AN ABSTRACT OF THE THESIS OF

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Title: Perinatal and Postweaning Effects of the Interaction between Maternal Ethanol Ingestion and Low Dietary Zinc in the Rat

Abstract Approved: _____

Florian L. Cerklewski

This research was designed to study the perinatal and postweaning effects of the interaction between ethanol and low dietary zinc during gestation and lactation in the rat.

Pregnant rats were fed liquid diets containing either 2 or 10 μg zinc/ml with or without 30% of kcal from ethanol throughout gestation and lactation. The liquid diet formulation was nutritionally adequate to insure offspring growth and survival during lactation. At weaning, dams and five of eight offspring from each litter were killed by exsanguination under sodium pentobarbital anesthesia. The remaining offspring were orally inoculated with Streptococcus mutans and fed a caries-promoting diet for six weeks.

The low zinc diet produced a moderate zinc deficiency in dams as evidenced by a decrease in tissue zinc content, serum alkaline

phosphatase activity, and urinary zinc concentration. Despite the presence of high zinc content in the diet, ethanol antagonized maternal zinc status to a level typical of that produced by the low zinc diet. The lowest zinc status, however, was found when low dietary zinc and ethanol were combined. The maternal interaction between ethanol and zinc also depressed offspring serum zinc and alkaline phosphatase activity in a similar manner but the magnitude was smaller. The maintenance of a lower than normal maternal tissue zinc and decreased maternal urinary excretion of zinc suggested a maternal attempt to support the growth and development of offspring despite zinc deficiency.

Physiological consequences of ethanol-antagonized zinc status were evidenced by depressed activity of maternal and offspring serum alkaline phosphatase, increased maternal urinary excretion of hydroxyproline, decreased offspring molar enamel and dentin zinc content, increased dental caries score, and decreased cross-linking structure of mandibular second molar enamel.

The liquid diet developed in the present study was nutritionally adequate and allowed for the investigation of a single nutrient deficiency, zinc, in ethanol fed rats during gestation and lactation without confounding effects of general malnutrition. Although the direction of interaction was predominately an effect of ethanol on zinc rather than the effect of zinc on ethanol, this study clearly indicates that zinc deficiency is an important consequence of maternal ethanol ingestion.

Perinatal and Postweaning Effects of the Interaction between Maternal Ethanol Ingestion and Low Dietary Zinc in the Rat

by

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A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

completed February 22, 1984

Commencement June 3, 1984

APPROVED:

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Date thesis is presented February 22, 1984

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ACKNOWLEDGEMENTS

Sincere appreciation and thanks are expressed to Dr. Florian L. Cerklewski, my major professor, for encouraging and supporting me to explore my area of interest throughout the graduate program. This research would not have been possible without his interest and guidance.

I would like to thank Dr. Donald R. Buhler for the use of the liquid feeding tubes, the Kelco Corp. for providing xanthan gum, Eastman Chemical Products for providing anti-foaming and dispersing agents, and A.E. Staley Co. for providing a sample of pre-gelatinized starch, Dr. Te-May Ching, Mr. Harvey T. Holmes, and Mr. Alfred Soeldner for their technical assistance, and the staff of the Laboratory Animal Resources Center for their care of the animals. Special thanks to the members of my graduate committee, Dr. Margy J.Woodburn, Dr. David R. Thomas, Dr. Ronald E. Wrolstad, and Dr. Paul Kopperman, for their time and energy.

I am grateful to Dr. Margy J. Woodburn, Dr. Cheryl Bittle, and Dr. Thomas R. Shearer for reviewing the three manuscripts presented in this thesis. In addition, I would like to thank the faculty and graduate students in the Department of Foods and Nutrition for their suggestions, encouragement, and friendship during the course of this study.

Finally, my deepest appreciation to my husband, my parents and family for their understanding, patience, and love throughout these past years. The help, encouragement, and emotional support from my friends are particularly appreciated. I am truly fortune for having had such wonderful people in my life to give me all the love, understanding, and help I needed to pursue an advanced degree - it is to them that I dedicate this thesis.

