

AN ABSTRACT OF THE THESIS OF

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Pregnant and weanling rats were fed liquid diets with or without 30 percent of total kcal from ethanol and varying levels of copper in order to determine if ethanol ingestion would exaggerate a marginal copper status to an obvious copper deficiency. Pregnant albino rats were fed either 0.75 or 3.75 mg Cu/L throughout gestation and the first 15 days of lactation while female weanling rats received 0.5 or 2.5 mg Cu/L for 5 weeks. Ethanol consumption exaggerated a marginal copper status during reproduction as evidenced by significant reductions in maternal liver copper concentration and enzymatic activity of the copper metalloprotein Copper-Zinc superoxide dismutase in offspring liver. Ethanol had little or no effect upon copper status in weanling rats. In addition, serum copper failed to reflect a developing depletion of liver copper when ethanol was being

consumed. Since it is known that the average American diet is just adequate in copper content, and that copper balance is difficult to achieve during times of increased metabolic demand, pregnant subjects may be at a great risk to develop a copper deficiency when ethanol is being consumed. This ethanol and copper interaction, however, will likely go undetected if only serum copper is used as an indicator of copper status.

Influence of Ethanol on Copper Utilization  
by Pregnant and Growing Rats

by

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INFLUENCE OF ETHANOL ON COPPER UTILIZATION  
BY PREGNANT AND GROWING RATS

INTRODUCTION

Alcoholism is the most common cause of malnutrition in the United States today (1). Extensive studies have shown, for example, that ethanol (commonly referred to as alcohol) antagonizes the metabolism of protein, thiamin, Vitamin B-6, folate, Vitamin A, zinc, calcium, magnesium, and iron (2-8). Furthermore, it is now understood that this reduction in nutritional status of the alcoholic can be caused in three different ways as classified by Lieber (9). Primary alcohol malnutrition results from a decrease in the actual amount of food ingested because of the appetite suppressant effect of ethanol and because of the negligible amounts of nutrients in most alcoholic beverages. Secondary alcohol malnutrition results from maldigestion and malabsorption of essential nutrients because of direct effects of ethanol on the integrity of the gastrointestinal tract (1,9,10). Tertiary alcohol malnutrition is related to alterations in activation and utilization of essential nutrients.

According to Lieber (11) nutrient metabolism may be

antagonized, in part, by the ability of ethanol to produce fatty liver. However, this type of liver dysfunction is most likely to occur in a person who regularly consumes more than 80 g of ethanol a day for several years (12) in contrast to the occasional alcohol consumer. In addition, research indicates that ethanol-induced fatty liver is directly related to the amount of fat in the diet and that many chronic alcoholics show no signs of fatty liver (13,14). Because of this, it is unfortunate that many of the experimental alcohol studies that have investigated nutrition and alcohol relationships have done so in the presence of alcoholic liver dysfunction rather than in its absence.

The antagonistic effects of ethanol on nutrient metabolism may be exacerbated during two stages of life when there is an increase in metabolic rate. The first of these is during pregnancy (15). It is a well-known fact that a pregnant woman must consume more calories, vitamins, and minerals than a non-pregnant woman of the same age (15). Because alcohol is known to antagonize the metabolism of vitamins and minerals, alcohol consumption during pregnancy may compound the situation by causing an even greater demand for essential nutrients. In addition, it is probable that this situation exists during the rapid growth phase of adolescence when metabolic rate also increases (16). It may be assumed that the effects of alcohol consumption on nutrient metabolism during the rapid growth phase will be similar to the effects seen during pregnancy. However,

because the demand for most nutrients is greater during pregnancy than for normal growth (15,16), the antagonistic effects of ethanol should be more pronounced during pregnancy.

Although there are numerous studies on the effects of ethanol on specific vitamins and minerals, little information is currently available about a copper and alcohol relationship. Copper is a necessary component of several metalloenzymes involved in important metabolic processes (17,18). These include ceruloplasmin which plays a role in copper transport and iron reutilization, lysyl oxidase which is important for cross-linking of the structural proteins collagen and elastin, and copper-zinc superoxide dismutase which protects cells from cellular oxidants (17). In these metalloenzymes, copper is firmly associated with the protein and cannot be replaced by any other metal (17). If alcohol does disrupt the bioavailability of copper there could be a significant depression of copper-dependent enzymes (17). Furthermore, there is very little information about a copper and ethanol relationship during periods of increased metabolic demand such as those represented by pregnancy and growth. It is also unknown if a copper and alcohol relationship is dependent upon the level of dietary copper and internal copper status.

Admittedly, a dietary copper deficiency is rare in the United States (19). However, it might be possible to see a depressive effect of alcohol on copper utilization if a marginal dietary copper intake was coupled to a period of high metabolic

demand such as that represented by either pregnancy or growth. Present knowledge, however, does suggest that the copper requirement is greater during pregnancy than for growth in rats (20) and possibly in humans. It is also known that the average American diet is just adequate in copper content (19).

With these thoughts in mind a research hypothesis was developed to test the possibility that an antagonistic effect of ethanol (30 percent of kcal) on copper utilization and function in the weanling rat will be less than the effects in a pregnant rat. Furthermore, it was hypothesized that an antagonistic effect of ethanol on copper utilization during either reproduction or growth will be dependent upon the level of copper in the diet.

The specific aims developed were:

- . To determine if ethanol antagonizes copper utilization, irrespective of physiological state in the rat, in the absence of liver dysfunction and primary alcoholic malnutrition.

- . To determine if ethanol-antagonized copper utilization is dependent upon the level of dietary copper in weanling and pregnant rats.

- . To determine if ethanol-antagonized copper utilization is expressed as a functional impairment of copper's role in metalloenzymes.

## LITERATURE REVIEW

The purpose of this review is to discuss ethanol consumption and malnutrition with specific emphasis on the potential interaction of dietary copper and ethanol. An interaction occurs when the effect of one factor is dependent upon the level of the other factor. For example, in the studies to be described the two factors involved are ethanol and copper. An interaction will exist if the antagonistic effect of ethanol is dependent upon the level of copper in the diet.

### Ethanol Consumption In The United States

Ethanol may be referred to as a drug because it is classified as a general central nervous system depressant (21). Ethanol is also rich in energy providing 7.1 kcal/g and, therefore, may be referred to as a food (22). Alcoholic beverages, however, are essentially devoid of important nutrients such as protein, vitamins, and minerals (1).

In the United States ethanol is ingested in the form of beer, wine, and distilled spirits with the ethanol concentration varying from approximately 4 percent by volume in beer, to 12 percent in wines, to 40-50 percent in distilled liquors (12). The amount of absolute ethanol (pure 100% ethanol) in any type of alcoholic beverage is calculated by multiplying the amount of

ethanol in the beverage by the total volume.

A drink is defined as the volume of an American alcoholic beverage containing 15 ml (0.5 oz) of absolute ethanol. This amount of ethanol is contained in 360 ml (12 oz) of beer, 120 ml (4 oz) of wine, or 30 ml (1 oz) of 86-proof liquor. The simplest definition of an alcoholic is a person who regularly consumes more than 80 grams of ethanol per day (12). This amount of ethanol is contained in nine single measures of spirits, one bottle of wine, or five pints of beer. In the case of the woman consuming 2,000 kcal per day, 80 grams of ethanol would contribute 29 percent of total calories. Therefore, studies to be described were designed to provide 30 percent of calories from ethanol.

#### Ethanol-Induced Malnutrition

Lieber has classified the etiology of alcoholic malnutrition as primary, secondary and tertiary (9,23). Primary alcoholic malnutrition refers to the failure of alcoholics to consume a nutritionally adequate diet. This form of malnutrition is complex because it includes the appetite suppressant effect of alcohol, the preference of the chronic alcoholic to spend limited resources on ethanol, and because, with the exception of beer, most alcoholic beverages are nearly devoid of essential vitamins and minerals (24). In experimental studies involving laboratory rats, this ability of ethanol to depress food intake, exemplified by the simple addition of alcohol to drinking water, can introduce an

undesirable variable into a nutrition and alcohol study. Fortunately the depressive effects of alcohol on food intake of rats can be overcome if alcohol is incorporated into a liquid diet (25,26).

