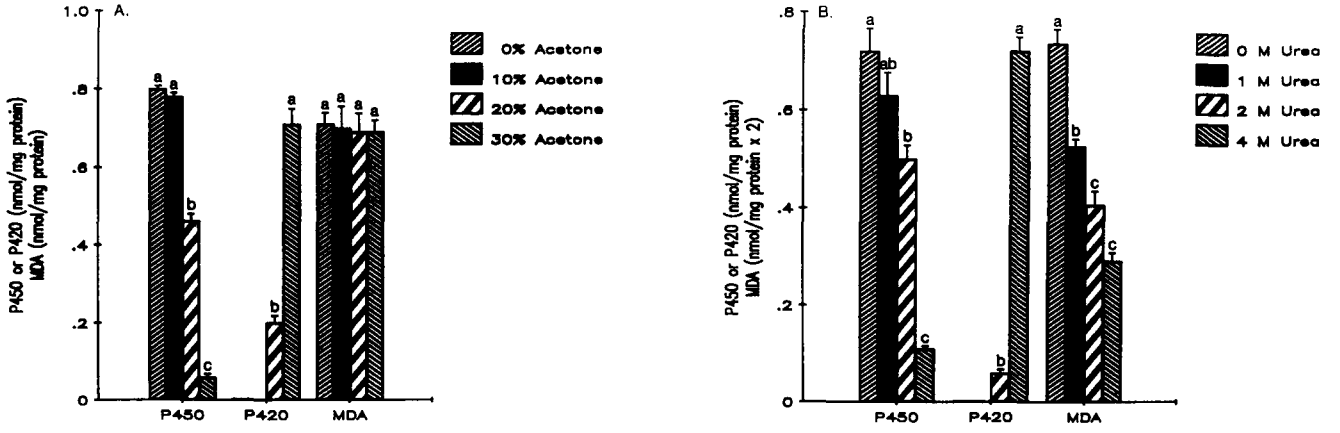


**Figure 2** The effects of in vitro treatment of (A) acetone and (B) urea on the stability of liver microsomal P450 in ZnAL rats. Incubations were as described in the Materials and methods and carried out at 25° C. Where indicated, acetone (0, 10%, 20%, or 30%, vol/vol, 3 min.) or urea (0, 1, 2, or 4 M for 4 hrs) was added. Values are means ± SEM, $n = 3$ liver microsomal preparations. Means of the same parameter having different superscript letters are significantly different ($P < 0.05$). P450 or P420, cytochrome P450 or P420. MDA, malondialdehyde.