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CHAPTER I

INTRODUCTION

It has been suspected since ancient times that alcohol (ethanol) consumption during pregnancy can be harmful (1), but it is only in the last few years that a characteristic pattern of central nervous system dysfunction, growth retardation, typical facial appearance, and organ malformations termed "fetal alcohol syndrome" (FAS) has been recognized (2-5).

Incidence of the fetal alcohol syndrome in children of chronically alcoholic women has been reported to range from 30 to 50% (6, 7) so that between one in 600 and one in 1,000 live births in the United States have this syndrome (8). It has been suggested that the fetal alcohol syndrome is one of the leading causes of birth defects associated with mental retardation (4, 9). The frequency of congenital functional anomalies and growth retardation was more than doubled in offspring born to heavy drinkers, as compared with those born to women who abstained or drank moderately (10-12). In addition, lower birth weight was also reported (6, 13, 14). Although drinking less alcohol may result in reduced risks for infants, a totally safe level of consumption in pregnancy has yet to

be ascertained. In the meantime, the National Institute on Alcohol Abuse and Alcoholism (NIAAA) warns that there is a definite risk in drinking 90 ml (3 oz) or more of ethanol a day (i.e., 6 drinks or more). Drinking one to three ounces of ethanol a day may be risky (15). These findings confirm an association between maternal drinking during pregnancy and increased risk of poor pregnancy outcome.

Although a mechanism for the fetal alcohol syndrome is ill-defined, it is likely to involve nutrition since ethanol is known to antagonize the metabolism of several vitamins and minerals (9, 16). The pattern of congenital malformations produced by dietary zinc deficiency for example has been noted to be similar to the fetal alcohol syndrome (16, 17). In addition, changes in the metabolism of zinc have long been related to chronic ethanol consumption (18-20) and zinc is involved in the metabolism of ethanol (21, 22). Since zinc is involved in the metabolism of carbohydrate, fat, protein, and nucleic acids (23, 24), it is possible that zinc deficiency produced by ethanol may account for a large part of the metabolic effects of ethanol.

The purpose of this study was to investigate the possible biochemical interaction of ethanol and low dietary zinc during pregnancy and lactation using the albino rat as an animal model. The hypothesis was based on the concept that the antagonistic effect of ethanol on zinc metabolism and function may be exaggerated when ethanol is combined with a low zinc diet. This study has been

organized into three areas. The first area (Chapter II) deals with the formulation of a nutritinally adequate liquid diet for ethanol studies involving gestation and lactation in rats. The use of this diet makes it possible to study a single nutrient deficiency without confounding effects of general malnutrition. The second area (Chapter III) deals with the interaction between ethanol and low dietary zinc during gestation and lactation in both dams and offspring. The last area (Chapter IV) deals with the developmental influences of maternal ethanol ingestion during molar pre-eruptive period on molar composition and dental health of offspring.

Before considering these areas, however, it will be necessary to review the metabolism and effects of ethanol as they relate to the essential functions of the trace element zinc.

ETHANOL - FOOD OR DRUG

Ethanol, commonly known as alcohol, is self-ingested orally in the form of beer, wine, and distilled spirits with the ethanol concentration varying from approximately 4% by volume in beer, to 12% in wines, to 40-50% in distilled spirits (25). The amount of absolute ethanol (i.e., pure 100% ethanol) in any type of alcoholic beverage is calculated by multiplying the percent 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 g of ethanol a day. This amount of ethanol is contained in nine single measure of spirits, one bottle of wine, or five pints of beer (25).

Ethanol provides 7.1 kcal/g and may be referred to as a food (26). Alcoholic beverages however are essentially devoid of important nutrients such as protein, vitamins, and minerals. Ethanol is also classified as a general central nervous system depressant and may be referred to as a drug (9). Ethanol in quantities that constantly approach or exceed the capacity for metabolic elimination can be deleterious (26).