Secondary alcoholic malnutrition refers to the direct effect of ethanol upon the gastrointestinal tract which may profoundly alter digestion and gastrointestinal absorption of essential nutrients (9,10). The stomach may be particularly vulnerable to acute damage from alcohol because the stomach is exposed to higher concentrations of ethanol than the lower gastrointestinal tract. Ethanol disrupts the gastric mucosal barrier and is an accepted cause of acute gastritis. Acute gastritis is accompanied by massive bleeding and may contribute to the development of an iron-deficiency anemia (27).

The duodenum is also exposed to high concentrations of ethanol. Experimentally in the rat, ethanol-induced lesions in the intestine appear to be related directly to the concentration of ethanol, with the greatest damage resulting from those solutions with the highest concentration of ethanol (28). These morphologic effects of ethanol on the small intestine are accompanied by functional changes. It has been demonstrated, for example, that prolonged contact between the brush-border membrane and ethanol can alter the fluidity of the membrane affecting both the microenvironment and the transport and activation of enzymes. In the latter instance, ethanol decreases the activity of the

brush-border enzymes lactase, sucrase, maltase, and alkaline phosphatase (29).

Lieber has also suggested that ethanol causes impaired utilization of nutrients by the liver leading to tertiary malnutrition (11,30). Alcohol can produce fat accumulation in the liver in both rats and humans even when dietary intake is adequate with respect to known nutritional requirements. The production of fatty liver, however, is directly related to the amount of fat in the diet (13,14). Jones and Green (13), for example, found that when ethanol was incorporated into the diets of rats at 36 percent of the total kcal, hepatic lipids increased only when dietary fat exceeded 20 percent of the total kcal. Within the ethanol-fed groups severe fatty liver only developed in rats fed the DeCarli-Lieber liquid diet (31) which contained 35 percent of kcal as fat.

Although hepatic fat accumulation itself is not necessarily harmful, it reflects a metabolic disturbance of the liver and may lead to irreversible damage of the hepatocyte (11). As mentioned previously, many consumers of alcohol do not develop fatty liver even after 25 years of chronic ethanol consumption and yet they still show signs of altered nutrient metabolism by the liver (32). Therefore, ethanol may have an indirect effect on the liver's ability to metabolize essential nutrients (2).

In the studies to be described in the methods section, effects of ethanol on liver copper status will be reported under experimental conditions that do not promote liver disease. This



goal was made possible by manipulating diet composition. A detailed description of the diet used will be given in the Methods section.

#### Ethanol Consumption During Growth and Pregnancy

According to the National Council on Alcoholism (33), the average age of taking the first drink is 13 years. Among high school students as many as 25 percent of young women can be classified as alcoholics (33) because they become drunk at least six times a year or otherwise experience negative consequences as a result of drinking.

In many cases, alcohol consumption by women continues into the reproductive years. It has been estimated that two-thirds of American women of reproductive age (21-34 years) consume alcohol (33). This consumption pattern does not significantly change during pregnancy, even though alcohol consumption during pregnancy can affect the nutritional status of the mother and developing fetus in potentially harmful ways (34-37). Ethanol readily crosses the placenta, and epidemiological studies clearly indicate an association between maternal ethanol intake and adverse outcome of pregnancy. Controversy still exists about how much ethanol, if any, is safe for a pregnant woman to consume.

The next sub-section will begin with a brief review of copper status in humans followed by a summary of what is presently known about the effects of ethanol on copper metabolism and utilization.

## COPPER

Copper, third in abundance among the essential trace elements in the human body, has been recognized as an essential dietary component for over four decades (38). However, human nutritional deficiency syndromes of copper have only been recognized since the early 1960's. Once it was discovered that copper was necessary to promote hematopoiesis (39), a wide variety of other biological processes were shown to depend on an adequate supply of copper, primarily by way of metalloenzyme function. For these reasons current research is directed toward elucidating the mechanisms that regulate the absorption, transport, detoxification, and excretion of copper in the mammalian body. Copper metabolism has been the subject of review articles (39,40).

### Sources of Copper

Copper is found in small amounts in nearly all plant and animal tissue. The copper content of foods varies greatly depending upon the age, genetics and environmental conditions affecting the plant and animal as well as by the methods of handling, processing and cooking the food (41). The foods in the human diet which contain the highest amount of copper are shellfish and organ meats. Other sources of copper are nuts and seeds, whole grains, legumes, dried fruits and mushrooms.

### Requirements for Copper

Based upon human metabolic studies and to allow for a margin of safety, a daily copper intake of 2-3 mg has been recommended for adults (42). This provisional Recommended Dietary Allowance (RDA) for copper also applies to pregnancy and lactation. Recent surveys of human dietaries, however, found that daily copper intake of humans may be lower than two mg per day and that some groups of people are not even consuming one mg per day (19,42-44). In contrast to human copper requirements, the requirement for normal growth in rats is 5 mg/kg of diet (45), whereas during pregnancy and lactation the copper requirement increases to 9 mg/kg (20).

Because ethanol is known to antagonize vitamin and mineral utilization in humans (46) and rats (47-50), an individual consuming alcoholic beverages and a diet low in copper may be at special risk to develop a copper deficiency. This situation may be further exaggerated by a period of high metabolic demand such as that represented by pregnancy and lactation as well as by growth.

### Copper Absorption and Utilization

When Cu-64 is orally administered to humans, copper rapidly appears in the blood which suggests that absorption occurs from

the stomach and upper small intestine (51). Depending on the dietary source and the level of intake, 25 to 40 percent of copper consumed is absorbed (51). Homeostasis is maintained by controlling hepatic biliary excretion of endogenous copper, the major route for copper excretion, and by the rate of intestinal copper absorption (51) which is regulated in part by the protein metallothionein. Very little copper is excreted by way of urine.

Metallothionein is a low molecular weight, cystein-rich intracellular metal-binding protein which can bind copper (52,53). Intestinal metallothionein may have at least two functions in maintaining copper homeostasis. Metallothionein probably has a passive role in copper absorption by providing binding sites within the intestinal mucosa to insure that an adequate supply of the metal is removed from the dietary source and temporarily stored for subsequent absorption. It may also serve as a mucosal block to protect against absorption of toxic levels of copper as well as other trace elements (53).

After absorption from the gastrointestinal tract, copper is transported to the liver through the portal bloodstream by the transport proteins albumin and transcuprein (54). The liver contains 8 to 10 percent of total body copper in humans (40) and rats (55) and is the major location of storage copper in both species. Using radioactive copper, Hazelrig et al (56) examined copper metabolism in the isolated perfused rat liver. They determined that the liver binds copper to metallothionein for

temporary storage, prepares copper for excretion in bile, and incorporates copper into copper containing proteins. Copper is transported from the liver to other tissues by the protein ceruloplasmin (39, 40).

The functional significance of copper in liver and other tissues relates to its role in metalloenzymes. Examples of copper-containing enzymes include the ferroxidase activity of ceruloplasmin (important for reutilization of cell iron), cytochrome C oxidase (the terminal oxidase of the electron transport chain in the mitochondria), lysyl oxidase (important for synthesis of cross-linking substances in the structural proteins collagen and elastin), and superoxide dismutase (40,51,57).