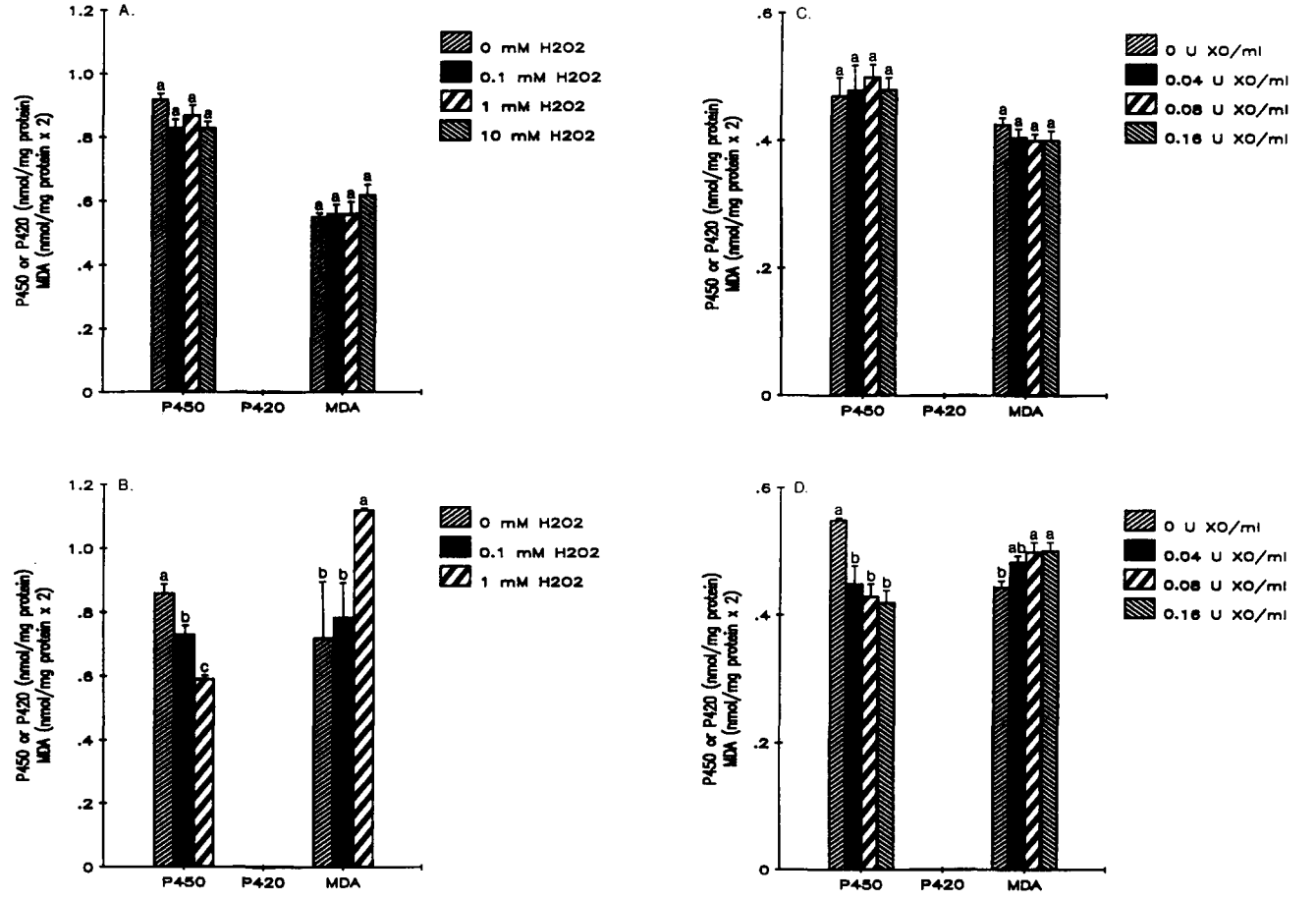


**Figure 3** The effects of in vitro treatment of (A) $H_2O_2$; (B) $H_2O_2$-$FeCl_2$/$FeCl_3$ system; (C) xanthine/xanthine oxidase system; and (D) xanthine/xanthine oxidase-$FeCl_3$ system on the stability of liver microsomal P450 in ZnAL rats. Incubations were as described in the Materials and methods and carried out at 37° C. Where indicated, xanthine (0.33 mmol/L) was added. The reaction was (A) initiated by adding $H_2O_2$ (0, 0.1, 1, or 10 mmol/L) and incubated for 20 min.; (B) initiated by adding $H_2O_2$ (0 or 0.1 mmol/L), $FeCl_2$ (0.2 mmol/L)/$FeCl_3$ (0.2 mmol/L) or $H_2O_2$ (1 mmol/L), $FeCl_2$ (2 mmol/L)/$FeCl_3$ (2 mmol/L); (C) initiated by adding xanthine oxidase (0, 0.04, 0.08, or 0.16 units for 30 min.; and (D) initiated by xanthine oxidase (0, 0.04, 0.08, or 0.16 units) and ADP (1.7 mmol/L)-$FeCl_3$ (0.1 mmol/L). The P420 concentration was undetectable in all treatments. Values are means ± SEM, $n = 3$ liver microsomal preparations. Means of the same parameter having different superscript letters are significantly different ($P < 0.05$). P450 or P420, cytochrome P450 or P420. MDA, malondialdehyde.