ABSORPTION AND METABOLISM OF ETHANOL

Ethanol is absorbed mainly from the small intesine and distributed uniformly throughout total body water. Ethanol is a lipid-soluble nonelectrolyte, and as such it is rapidly absorbed into the circulation by diffusion across the gastrointestinal mucosa (27). Factors which affect the absorption of ethanol from the gastrointestinal tract include the concentration of ethanol, the nature and surface area of mucosa, the blood flow through the mucosal capillaries, the rate of stomach emptying, and the presence or absence of food in the gastrointestinal tract (27, 28). For example, high concentration of ethanol (>30%) tends to reduce gastric motility which significantly slows down absorption. Food in the stomach reduces diffusion of ethanol by acting as a barrier to

ethanol's contact with mucosa. High carbohydrate, high fat, and glucose all tend to decrease ethanol absorption.

Over 70% of absorbed ethanol is metabolized or eliminated in the liver by metabolic conversion to carbon dioxide and water. As shown in figure I.1, ethanol is first oxidized to acetaldehyde. The oxidation of ethanol to acetaldehyde is catalyzed by three enzyme systems: (a) alcohol dehydrogenase (ADH), (b) catalase, and (c) microsomal ethanol-oxidizing systems (MEOS) (26, 28, 29). Only the reaction catalyzed by alcohol dehydrogenase causes a reduction in NAD/NADH ratio. It is generally believed that the rate-limiting step in the metabolism of ethanol is its oxidation to acetaldehyde by alcohol dehydrogenase. Liver alcohol dehydrogenase, a cytosolic NAD-dependent and zinc-dependent enzyme, is responsible for the major production of acetaldehyde from ethanol under low or moderate levels of intake. Catalase and MEOS may function as alternate pathways of ethanol oxidation. Their contribution is normally minor, but can become important when blood ethanol concentrations reach very high levels (26, 28, 29).

Acetaldehyde is cytotoxic (30) with an inhibitory effect on liver alcohol dehydrogenase activity. It is however quickly removed by further oxidation to acetate. The oxidation of aetaldehyde to acetate is catalyzed by acetaldehyde dehydrogenase which is also NAD-dependent but not zinc-dependent. On the average, 75% of the ethanol taken up by the liver is released as acetate into the circulation. Acetate is available for most tissues to produce

Fig. I.1 The principal pathway of ethanol metabolism in the liver cell. ADH = alcohol dehydrogenase, ALDH = aldehyde dehydrogenase, X = oxidized intermediate of hydrogen shuttle, XH_2 = reduced intermediate of hydrogen shuttle. (Taken from Dawson, A.G. 1983 What governs ethanol metabolism? Trends Biochem. Sci. 8, 196.)