#### Copper-Zinc Superoxide Dismutase

Copper-Zinc Superoxide Dismutase (Cu-Zn SOD) is ubiquitous among aerobic organisms, and is essential for defense against oxygen toxicity in erythrocytes, brain, and liver cells. It is a copper-and zinc-containing enzyme that catalyses the dismutation of the superoxide radical ( $O_2^-$ ). The superoxide radical normally produced during aerobic metabolism, is dismutated by the reaction:  $O_2^- + O_2^- + 4H = 2H_2O + O_2$ . This reaction can proceed spontaneously at a relatively rapid rate, but Cu-Zn SOD increases this rate more than 10,000 fold. Because of this catalytic function, Cu-Zn SOD is thought to be involved in the protection of cells against damage from lipid peroxidation (57,58) brought about by high levels of superoxide radicals. Lipid peroxidation has

been associated with numerous pathological conditions including anemia, lung damage, liver necrosis and atherosclerosis (59,60). Cu-Zn SOD may be important as an intracellular storage form of copper and zinc (57). It has also been proven to be a sensitive indicator of copper status in rats (61) and humans (62).

#### Copper Deficiency

Manifestations of copper deficiency vary with age, sex, and species of the animal and with severity and duration of the deficiency (52). Examples of signs of copper deficiency found in experimental animals include anemia, reduced plasma copper concentration, reduced oxidase activity of ceruloplasmin, and reduced liver copper content (52). In addition, recent observations in human subjects and in experimental animals have revealed a series of additional manifestations of copper deficiency. These include decreased oxidative protection of tissues and increased lipid peroxidation damage to the liver of copper deficient rats (63,64), cardiac muscular dysfunction, skeletal malformations, and hypercholesterolemia (65,66). These metabolic disorders arising from copper deficiency can be attributed to a reduction in one or more of the copper enzymes previously discussed.

#### Ethanol and Copper

Ethanol significantly alters copper utilization evidenced

by decreased liver copper levels in alcoholic rats (47,48,61). However, the effects of ethanol on liver copper concentration differs markedly in severity between investigations. Bogden et al (48) demonstrated that liver copper concentration significantly decreased (32 percent relative to non-alcohol controls) after 15 weeks in rats consuming 35 percent of their total kcal as ethanol. Ethanol was provided in the drinking water and the dietary fat content was 12 percent of total kcal. Zidenberg-Cherr et al (65) noted a non-significant 17 percent reduction in liver copper in rats receiving 20 percent (w/v) ethanol in their drinking water for 14 days. The fat content of the diet was 18 percent of total kcal. Dreosti et al (47) noted a reduction in liver copper when adult female rats were given a diet containing 12 percent of the diet as fat and 20 percent ethanol as part of their drinking water for 12 months. Their study showed a 15 percent ( $P < 0.005$ ) reduction of liver copper in the ethanol-fed group, but they did not include a pair-fed group or give any information relating to dietary copper intake. Therefore, reductions in measurable liver copper may have been simply due to poor dietary intake. Hopf et al (50) showed a non-significant 15 percent reduction in liver copper in adult female mice after 12 weeks of ethanol ingestion. In their experiment the experimental group was fed ad libitum with a liquid diet containing 5 percent (w/w) ethanol and 43 percent of kcal as fat. The control animals were pair-fed with a similar diet in which ethanol was substituted by an isocaloric amount of

sucrose.

When liver copper is reduced to a low enough level, copper-containing enzymes are depressed in experimental animals (47,50,63). Animal studies have shown, for example, that Copper-Zinc Superoxide Dismutase (Cu-Zn SOD) activity in pigs (66), chicks (67), and rats (61,65,68) is directly related to copper nutrition. Paynter et al (63) used male weanling rats to determine the effect of dietary copper level on tissue activity of Cu-Zn SOD. Rats were fed diets containing either 0.8 or 4.8 mg Cu/kg. Liver, which has the greatest activity of Cu-Zn SOD activity of all tissues (68), showed the greatest relative decrease in enzyme activity with copper depletion. Under these conditions, enzymatic activity of Cu-Zn SOD diminished to 56, 24, and 14 percent ( $P < 0.001$ ) of control activities at weeks 1, 3, and 6 respectively. The magnitude of the decrease in liver Cu-Zn SOD was greater than that observed in liver copper content.

Ethanol has also been shown to decrease liver Cu-Zn SOD activity (49,61,65). Dreosti et al (61) fed ethanol (20 percent ad libitum in drinking water) to adult female rats for 12 months while controls were given water. They noted a 40 percent reduction in liver Cu-Zn SOD in the ethanol-fed group relative to controls. Keen et al (49) also noted a reduction in liver Cu-Zn SOD in monkeys fed a liquid diet containing 50 percent of the energy intake as ethanol for four years. This reduction, however, was not statistically significant.

In a classical copper deficiency a reduction in liver copper



should be mirrored in a decrease in serum copper concentration (64). Ethanol ingestion, however, has been reported to increase serum copper levels. Hopf et al (50), for example, produced a 48 percent increase ( $P < 0.05$ ) in serum copper levels in adult female mice fed a liquid diet containing 5 percent (v/v) ethanol and 43 percent of diet energy as fat for 12 weeks. Wu et al (69) also noted an elevation of serum copper in twenty male alcoholics. Their study, however, was under uncontrolled conditions and results were statistically non-significant. Nevertheless, the results of these studies suggest that ethanol-antagonized copper metabolism does not represent a simple copper deficiency when serum copper is used as the indicator of copper status.

Although studies have been undertaken to determine liver Cu-Zn SOD, liver copper, and serum copper during ethanol ingestion, they did not determine if ethanol-antagonized copper utilization is dependent upon the level of dietary copper. In addition, many previous studies utilized a diet that promoted liver dysfunction. Furthermore, the possible influence of physiological state was not considered. For these reasons the present research was designed to determine if the antagonistic effect of ethanol (30 percent of kcal) on copper utilization and function in the weanling rat is further exaggerated by pregnancy. It was also the intent of this research to determine if the antagonistic effect of ethanol on copper utilization and function is dependent upon the level of copper in the diet.

## METHODS

This section covers the experimental design, methods of data collection, and analyses conducted in this project. Methods have been subdivided into three sections: 1) Experiment One (pregnancy and lactation), 2) Experiment Two (growth), and 3) Procedures Common To Both Experiments.

The experimental design for both experiments involved two factors, ethanol (30 percent of kcal) and copper, with two levels of each factor (2 X 2 factorial design, 4 treatments). In Experiment One, each treatment had 6 replications for a total of 24 rats. In Experiment Two each treatment had 8 replications for a total of 32 rats.

### EXPERIMENT ONE

The purpose of this experiment was to define the possible interaction of ethanol and low dietary copper during pregnancy and lactation in the rat. This section has been subdivided into general protocol, diet formulation, and feeding procedures.

#### General Protocol

Forty proven-breeder female Sprague-Dawley outbred rats (CR:1 CD(SD)BR, Charles River Laboratories, Wilmington, MA),

weighing 200 to 300 grams, were received two days prior to breeding. During this two-day period rats were fed Oregon State University lab chow and tap water. Rats were bred overnight with adult males of the same age and species in breeding cages (51 x 41 x 20 cm. L x W X D). Presence of spermatozoa in a vaginal smear taken the following morning was considered to be day 0 of gestation. The 24 pregnant rats obtained by breeding were weighed, assigned to one of the four experimental diet groups, and individually housed in polycarbonate cages (48 x 27 x 20 cm., L x W x D) equipped with stainless steel wire tops and raised wire floors (Lab Products, Inc., Maywood, NJ).

On day 20 of gestation, rats were transferred to clean cages with enough bedding material for delivery. They were allowed to normally deliver the pups, ingest the placenta, and clean the young. On the first day of lactation, each litter was reduced in size to eight. Dams were allowed to nurse the pups only to the 15th day of lactation to prevent diet consumption by pups. Procedures of the termination are outlined under Procedures Common To Both Experiments.