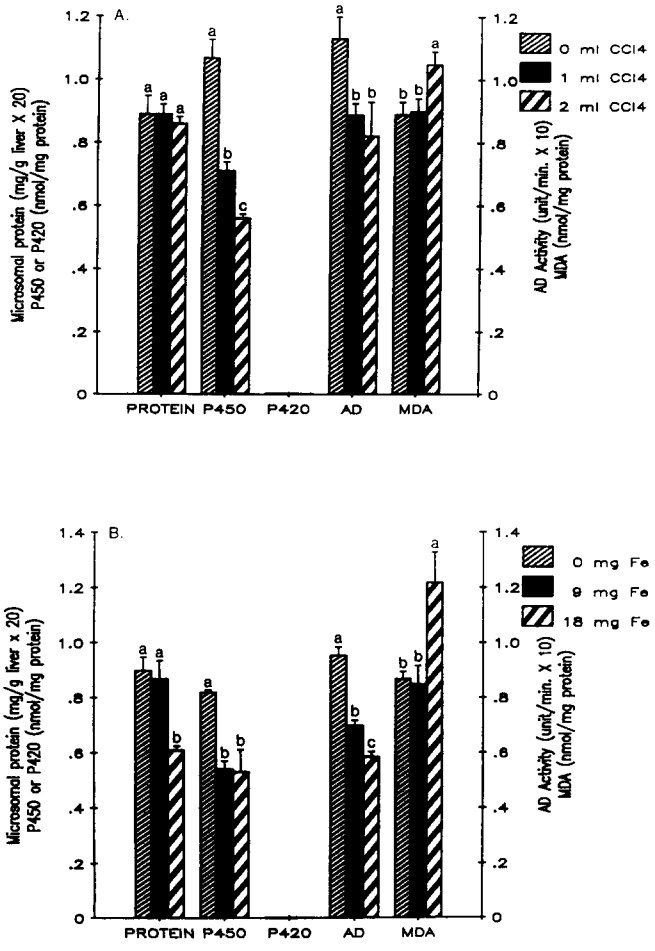


**Figure 4** The effects of (A) $CCl_4$ and (B) FeNTA on the function and the stability of liver microsomes isolated from ZnAL rats. The P420 concentration was undetectable in all treatments. Values are means ± SEM, $n$ = 6 rats. Means of the same parameter having different superscript letters are significantly different ($P < 0.05$). Protein, total microsomal protein. P450 or P420, cytochrome P450 or P420. A.D. activity, aminopyrine demethylase activity (1 unit activity = 1 nmol/mg microsomal protein). MDA, malondialdehyde.

the microsomes it had no effect on the concentration of P450 and MDA *(Figure 3A)*. However, when $H_2O_2$-$FeCl_2/FeCl_3$, a free radical generating system, was added to the microsomes, the P450 concentration was decreased when we increased the concentration of the free radical generating system in the incubations without measurable change in the amount of P420. This indicated that a decrease in the P450 concentration was not accompanied by the conversion of P450 to P420. MDA concentration, on the other hand, was significantly increased in the incubations containing the highest concentration of the free radical generating system *(Figure 3B)*. Similarly, the concentrations of P450 and MDA were not affected by the xanthine/xanthine oxidase (X/XO) system *(Figure 3C)*, but were affected by the X/XO-$FeCl_3$ system *(Figure 3D)*. With the increased XO concentration in the free radical generating system, P450 concentration was decreased without conversion to P420 (as there was unmeasurable amount of P420), but MDA production was increased.

The effects of an in vivo challenge with chemicals, which are known to induce free radicals, on the stability of microsomal membranes, are shown in *Figure 4*. Acute $CCl_4$ challenge had no effect on the total microsomal protein concentration *(Figure 4A)*. However, the P450 concentration was decreased with an increasing dose of $CCl_4$, but there was no conversion of P450 to P420. The decrease in P450 concentration was accompanied by a significant decrease in aminopyrine demethylase activity. MDA concentration was increased only in the microsomes isolated from the rats treated with the highest oral dose of $CCl_4$. After a 24-hr challenge of 18 mg Fe/kg body weight, the total microsomal protein concentration was significantly decreased *(Figure 4B)*. The P450 concentration was significantly lower in both Fe-treated groups as compared with the control. Again, this decrease in P450 was not accompanied by a conversion of P450 to P420. The aminopyrine demethylase activity was also significantly decreased as a result of Fe treatment. MDA concentration was significantly increased only in the microsomes isolated from the rats treated with the highest concentration of Fe. Intraperitoneal injection of FeNTA at 9 or 18 mg/kg body weight successfully loaded Fe in the liver, but the plasma Fe concentration was not significantly different due to large variations in the rats treated with 18 mg Fe/kg body weight *(Table 3)*.