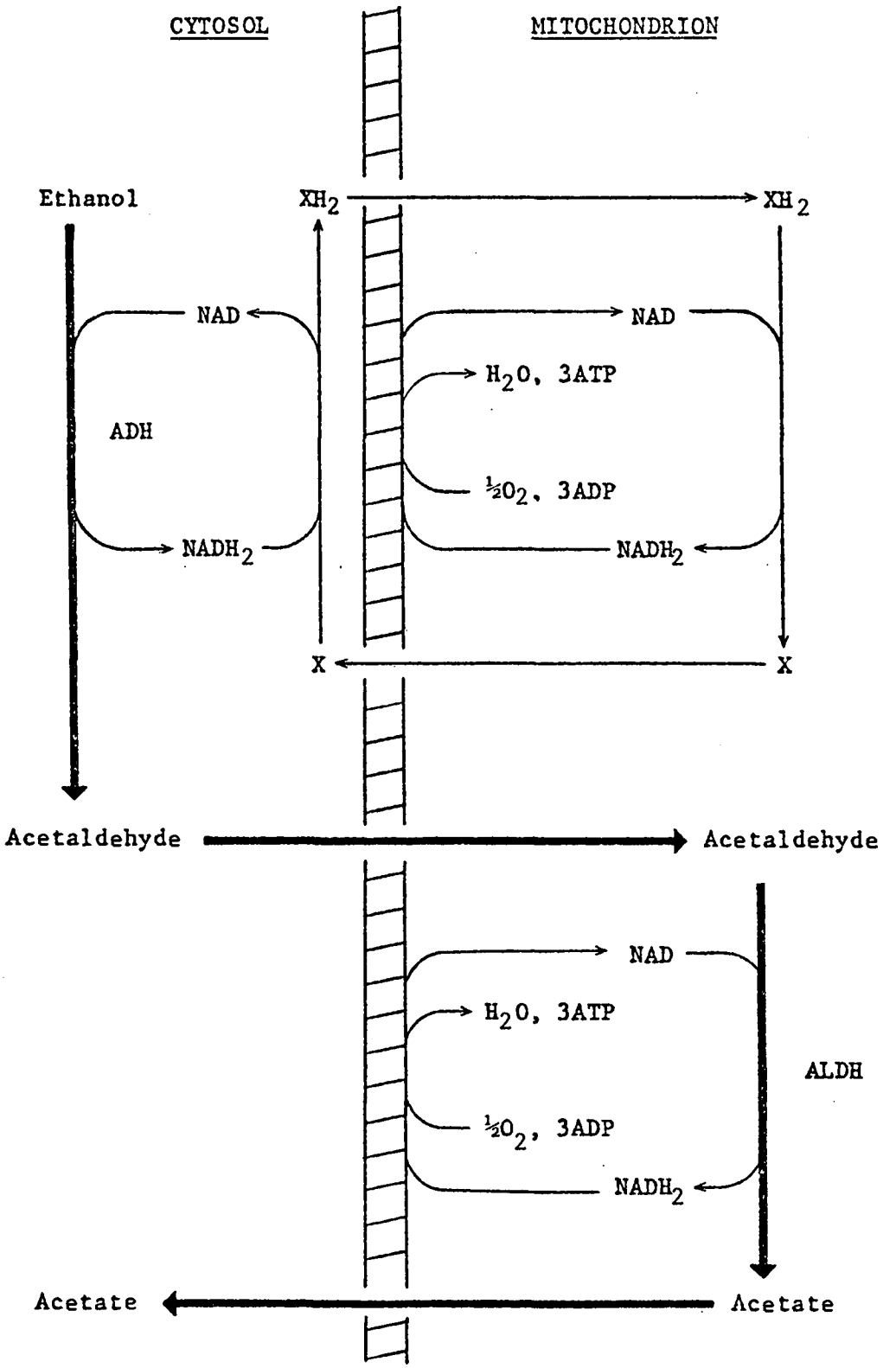
CYTOSOL
MITOCHONDRION
Ethanol
XH2
XH2
NAD
NAD
H2O, 3ATP
ADH
½O2, 3ADP
NADH2
NADH2
X
X
Acetaldehyde
Acetaldehyde
NAD
H2O, 3ATP
ALDH
½O2, 3ADP
NADH2
Acetate
Acetate

Fig. I.1

energy by way of the tricarboxylic acid cycle (26). In addition, ethanol oxidation is a source of energy because the production of NADH supplies the electron transport system with hydrogen equivalents and yielding high energy phosphate bonds for ATP synthesis.

The rate of ethanol metabolism can be regulated by three important factors (31) which include the level of liver alcohol dehydrogenase (32), the NAD/NADH ratio (33), and/or the level of acetaldehyde (34). It is also important to point out that the principal pathway of ethanol metabolism is integrated with other metabolic processes such as hydrogen shuttles, which means that ethanol oxidation can be controlled to a greater or lesser extent by factors that regulate these other processes (31).