#### Diet Formulation

Individual liquid diets containing either 0.75 or 3.75 mg Cu/L as cupric chloride dihydrate with or without 30 percent of kilocalories from ethanol, were assigned at day 0 of gestation and fed until the experiment was terminated. This liquid diet was

designed to provide 1 kcal/mL during gestation in contrast to a solid diet which would have an average of 4 kcal/g. Therefore, 0.75 mg Cu/L was equivalent to a solid diet containing 3 mg Cu/kg or about 40 percent of copper required for growth and reproduction in the rat (20). Similarly 3.75 mg Cu/L was equivalent to a solid diet containing 15 mg Cu/kg or approximately two times the required amount (20). During lactation the energy value of the liquid diet was increased to 1.5 kcal/mL.

Composition of the control diet (without ethanol), which has been previously published (26), contained, in g/L, micropulverized casein, 69.75; DL-Methionine, 0.5; cellulose powder, 10; dextrose, 144.0; corn oil, 13.00; vitamin mix, 12.5; mineral mix, 10.00; xanthan gum, 2.00; and cold distilled-deionized water to give one liter when blended (Waring, New Hartford, CT). To prepare one liter of the alcohol diet, dextrose was isocalorically replaced with 95 percent ethanol in an amount equal to 30 percent of total kcal. The mineral mixture originally published (26) was modified to yield a copper content of 0.75 mg/L.

Copper-supplemented diets were prepared by adding cupric chloride dihydrate to control diets to give the desired higher copper content (3.75 mg/L). This was accomplished by preparing a 1,000 mg/L copper stock solution and pipetting the amount needed directly into the blender when preparing the diet. Analysis of the diets prior to the start of the study showed that copper

concentrations were within 5 percent of calculated values.

The diet described above was designed to study the effects of ethanol on copper status essentially in the absence of liver disease. As stated previously, the production of fatty liver is directly related to the amount of fat in the diet. The DeCarli-Lieber diet (31), which is commonly used in experimental alcohol studies, incorporates 35 percent of kcals as fat into the diets of laboratory rats and was designed to study alcohol effects in the presence of alcoholic fatty liver. The diet used in this study provided only 11.5 percent of kcal as corn oil and, therefore, will not produce excessive fat accumulation in the liver (13,14). This level of fat is equivalent to that recommended by the American Institute of Nutrition for nutritional studies involving rats (45).

#### Feeding Procedures

The liquid diet feeding tubes used in this experiment were made by a glassblower at Oregon State University but are available commercially (Bio-Serve, Frenchtown, NJ). These glass tubes were mounted on the outside of each cage with a stainless steel clip and entered the cage by way of a hole drilled in the side. Each morning all tubes and stoppers were brushed with soapy water, rinsed three times with distilled-deionized water, and allowed to air-dry before being filled with fresh diet.

Liquid Diets were prepared daily from the powdered formula described previously and were fed throughout pregnancy and lactation. Our goal was to equilibrate energy intake for all groups and to feed an amount of liquid diet that would be typical of the energy value expected from a conventional solid diet (70). Fresh liquid diet was measured in a graduated cylinder and poured into the feeding tubes. If any diet remained from the following day, it was also measured in a graduated cylinder and recorded.

#### EXPERIMENT TWO

The purpose of this experiment was to determine if there was an interaction between ethanol and low dietary copper during a rapid growth phase. Thirty-two weanling female Sprague-Dawley outbred rats (CR:1 CD(SD)BR, Charles River Laboratory, Wilmington, MA ), initially weighing 70 to 78 grams, were assigned to one of four experimental diets (n=8/group), weighed and individually housed in a polycarbonate cage as described under Experiment One. Liquid diets were assigned and feeding began the following morning.

The liquid diet used and feeding procedures were identical to Experiment One with the exception of copper content. Diets containing either 0.50 or 2.5 mg Cu/L with or without 30 percent of kcal from ethanol, were fed for 35 days. As with Experiment

One, the diet was designed to provide 1 kcal/mL of the diet in contrast to a solid diet which would have an average of 4 kcal/g. Therefore, 0.5 mg Cu/L was equivalent to a solid diet containing 2 mg Cu/kg which is 40 percent of the recommendation for copper for growth in the rat (45). Likewise, 2.5 mg Cu/L was equivalent to a solid diet containing 10 mg Cu/kg which is approximately two times the recommended amount (45). Diet analysis prior to the start of this study showed that copper concentration of each liquid diet was within 5 percent of expected values.

#### PROCEDURES COMMON TO BOTH EXPERIMENTS

##### Animal Care

All rats were housed in a ventilated, temperature-and light-controlled room used for trace element studies only. The room was located at the Laboratory Animal Resource Center at Oregon State University. Rats were kept in accordance with "Guide for The Care and Use of Laboratory Animals" (71).

##### Sample Collection

Humane euthanasia of all experimental animals was ensured by following recommendations of the American Veterinary Medical Association panel on euthanasia (72). Blood was drawn from dams and pups (Experiment One) and from weanling rats (Experiment Two) from the abdominal aorta under light sodium pentobarbital

anesthesia (3 mg/100 g body weight) into a plastic syringe (12 ml. Monoject, Sherwood Medical Industries, Inc., Deland, Fl.) with a 22 guage x 1 inch needle. After blood was collected, rats were killed by decapitation. The blood was then transferred to a silicon-coated tube, allowed to clot at room temperature for 30 minutes, and centrifuged for 10 minutes at 700 x g. Serum was collected with a serum separator, transferred to a plastic vial and placed on ice for transport to the laboratory. Serum samples were frozen at zero degrees C until serum copper assays were performed.

Liver samples were removed and weighed immediately after the blood was withdrawn. The sample was placed in a plastic bag, sealed, and stored on ice until it could be frozen at zero degrees C.

All blood and liver taken from the pups in Experiment One were pooled by litter. This was done to ensure that an appropriate amount of sample was available for analysis.

#### Precautions to Minimize Trace Element Contamination

Precautions to minimize trace element contamination included preparation of liquid diets with distilled-deionized water, use of glass feeding tubes with silicon rubber stoppers, and segregation of feeding tubes by groups for daily cleaning purposes. Crystalline vitamins were used in the preparation of vitamin mixes. All glassware used, including glassware used for



laboratory analysis, was soaked in 10 percent nitric acid for at least three hours, rinsed with distilled-deionized water, dried and stored in polyethylene bags until used (73). Reagent grade chemicals were used in all laboratory analyses and for the preparation of the mineral mix.

#### LABORATORY ANALYSIS

The experimental procedures followed were practiced and revised prior to the onset of each study. All analyses were performed in duplicate with the mean value being accepted as the true value if the two values were within 5 percent of the mean.

#### Liver Copper and Zinc

Liver copper and zinc concentrations were determined using Atomic Absorption Spectrophotometry (Perkin-Elmer 2380, Norwalk, CT). The liver was wet-ashed with concentrated nitric acid followed by 30 percent hydrogen peroxide on a hot plate in a fume hood. After complete digestion, the ash was dissolved in 3 mL of 3N HCl and diluted to 10 mL with redistilled water. This solution was aspirated into the burner of the spectrophotometer and analyzed according to standard conditions described by Perkin-Elmer (74).

### Serum Copper

Serum copper was determined by Atomic Absorption Spectrophotometry (74). Equal volumes of serum and trichloroacetic acid (8% w/v TCA) were added to a 5 mL Pyrex conical centrifuge tube, thoroughly mixed, and then allowed to stand for 5 minutes before being centrifuged at 700 x g for 10 minutes. The TCA acted to precipitate any proteins in the sample and to free the copper from the protein (75). The supernatant was directly aspirated from the centrifuge tube into the burner of the spectrophotometer and analyzed according to standard conditions as described by Perkin-Elmer (74).