## Discussion

The present study demonstrated that severe dietary Zn deficiency affects liver microsomal membrane, resulting in a decrease both in vitro and in vivo of drug metabolizing capacity as indicated by a decreased aminopyrine demethylase activity and a prolonged pentobarbital-induced sleeping time, respectively. Although the mechanism was unknown, this effect of dietary Zn deficiency on the liver microsomal P450 has also been observed by Becking and Morrison.[16] This impaired function of liver microsomal P450 in dietary Zn-deficient rats is likely not due to a general decrease in protein synthesis because the total microsomal protein concentration was the same in all the dietary treat-

**Table 3** Liver and plasma Fe concentration of rats challenged with Fe-NTA*

| Treatment† (mg Fe/kg BW) | Plasma Fe (μg/mL) | Liver Fe (μg/g) |
|---|---|---|
| 0 | 3.1 ± 0.4[a] | 334.7 ± 11.1[c] |
| 9 | 3.1 ± 0.5[a] | 542.9 ± 27.2[b] |
| 18 | 11.0 ± 4.4[a] | 827.2 ± 76.4[a] |

*Values are means ± SEM, $n$ = 6 rats. Means within the same column having different superscript letters are significantly different ($P < 0.05$).
†Fe was injected intraperitoneally as FeNTA at a dose of 0, 9, or 18 mg Fe/kg body weight.

ments in the present study. There are two possible mechanisms by which dietary Zn deficiency causes impaired function of this membrane enzyme system. The first possible mechanism is that Zn functions as an antioxidant to protect the sulfhydryl groups against oxidation damage of protein.[1,4] It has been shown that there are six cysteine residues in each P450 molecule.[17] The sulfydryl group from the cysteine residues is critical to the structure and function of microsomal P450. Modification of one or more of these cysteine residues, as in the presence of acetone or urea, results in the conversion of P450 to P420.[18] Cytochrome P420 can be effectively reconverted to P450 with full enzymatic activity by sulfhydryl compounds, such as cysteine, glutathione, or dithioerythriol.[18] However, there is no measurable amount of P420 in the liver microsomes of the ZnDF rats, indicating there is no conversion of P450 to P420. It is not likely that this mechanism is responsible for the impaired function of liver microsomal P450 in the ZnDF rats.

The second possible mechanism is that dietary Zn deficiency results in an increase of free radical generation that causes structural damage and consequently functional impairment in biomembranes. In the present study, the effect of dietary Zn deficiency on the microsomal P450 was very similar to the effect of the free radical generating systems/agents on P450, especially at the lower concentrations of the free radical generating systems/agents. It has been previously shown that lipid peroxidation in the microsomal membranes causes a loss of P450 when rat liver microsomes were incubated with NADPH[19] or an in vitro free radical generating system.[20] This loss of microsomal P450 results from a breakdown of the heme moiety of this hemeprotein.[19] Thus, the loss of microsomal P450 will not be accompanied by the appearance of P420. Dietary Zn deficiency stimulates the production of $H_2O_2$.[5] It was suggested that $H_2O_2$ arises from the ferrous-$O_2$ complex in the P450 electron transport chain[21] due to uncoupling in the P450 electron transport chain.[22] Therefore, it is possible that the $H_2O_2$ that leaked out from the electron transport chain is localized at the catalytic site. This leaked $H_2O_2$ may cause an increase of free radical generation because there is a significant elevation of Fe in the microsomal membranes isolated from the ZnDF rats.[5] Consequently, free radicals generated in this system may attack the nearby heme moiety or the protein moiety of P450, causing structural damage and functional impairment of P450. It is worthwhile to notice that under the presence of oxidative stress, either in vitro *(Figure 3B and 3D)* or in vivo *(Figure 4B and 4D)*, loss of microsomal P450 occurred at the lower level of the free radical generating system/agents, while the increase in MDA production only occurred at the higher level of the free radical generating systems/agents. It appears that the decrease in microsomal P450 concentration was an early event as compared with membrane lipid peroxidation under the oxidative stress, suggesting that P450 hemeprotein is more prone to oxidative stress than the surrounding membrane lipids or the oxidative stress is very localized at the catalytical site of the microsomal P450. Despite the similarity in the effect on P450 structure, dietary Zn deficiency caused an impairment of the function of P450 in a way similar to in vivo $CCl_4$ and FeNTA. Therefore, the effect of dietary Zn deficiency on the structural damage and functional impairment of microsomal P450 may be through a free radical-mediated mechanism.

In conclusion, severe dietary Zn deficiency in rats resulted in a structural and functional impairment of liver microsomal P450, as shown by decreased P450 concentration, decreased enzymatic activity, and decreased in vivo drug metabolism capacity. Apparently these damages occurred with the liver microsomal P450 through a free radical-mediated mechanism.

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