Changes in rate of ethanol metabolism have also been shown to vary with time of day, previous drinking history, nutritional status, and genetic factors (9). Many attempts have been made to increase the metabolic rate of ethanol oxidation. It has been shown for example that fructose, pyruvate, and glyceraldehyde can stimulate ethanol oxidation indirectly by increasing the rate of NADH oxidation and energy consumption by the liver. Pyrazol and 4-methylpyrazol, potent inhibitors of liver alcohol dehydrogenase, have been reported to be effective in inhibition of ethanol metabolism (26). Disulfiram (Antabuse, tetramethylthiuram disulfide) and cyanamide, inhibitors of acetaldehyde dehydrogenase, have been found to stop ethanol metabolism by increasing

acetaldehyde levels (34). Castration (32) and thyroidectomy (35) have been reported to increase alcohol dehydrogenase activity.

HEALTH HAZARDS ASSOCIATED WITH ETHANOL CONSUMPTION

Ethanol significantly impairs performance of sensory-motor and cognitive tasks at blood ethanol levels as low as 40 mg/dl (8). Non-disease health hazards associated with ethanol consumption include suicides, traffic fatalities, industrial accidents, drownings, burns, assault, rape. child abuse, and family violence in general.

Ethanol has potentially detrimental effects on the gastrointestinal tract, liver, and pancreas (9). Liver however is the organ most significantly damaged and physiologically deranged as a result of ethanol ingestion. Chronic ethanol ingestion results in numerous aberrations in intermediary metabolism of carbohydrate, fat, and protein in liver. Those alterations result from a direct effect of ethanol itself as well as indirect effects arising from the oxidation of ethanol (36). The most profound effect of ethanol metabolism in the liver is related to increased formation of NADH, which alters the redox state of the liver. A highly reduced state of the liver results in decreased gluconeogenesis, tricarboxylic acid cycle activity and ATP production; increased conversion of pyruvate to lactate; and fatty liver (29, 30). Fatty liver, hepatitis, and hepatic cirrhosis are the three most common hepatic complications of alcoholism (37).

Ethanol ingestion during pregnancy is hazardous not only to the expectant mother, but also to the unborn child since ethanol can cross the placental barrier and reach the developing fetus (38). The consumption of alcoholic beverages by pregnant women and the concern about adverse effects on offspring are as ancient as recorded history (1). For example, a newly married couple was forbidden to drink wine on their wedding night for fear that a child may be conceived in drunkenness.

In 1968, a pattern of prenatal and postnatal growth deficiency, developmental delay, mental retardation, microcephaly, and facial defects in offspring of chronic alcoholic women was described and attributed to the effects of ethanol in utero (39). It wasn't until five years later however that Jones and Smith and their coworkers (2, 3) coined the phrase fetal alcohol syndrome (FAS). Since then, many cases of fetal alcohol syndrome in infants born to alcoholic mothers have been identified both retrospectively and prospectively (40, 41). The characteristic features of fetal alcohol syndrome have been classified into four categories: (a) central nervous system dysfunction, (b) growth retardation, (c) typical facial appearance, and (d) various malformations (5, 6). The variability in the pattern of defects is probably related to differences at critical stages of pregnancy in terms of blood ethanol concentration; frequency and duration of drinking; types of beverages; nutritional status; synergistic effects of smoking, drug abusing, coffee drinking; and genetic differences (42). Age,

parity, race and socio-economic status also influence the incidence of ethanol-related fetal defects (43). Confounding factors can be controlled by experimental animal models (42, 44, 45) to determine the role of ethanol in the fetal alcohol syndrome. Rat studies for example have confirmed that litter size, birth weight of pups (43), postnatal growth and development were all depressed in offspring nursed by dams fed ethanol during pregnancy (46-53).

Maternal ethanol ingestion during lactation is also hazardous to nursing infants since ethanol can readily enter the neonates through breast milk (54, 55), decrease the milk injection (56), and impair the newborn's ability to suck (57). In laboratory rats, the body weights of offspring nursed by dams fed 10% (v/v) ethanol throughout lactation were significantly lower (13%) than that of control (58). Impaired physical growth and development has also been reported in offspring nursed by dams fed a liquid diet with 35% of kcal from ethanol (48).