### Superoxide Dismutase

Copper Superoxide Dismutase (Cu-Zn SOD) activity was determined by its ability to inhibit the autooxidation of pyrogallol (Catalog No. P-0381, Sigma Chemical Co., St. Louis, MO) by the method of Marklund and Marklund (76). One gram of liver was removed from the right lobe and placed in a test tube containing 4 mL of 0.25M sucrose. Liver was then homogenized (Cencostirrer NS 1-12, Central Scientific Co., Chicago, Ill.) by slowly moving the homogenizing tube over the pestle 10 times. Homogenates were centrifuged at 10,000 x g for 30 minutes (Beckman Model J-21C, Beckman Instruments, Inc., Palo Alto, CA). The pellet was discarded and equal volumes of supernatant and glycerol were mixed and stored at zero degrees C (77). The glycerol acted

to produce a more hydrophobic environment for the protein and to allow super-cooling of the protein without denaturation by freezing (77). The following day, preparations were removed from the freezer and allowed to come to room temperature before the assay was conducted. Cu-Zn SOD activity was determined in an assay medium of 50 mM triscacodylic acid (catalog no. D-6518, Sigma Chemical Co.), pH 8.3 at 25 degrees C. One unit of Cu-Zn SOD was defined as the amount of enzyme needed to obtain 50 percent inhibition of pyrogallol autooxidation (76). A Beckman Spectrophotometer (Model Du, National Technical Laboratories, South Pasadena, Ca.) was used to follow the rate of autooxidation of each sample. Rates were recorded once a minute for five consecutive minutes. Cu-Zn SOD units/g of liver were calculated by first comparing the change in absorbance of pyrogallol plus sample to the change in absorbance of pyrogallol alone. The percent inhibition caused by Cu-Zn SOD in the sample was then determined and, from that percentage, the number of units was calculated. The final number of Cu-Zn SOD units/g of liver was determined by dividing the number of units per microliter used by the actual number of grams of liver in the sample.

#### Statistical Analysis

The results for individual treatments are reported as mean + one standard deviation. Treatment effects were partitioned into effects due to copper, ethanol, and the interaction of the two

factors if a significant ( $P < 0.05$ ) F-value was found for treatments (78). Differences between planned comparisons of means were evaluated by Fishers' least significant difference and considered to be significant at  $P < 0.05$  (78).

## RESULTS

### EXPERIMENT ONE

The purpose of this experiment was to determine the possible interaction of ethanol and low dietary copper during pregnancy and lactation in the rat under experimental conditions that did not promote liver dysfunction. Results for experiment One have been divided into 5 sections: Energy Intake and Weight Gain, Litter Size and Weight Gain of Pups, Serum Copper, Liver Copper and Zinc, and Liver Copper-Zinc Superoxide Dismutase activity.

#### Energy Intake and Weight Gain

The average dietary intake during gestation and lactation and weight gain of dams are summarized in Table 1. Energy intake, which was similar for all groups, was comparable to those reported for rats consuming a conventional solid diet during gestation and lactation (70). No significant differences were found among groups in terms of maternal weight gain during either gestation or lactation.

#### Litter Size and Weight Gain of Pups

Litter size for the four treatment groups did not differ significantly from each other (Table 1). In addition, weights for the four treatment groups were not significantly different from

TABLE 1

Effect of maternal copper and ethanol (EtOH) ingestion on maternal energy intake and weight gain, litter size and offspring growth<sup>1,2</sup>

Measure	Diet, % kcal from Ethanol				Significance Levels		
	3.75 mg Cu/L		0.75 mg Cu/L		EtOH	Cu	Cu+EtOH
	0	30	0	30			
Energy Intake, ave. kcal/100 g body wt.							
Gestation	32 ± 2	31 ± 2	33 ± 1	31 ± 2	NS	NS	NS
Lactation	41 ± 2	39 ± 3	43 ± 2	41 ± 3	NS	NS	NS
Maternal Wt. Gain day 0 to 20 gestation	154 ± 15	134 ± 20	145 ± 21	135 ± 17	NS	NS	NS
Litter Size, n (parturition)	14 ± 2	12 ± 3	13 ± 4	14 ± 2	NS	NS	NS
Offspring wt. (g)							
Lactation day 1	7.7 ± 0.5	7.5 ± 0.4	7.6 ± 0.4	7.1 ± 0.3	NS	NS	NS
Lactation day 15	33.7 ± 3.7	31.1 ± 2.6	34.4 ± 4.2	28.6 ± 1.7	<0.005	NS	NS

<sup>1</sup> Values are means ± SD (n=6). Different superscripts along a horizontal line indicates a significant difference (P<0.05) of means. If any letter combination matches, the difference between means is not significant.

<sup>2</sup>

each other at day one of lactation. By day 15 of lactation, however, a significant decrease ( $P < 0.005$ ) in body weight was observed in the low copper and ethanol-fed group as compared to either the high copper or low copper groups not receiving ethanol.

#### Serum Copper

Serum copper concentration in dams was depressed by low dietary copper (Table 2). Rats consuming a low copper diet without ethanol showed a 27 percent reduction ( $P < 0.001$ ) in serum copper as compared to the high copper, non-ethanol group. This reduction was consistent with previous studies (47-49). Incorporation of ethanol into the diet increased serum copper at both levels of dietary copper but this effect was more pronounced when the dietary copper level was high rather than when it was low.

The level of copper in the maternal diet did not significantly influence the level of serum copper in pups (Table 2). In addition, differences in pup serum copper were non-significant when ethanol was incorporated into the maternal diet.

#### Liver Copper and Zinc

In the non-ethanol groups, liver copper in dams was decreased by 14 percent ( $P < 0.001$ ) in the low copper group as

TABLE 2

Effect of maternal copper and ethanol(EtOH) ingestion on maternal and offspring  
serum copper and liver zinc concentration <sup>1,2</sup>

Measure	<u>Diet, % kcal from Ethanol</u>				<u>Significance Level</u>		
	<u>3.75 mg Cu/L</u>		<u>0.75 mg Cu/L</u>		EtOH	Cu	Cu+EtOH
	0	30	0	30			
Serum Cu,ug/100 ml							
Maternal	129 ±12.7 <sup>b</sup>	158 ± 13.7 <sup>a</sup>	102 ±10.7 <sup>c</sup>	106 ±30.1 <sup>c</sup>	<0.05	<0.001	NS
Offspring	44.3± 6.4	40.6± 0.3	47.7± 6.3	42.2± 3.8	NS	NS	NS
Liver Zinc ug/g (wet weight)							
Maternal	25.6± 3.0	24.8± 2.1	28.4± 1.8	25.7± 1.8	NS	NS	NS
Offspring	37.4± 6.2	32.9± 9.9	38.1± 4.4	41.6± 8.2	NS	NS	NS

<sup>1</sup> Values are means ± SD (n=6). <sup>2</sup> Different superscripts along a horizontal line indicates a significant difference (P<0.05) of means.



compared to the high copper group (Figure 1). Incorporation of ethanol into the diet further depressed dam liver copper when the copper level was low but not when dietary copper was high (Figure 1). This interactive effect was demonstrated by a significant reduction ( $P < 0.05$ ) in the low copper and ethanol-fed group as compared to all other groups.

The level of copper in the maternal diet failed to significantly influence the concentration of liver copper in pups (Figure 1). Ethanol produced a significant decrease ( $P < 0.001$ ) in pup liver copper regardless of the level of copper in the maternal diet. Maternal ethanol consumption reduced pup liver copper by 38 percent in the high copper group and by 48 percent in the low copper group as compared to their respective non-ethanol control groups.

Differences in liver zinc values were not significant in this experiment for either dams or offspring (Table 2).

#### Copper Superoxide Dismutase

The enzymatic activity of liver Cu-Zn Superoxide dismutase (Cu-Zn SOD) in dams was significantly reduced by low dietary copper regardless of ethanol level (Figure 2). Ethanol did tend to reduce maternal Cu-Zn SOD activity at each copper level (main effect of ethanol  $P < 0.025$ ), but individual mean comparisons were not different. In pups, liver Cu-Zn SOD activity was

Figure 1.

Effect of ethanol (EtOH) and low dietary copper (hatched bars) on maternal and offspring liver copper concentration. Values are means  $\pm$  SD (n=6). Different superscripts in each figure represent a significant difference between means ( $P < 0.05$ ). If any letter combination matches, the difference between means is not significant. The main effects were EtOH ( $P < 0.05$ ), copper ( $P < 0.001$ ), and EtOH + copper ( $P < 0.05$ ) for dams and EtOH ( $P < 0.005$ ), copper (NS) and EtOH + copper (NS) for pups.

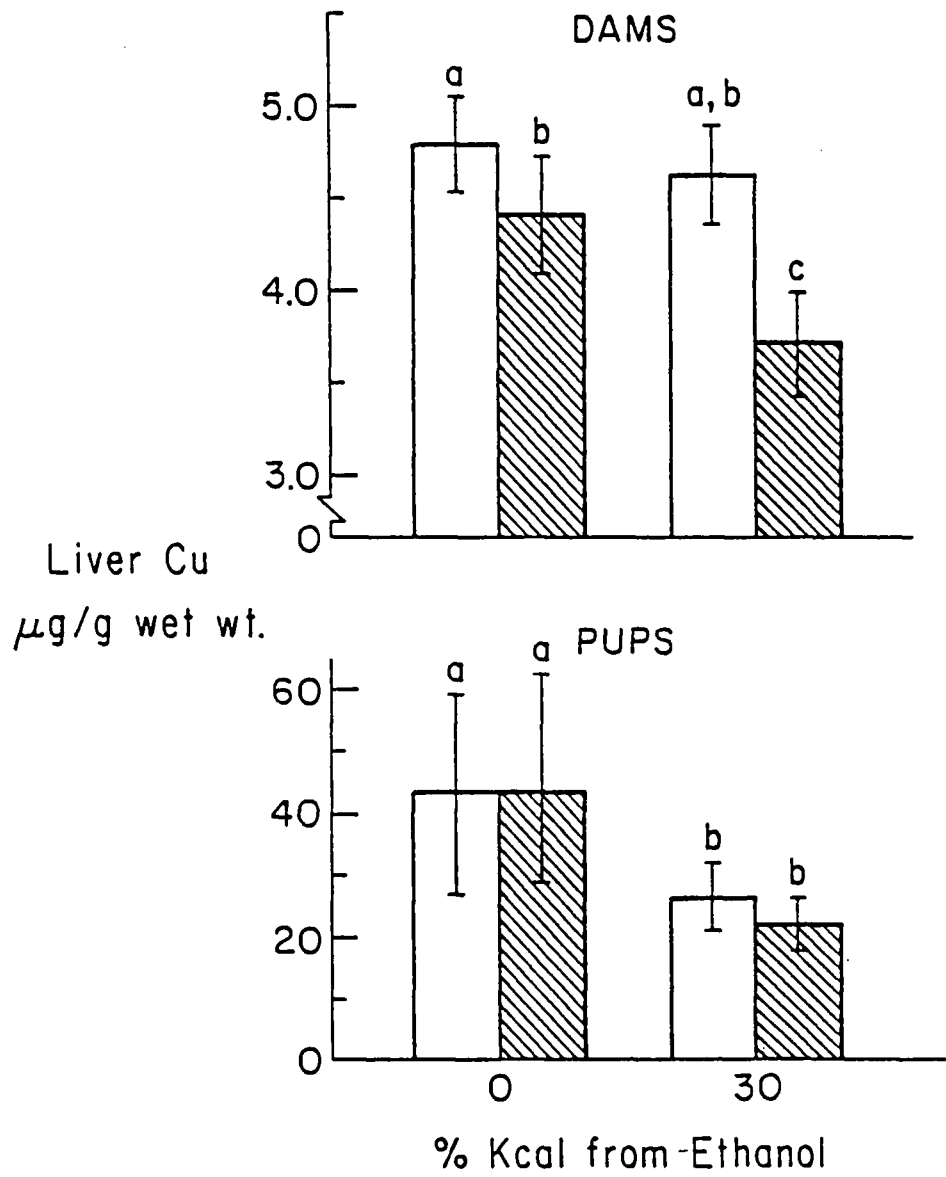


Figure 1

Figure 2.

Effect of ethanol (EtOH) and low dietary copper (hatched bars) on maternal and offspring Cu-Zn Superoxide Dismutase (SOD) activity. Values are means  $\pm$  SD (n=5). Different superscripts in each figure represent a significant difference between means (P<0.05). If any letter combination matches, the difference between means is not significant. The main effects were EtOH (P<0.025), copper (P<0.001) and EtOH + copper (NS) for dams and EtOH (P<0.05), copper (P<0.005) and EtOH + copper (NS) for pups.

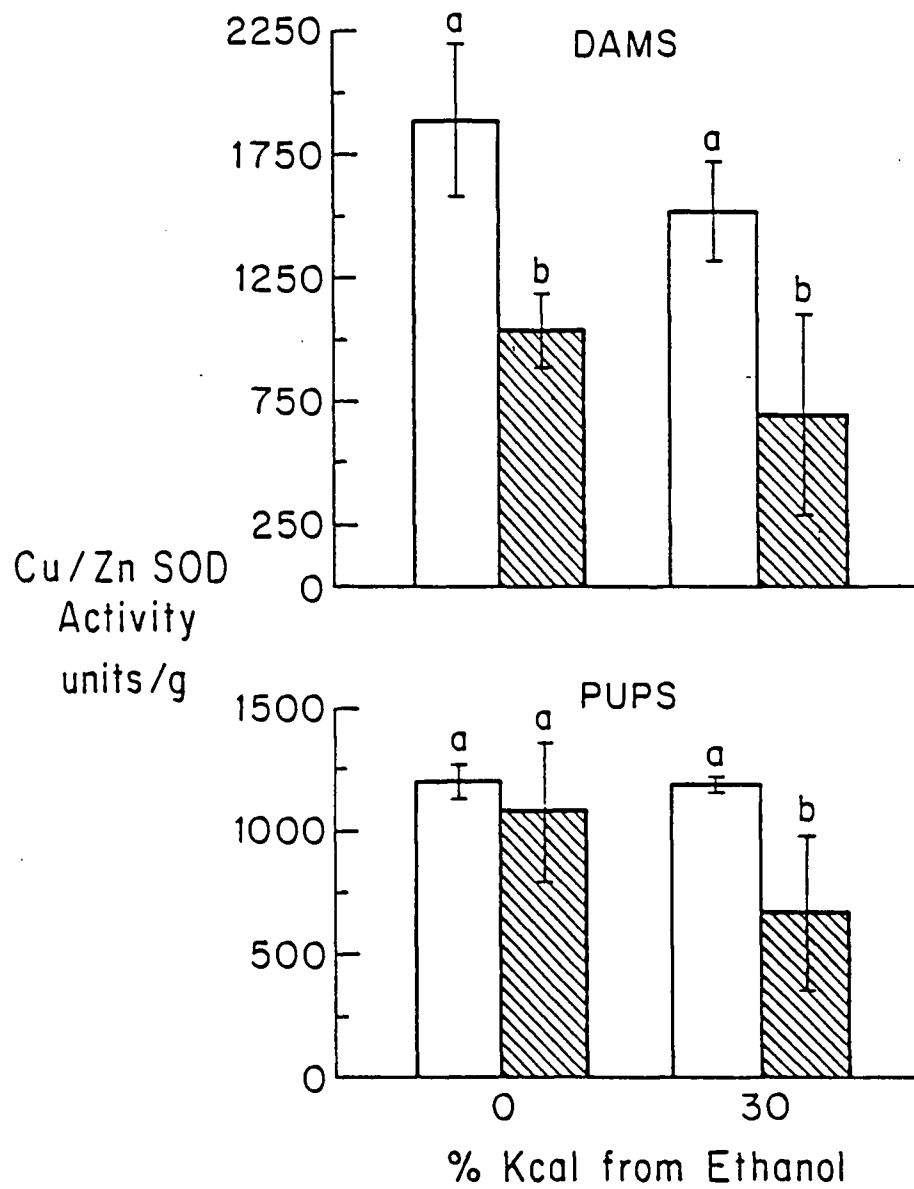


Figure 2

significantly depressed ( $P < 0.05$ ) in the low copper plus ethanol-fed group relative to all other treatments. This interactive effect just missed achieving significance ( $P < 0.06$ ).