Although maternal ethanol ingestion during gestation and lactation is known to have detrimental effects on both mother and child, the actual mechanisms underlying these hazards are ill-defined. Since both gestation and lactation are periods of high metabolic demands, it is likely that deficiency of one or more nutrients may account for a part of the metabolic effects of ethanol.

ETHANOL-INDUCED MALNUTRITION

Chronic ethanol ingestion leads to primary and secondary malnutrition. Primary malnutrition is caused mainly by impaired appetite due to gastrointestinal and liver disorders and decreased food intake. Secondary (or conditioned) malnutrition is caused by multiple factors which include ethanol-induced gastrointestinal damage, deficiency-induced maldigestion and malabsorption, and decreased activation or increased inactivation of nutrients (59). In addition, inefficient utilization of energy has been reported in alcoholics as another contributory factor to malnutrition (60). For example, 20 oz of 86-proof alcoholic beverage represent about 1,500 kcal, or one-half to two-thirds of the normal daily caloric requirement. Therefore, alcoholics have a much reduced demand for food to fulfill their caloric needs. Because alcoholic beverages do not contain significant amounts of protein, vitamins, and minerals, the intake of these nutrients may become insufficient. Vomiting, diarrhea, anorexia, and liver damage are often complicating factors (29, 61).

Alcoholism has been suggested as the most common cause of undernutrition and the chief cause of vitamin and mineral deficiency in adults in the United States (62). Nutritional status for a number of nutrients such as folic acid (63, 64), thiamin (65, 66), vitamin B-6 (67), vitamin A (20), calcium (61), magnesium (61), and zinc (18-20) has been found to be suboptimal in alcoholics. The next section will summarize what is presently known about the

effects of ethanol on zinc metabolism and status.

EFFECTS OF ETHANOL ON ZINC METABOLISM

Ethanol significantly antagonizes zinc status as evidenced by decreased serum zinc levels and increased urinary excretion of zinc in alcoholic patients (18, 19, 68). Possible mechanisms for the ethanol-antagonized zinc status may involve a decreased intestinal zinc absorption related to decreased functional integrity of the small intestine (69, 70), or an increased urinary zinc excretion (68), or a combination of these effects. Ethanol for example is also known to increase release of cellular zinc into the circulation coupled with a change in the binding of zinc to plasma protein resulting in greater than normal loss of zinc by the renal excretion (20, 68). The negative zinc balance caused by ethanol has been in fact confirmed in laboratory rats by decreased tissue zinc content and increased zinc loss from urine and feces (71, 72).

The relevance of ethanol-antagonized zinc status becomes clear when one considers the fact that zinc is an essential trace element in many metabolic pathways and is present in almost all tissues (23, 24). Zinc metalloenzymes participate not only in carbohydrate, protein, and nucleic acid metabolism, but also in ethanol metabolism as alcohol dehydrogenase. At the molecular level, zinc appears to function as an essential component of enzymatic catalysis and as a determinant in the structural configuration of certain nonenzymatic macromolecules (23, 24).

Zinc, which is absorbed mainly in the small intestine, is homeostatically controlled by the body zinc status. This accounts for the fact that fecal zinc represents the major route of zinc excretion. Fecal zinc consists mostly of unabsorbed dietary zinc with a small amount of endogenous origin secreted into the small intestine. The fact that the quantity of zinc excreted in the urine is small and does not vary greatly with dietary zinc levels (23) further emphasizes the importance of intestinal control on zinc homeostasis. The effect of ethanol on zinc status is to decrease intestinal zinc absorption and increase loss of both fecal and urinary zinc. Absorbed zinc is carried in the portal plasma bound to transferrin and metabolized mainly in the liver. The liver cytosol contains zinc-binding components of different molecular weight and lability (such as metallothionein), the amounts and proportions of which vary with the zinc status. This zinc is incorporated at differing rates into different tissues which reveal varying rates of zinc turnover. The most rapid accumulation and turnover of retained zinc occurs in the liver, kidney, pancreas, and spleen. On the other hand, turnover of bone zinc is relatively slow but it represents a major body pool of zinc (23, 24). Since ethanol is antagonistic to zinc status, it may become important to skeletal health because zinc may aid in calcification as an inorganic constitutent of bone or as an activator of the calcification process itself. Zinc is found in small but constant amounts in insoluble organic fraction of bone even when bone is demineralized (73).