## EXPERIMENT TWO

The purpose of Experiment Two was to determine the possible interaction of ethanol and low dietary copper during growth. This section has been divided into four sections: Energy Intake and Weight Gain, Serum Copper, Liver Copper and Zinc, and Liver Cu-Zn SOD activity.

### Energy Intake and Weight Gain

Rats fed diets containing high copper without ethanol grew significantly better than those fed diets containing either ethanol or low copper despite similar energy intake for all groups (Table 3). Ethanol depressed weight gain only at the higher level of dietary copper (interactive effect  $P < 0.05$ ).

### Serum Copper

Serum copper concentration was unaffected by either the level of dietary copper or ethanol (Table 3). Rats fed diets containing high copper plus ethanol did tend to have the highest serum copper concentration when compared to all other experimental

TABLE 3

Effect of dietary copper and ethanol (EtOH) on energy intake,  
weight gain, serum copper, liver copper, liver Cu-Zn Superoxide  
Dismutase (Cu-Zn SOD) activity, and liver zinc of young  
growing female rats<sup>1,2</sup>

TABLE 3

Measure	<u>Diet, % kcal from Ethanol</u>				<u>Significance Level</u>		
	<u>2.5 mg Cu/L</u>		<u>0.5 mg Cu/L</u>		EtOH	Cu	Cu+EtOH
	0	30	0	30			
Energy Intake (ave.) kcal/100 g body wt.	41 ± 5	43 ± 3	43 ± 5	42 ± 4	NS	NS	NS
5-Week wt. gain (g)	140 ± 7 <sup>a</sup>	113 ± 10 <sup>c</sup>	127 ± 13 <sup>b</sup>	119 ± 19 <sup>b,c</sup>	<0.01	NS	<0.05
Serum Cu ug/100 ml	132 ± 16	152 ± 29	130 ± 13	134 ± 11	NS	NS	NS
Liver Cu ug/g wet wt.	5.84 ± 0.43 <sup>a</sup>	5.19 ± 0.24 <sup>b</sup>	5.45 ± 0.33 <sup>a,b</sup>	5.53 ± 0.64 <sup>a,b</sup>	NS	NS	<0.025
Liver Cu-Zn SOD activity, units/g	1752 ± 138 <sup>a</sup>	1662 ± 213 <sup>a</sup>	1378 ± 290 <sup>b</sup>	1311 ± 227 <sup>b</sup>	NS	<0.001	NS
Liver Zinc ug/g wet wt.	29.5 ± 1.9	29.6 ± 1.3	31.5 ± 2.6	31.1 ± 1.9	NS	NS	NS

<sup>1</sup> Values are means ± SD (n=8). <sup>2</sup> Different superscripts along a horizontal line indicates a significant difference (P<0.05) of means. If any letter combination matches, the difference between means is not significant.



groups, but this effect was not significant.

#### Liver Copper and Zinc

Ethanol ingestion caused a small but significant reduction in liver copper concentration (Table 3) but only when the dietary copper level was high (interactive effect  $P < 0.025$ ). Liver copper concentration of rats fed low copper-containing diets was not significantly different from high copper groups regardless of the dietary ethanol level. Liver zinc concentration was unaffected by either the level of dietary copper or ethanol.

#### Liver Cu-Zn Superoxide Dismutase Activity

Cu-Zn SOD activity was significantly depressed in rats fed low copper-containing diets compared to high copper-containing diets regardless of the dietary ethanol level (Table 3). Ethanol did tend to depress Cu-Zn SOD activity at either dietary copper level, but the effect was not significant.

## DISCUSSION

The major hypothesis of this study was that ethanol ingestion during a period of high metabolic demand, represented by pregnancy and lactation (Experiment One) versus growth (Experiment Two), would exaggerate a marginal copper status to an obvious copper deficiency. The results obtained support this hypothesis evidenced by significant reductions in maternal liver copper concentration and enzymatic activity of the copper metalloenzyme Cu-Zn superoxide dismutase (SOD) in offspring liver. One dissenting piece of information in this regard was that maternal ethanol ingestion depressed pup liver copper regardless of dietary copper level. The reason why pup liver copper did not mimic the interactive effect ( $P < 0.05$ ) seen in maternal liver is not defined by present results. One explanation may be related to the fact that copper utilization is incompletely developed during the neonatal period (51). It is also possible that a measurement of pup liver copper was too insensitive to uncover the interactive effect between ethanol and copper level seen in maternal liver. A solution to this possibility might be to redefine the effect of maternal ethanol ingestion on pup liver copper in terms of copper content of the cytosolic metal-binding protein, metallothionein (79). On the other hand, ethanol had little or no effect upon

copper status in growing rats despite the fact that the levels of dietary copper deficiency in both the reproductive and growth study was 40 percent of that recommended (20,45).

At least two factors may have influenced our inability to see a depressive effect of ethanol on copper status of weanling rats in contrast to the reproductive study. It is possible, for example, that weanling and pregnant rats would have responded similarly to ethanol if the time-frame of the weanling rat study had been extended. This contention is supported by Dreosti et al. (47) who reported a 40 percent reduction in liver copper in ethanol-fed rats after 12 months. The length of the weanling rat study reported here, however, was only 35 days to approximately match the experimental time-frame of the reproduction study. The second factor which might explain differences in liver copper concentration between pregnant and weanling rats is the amount of dietary copper needed to achieve copper balance. It has been noted that balance studies are limited by the precision in which intake and output can be measured. For an element such as copper which has a slow rate of turnover, a variable degree of intestinal absorption, a strong homeostatic mechanism and an almost exclusive output via the feces, interpretations of balance studies becomes very difficult (39). However, recent studies in humans indicate that infants can maintain a positive balance with 0.5-1.0 mg Cu/day. Young children and adolescents require a minimum of 1.0-2.5 mg/day to maintain positive balance whereas pregnant and

lactating women are able to meet normal needs with a minimum of 2.0-3.0 mg Cu/day (42). Thus, it is easy to see that a pregnant woman must consume more copper to meet the requirements of increased metabolic demand. The present studies suggest that this is also true in the rat. Furthermore, when ethanol is combined with a diet low in copper, copper balance appears to be even more difficult to achieve during pregnancy.

Exactly why ethanol would only depress maternal liver copper concentration and enzymatic activity of the copper metalloenzyme Cu-Zn SOD in pups when dietary copper was low is unclear from present results. The present studies, however, do show that this ethanol effect cannot be explained on the basis of food intake because caloric intake was similar for all groups. This contention is supported by the fact that gestational weight gain, litter size at parturition and offspring body weight on the first day of lactation were similar for all groups. For undefined reasons, offspring body weight was slightly but significantly depressed by the 15th day of lactation in the low copper plus ethanol group relative to groups not fed ethanol. Other possible mechanisms that could explain the observed ethanol and copper interaction include an effect of ethanol on copper absorption and an enhanced effect of ethanol on removal of copper from the liver. In the former case, an induction of the metal-binding protein metallothionein by ethanol (80) could have been a factor. In the

latter case, ethanol may have enhanced copper removal from liver by way of either biliary excretion or by way of enhanced ceruloplasmin synthesis and secretion. These respective possibilities are supported by the fact that ethanol has been shown to stimulate secretion of glucocorticoids (81) which would enhance biliary copper excretion (79) and to stimulate estrogen secretion (82,83) which would stimulate ceruloplasmin synthesis (51). In both instances, liver copper depletion would be expected to be more pronounced in the low versus high copper group because the degradative turnover of liver copper is known to be accelerated in copper-deficient rats (84). Failure to see the ethanol and copper interaction in weanling rats unlike that for pregnant rats would be consistent with these hormone events because a simple copper deficiency was not achieved in the weanling rat study. Obviously, further research will be necessary to test the mechanisms proposed. The ability of ethanol to stimulate oxidase activity of ceruloplasmin and serum copper in mice (50) humans (69), and rats in the present studies, however, is consistent with an ethanol and hormone effect which suggests that the mechanism is worthy of further study.

With regard to significance, results of the reproductive study showed that the ethanol and copper interaction was of sufficient magnitude to significantly depress enzymatic activity of the copper metalloprotein Cu-Zn SOD in offspring. In weanling rats, on the other hand, ethanol failed to have a significant

effect upon Cu-Zn SOD enzymatic activity at either copper level. This observation again suggests that a critical level of copper deficiency must be achieved before an antagonistic effect of ethanol on copper utilization can be seen. Observed effects of ethanol and low dietary copper on Cu-Zn SOD activity was most likely the result of reduced availability of hepatic copper and not due to reductions of hepatic zinc because liver zinc was not reduced in the present studies. In addition, it is now known that copper's role in Cu-Zn SOD is catalysis while zinc is involved in stability of the enzyme. Recent experiments also show that a zinc deficiency does not reduce the enzymatic activity of Cu-Zn SOD in either liver or erythrocytes because it can be replaced by other metals (57).

The consequence of a reduction in Cu-Zn SOD is impaired tissue function due to increased lipid peroxidation (57). The primary function of Cu-Zn SOD appears to be that of scavenging the intermediates of oxygen reduction in aerobic organisms, namely superoxide anion radicals. Cu-Zn SOD catalyzes the conversion of the superoxide radical to hydrogen peroxide plus oxygen and the hydrogen peroxide is removed by catalase and peroxidases. Thus, Cu-Zn SOD is thought to help protect the cell from damaging effects of oxygen toxicity (57). Relating diminished Cu-Zn SOD to peroxidation damage is still difficult, however, because minimum requirements of this enzyme have not yet been established. Once these levels have been determined, Cu-Zn SOD activity will prove

to be an easy and sensitive measure of peroxidation damage within the liver.

One other thing that was learned from the present studies was that serum copper failed to reflect a developing depletion of liver copper when ethanol was ingested. On the other hand, assesment of copper status with superoxide dimutase activity did, for the most part, reflect liver copper status. Although measurement of superoxide dimutase activity in liver of human subjects would be impractical, the same copper-responsive enzyme also occurs in the much more accessible erythrocyte (67). It is therefore possible that measurement of erythrocyte Cu-Zn SOD could help to uncover previously unrecognized cases of copper deficiency in alcoholics. Further research, however, will be needed to test this possibility.

In conclusion, results of the present studies indicate that meternal ethanol consumption exaggerates a marginal dietary copper status into an obvious copper deficiency. Because it is known that the average American diet is just adequate in copper content, and that copper balance is more difficult to achieve during times of increased metabolic demand, pregnant subjects may be at special risk to develop a copper deficiency when ethanol is consumed. This ethanol and copper interaction, however, will likely go undetected if only serum copper is used as an indicator of copper status.

## SUMMARY

Alcoholism is the most common cause of malnutrition in the United States today. During times of increased metabolic demand, such as during pregnancy, the antagonistic effects of ethanol on nutrient utilization become even more pronounced (36). This is evidenced by the fact that ethanol consumption during pregnancy causes an even greater demand for many nutrients. It is still undetermined, however, if ethanol will antagonize nutrient utilization during a rapid growth phase. During this phase metabolic rate is also higher than normal adult levels. Therefore, it may be assumed that the effects of alcohol on nutrient metabolism during a rapid growth phase will be similar to the effects seen during pregnancy. However, because the demand for most nutrients is greater during pregnancy than for simple growth, the antagonistic effects of ethanol should be more pronounced during pregnancy.

In the present study it was of interest to determine if ethanol ingestion during a period of high metabolic demand, represented by pregnancy and lactation (Experiment One) versus growth (Experiment Two), would exaggerate a marginal copper status to an obvious copper deficiency. Very little information is currently available about a copper and alcohol relationship in non-pregnant adults and even less is known about this relationship during pregnancy and growth. Copper is a necessary component of



several metalloenzymes involved in important metabolic processes. Therefore, if ethanol did disrupt the bioavailability of copper, there could be a significant depression of copper-dependent enzymes such as ceruloplasmin which plays a role in copper transport and iron reutilization, lysyl oxidase which is important for cross-linking of the structural proteins collagen and elastin, and copper-zinc superoxide dismutase which protects cells from cellular oxidants (17,18).

Results of the present study support the hypothesis that ethanol ingestion during a period of high metabolic demand exaggerates a marginal copper status to an obvious copper deficiency during pregnancy. This is evidenced by significant reduction in maternal liver copper concentration and by a reduction in the enzymatic activity of the copper metalloenzyme copper-zinc superoxide dismutase (Cu-Zn SOD) in offspring liver. On the other hand, ethanol had little or no effect upon copper status in growing rats despite the fact that the levels of dietary copper deficiency in both the reproductive and growth studies was 40 percent of that recommended. Exactly why ethanol would produce these varied effects is not clear from the present study.

Possible mechanisms that could explain the observed ethanol and copper interaction during pregnancy include an effect of ethanol on copper absorption and an enhanced effect of ethanol on removal of copper from the liver. In the former case, an induction of the metal-binding protein metallothionein by ethanol

(80) could have been a factor. In the latter case, ethanol may have enhanced copper removal from liver by way of either biliary excretion or by way of enhanced ceruloplasmin synthesis and secretion. Enhanced ceruloplasmin synthesis could explain the observed increase in maternal serum copper concentration. These respective possibilities are supported by the fact that ethanol has been shown to stimulate secretion of glucocorticoids (81) which would enhance biliary copper excretion (79) and to stimulate estrogen secretion (82,83) which would stimulate ceruloplasmin synthesis (51). In both instances, liver copper depletion would be expected to be more pronounced in the low versus high copper group because the degradative turnover of liver copper is known to be accelerated in copper-deficient rats (84). Failure to see the ethanol and copper interaction in weanling rats unlike that for pregnant rats would be consistent with these hormone events because a simple copper deficiency was not achieved in the weanling rat study. In addition, two factors could explain why pup liver copper did not mimic the interactive effect ( $P < 0.05$ ) seen in maternal liver. The first factor may be related to the fact that copper utilization is incompletely developed during the neonatal period (51). The second factor may be that the measurement of pup liver copper was too insensitive to uncover the interactive effect between ethanol and copper level seen in maternal liver. Obviously, further research is necessary to test the mechanisms proposed.

In conclusion, the present studies indicate that ethanol antagonizes copper utilization during pregnancy and lactation and, to a lesser degree during simple growth. The effects during pregnancy, however, were severe enough to cause reductions in pup liver copper concentration regardless of the level of copper in the maternal diet. Possible reasons for these observed differences between pregnant and growing rats may include an ethanol-hormone interaction during pregnancy that results in increased biliary excretion and increased synthesis and secretion of the copper-dependent enzyme ceruloplasmin. Further research will be necessary to test this possibility. It is known, however, that the average American diet is just adequate in copper content, and that copper balance is more difficult to achieve during times of increased metabolic demand. Therefore, pregnant subjects may be at a special risk to develop a copper deficiency when ethanol is consumed. Because serum copper concentration increases when ethanol is being consumed, however, it is likely that this ethanol and copper interaction will go undetected if only serum copper is used as an indicator of copper status.

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