Since the importance of zinc-dependent serum alkaline phosphatase appears to be involved in bone formation and modeling, the decreased activity of serum alkaline phosphatase in ethanol-fed rats (74) may indicate an association between ethanol and skeletal health.

The recommended dietary allowance (RDA) for adult men and women is 15 mg/day. A higher intake is recommended during pregnancy (20 mg/day) and lactation (25 mg/day). The fact that more zinc is required during pregnancy and lactation may make the zinc status of alcoholic women even worse. In addition, the pattern of congenital malformations produced by dietary zinc deficiency has been noted to be similar to the fetal alcohol syndrome (16, 17). Although the actual mechanisms underlying the adverse effect of ethanol consumption during pregnancy and lactation are still unknown, it is likely that the fetal alcohol syndrome may arise as a consequence of secondary zinc deficiency and the ethanol-antagonized zinc status may play an important role in the impaired growth and development.

The possible role of zinc deficiency in fetal alcohol syndrome has been suggested by several investigators (1, 75-78). A higher incidence (37%) of birth defects has been related to low zinc status of pregnant women who consumed ethanol during pregnancy (75). The mean maternal plasma zinc of 25 alcoholic women was significantly lower than that of 25 non-alcoholic women (50.7 versus 72.2 μg/dl) and suggested to be a clue to the mechanism of ethanol-induced defects. However, information on age, race, nutritional status of these pregnant women was unavailable.

In pregnant rats, it has been shown that fewer (34%) and smaller (16%) fetuses were produced in the group fed 24% (v/v) ethanol eight weeks prior to and during pregnancy (79). The zinc content of maternal muscle and fetal carcass was lower in the ethanol-fed rats than in the pair-fed controls (18 and 8%, respectively). The inhibition of placental zinc transport to fetus as a potential mechanism for fetal growth retardation (80) has been suggested in pregnant rats fed a liquid diet with 35% of kcal from ethanol from day 4 to day 20 of gestation. The significantly lower zinc content of maternal serum, placenta, and fetal carcass (23, 9, and 13%, respectively) in ethanol-fed rats indicated that the availability of zinc to fetus was decreased by ethanol. However, supplementation of the ethanol containing liquid diet with zinc (40 μg/ml) did not overcome the inhibitory effect of ethanol on placental zinc transport (81).

In adddition, the rate of protein synthesis by livers of fetal and neonatal rats was significantly lower in offspring of the ethanol-fed rats compared with the control group (82). Because zinc is an essential trace element for RNA and DNA synthesis, protein synthesis, and nucleic acid synthesis (16), it has been postulated that the decrease in hepatic RNA content, the inhibition of protein synthesis, and the subsequent retarded growth of offspring of alcoholic mothers may be related to secondary zinc deficiency.

An increased retention of zinc during pregnancy and lactation to meet the demand made by the developing fetus and postnatal

offspring was reported in laboratory rats (83). Zinc deficiency during lactation has been shown to cause impaired postnatal growth and development of offspring (84), increased dental caries following a caries-test challenge than those fed a zinc-adequate diet (85, 86), and decreased zinc content of whole molar (85) or separated enamel and dentin (86). Present knowledge however does not provide any information about offspring dental health in relation to the ethanol-antagonized zinc status during lactation.

The first substantial evidence that zinc deficiency may contribute to ethanol-induced teratology was performed recently in pregnant rats (87). An interaction between transient dietary zinc deprivation and acute ethanol intoxication during pregnancy was presented in terms of teratogenesis when compared to either ethanol injection or zinc deprivation alone. However, little information is now available in terms of biochemical effects of the interaction between chronic ethanol ingestion and low dietary zinc during gestation and lactation and physiological consequences of the ethanol-antagonized zinc status. Therefore, it is necessary to study the biochemical interaction between ethanol and low dietary zinc during gestation and lactation in order to elucidate the metabolic effects of ethanol on zinc status and lead to a reduction of health hazards associated with ethanol.

Although malnutrition induced by ethanol ingestion makes the investigation of a single nutrient deficiency complicated and the interpretation of experimental results difficult, most studies

involving ethanol were unable to exclude the possibility of malnutrition. The next chapter describes the formulation of a nutritionally adequate liquid diet which allows for the study of biochemical effects of ethanol without confounding effects of general malnutrition.

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CHAPTER II

FORMULATION OF A LIQUID DIET FOR ETHANOL STUDIES INVOLVING GESTATION AND LACTATION IN THE RAT[1,2]

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ABSTRACT

This report describes the preparation of a liquid diet for rat studies involving ethanol ingestion during gestation and lactation. The control diet was formulated to contain 30% of energy from casein including methionine at 0.5 mg/kcal, 11.5% of energy from corn oil, 58.5% of energy from dextrose, cellulose, and adequate vitamins and minerals. Calculations for the isocaloric substitution of dextrose with ethanol are described as well as the use of xanthan gum to disperse and emulsify ordinary solid diet components. The use of this liquid diet as the sole source of nutrition for the rat during gestation and lactation produced energy intakes, gestational weight gains, litter sizes, percent survival to weaning, and weaning weights similar to those expected of a conventional diet even when ethanol was substituted for dextrose to provide 30% of total energy.

Indexing Key Words: Ethanol, alcohol, diet, pregnancy

INTRODUCTION

DeCarli and Lieber (1) have demonstrated that the depressive effect of ethanol on food intake of rats (2-4) can be overcome if ethanol is incorporated into a liquid diet. This diet is therefore useful for investigating metabolic effects of ethanol in a rat model including those involving the fetal alcohol syndrome (5-7). Questions however have arisen about the nutritional adequacy of this diet for studies involving ethanol ingestion during gestation and lactation. Wiener et al. (8) have reported low gestational weight gain in rats fed the DeCarli-Lieber formulation compared to the same diet supplemented with about twice as much protein. Our own unpublished results support this observation and we have also found cannibalism during lactation, poor survival of pups to weaning and less than normal weaning weights when the DeCarli-Lieber diet was used as a vehicle for ethanol.

The purpose of this report is to describe a liquid diet formulation that meets or exceeds recommended nutrient intakes for the rat during gestation and lactation (9) with higher protein content as suggested by Wiener et al. (8). Use of xanthan gum to disperse and emulsify ordinary solid diet components is described and evidence is presented for the adequacy of the diet in terms of survival, growth, and development of dams and their offspring despite the presence of ethanol at 30% of total kcal.

MATERIALS AND METHODS

Liquid diet formulation. Composition of the liquid diet for gestation and lactation in the rat is shown in table II.1. Components were mixed with a commercial food mixer (Hobart Mfg. Co., Troy, Ohio) equipped with stainless steel bowl, mixing paddle, and splash guard. To prepare one liter of liquid diet we dispersed 261.75 g of the diet into 880 ml of cold (<10°C) distilled-deionized water with a commercial blender (Waring, New Hartford, CT) set at low speed for 15 seconds. As summarized in table II.2, this formulation is designed to provide 1 kcal/ml of diet in contrast to a solid diet which would have an average 4 kcal/g (9). For lactation the energy content of the diet was increased to 1.5 kcal/ml. This was accomplished by increasing diet components by 1.5 times except xanthan gum. Using this modification, 391.63 g of diet were dispersed into 760 ml of distilled-deionized water. Energy values of diet ingredients are based upon published data (10). Other formulations tested used spray-dried egg white in place of casein plus methionine and pre-gelatinized starch (Dura-gel, A.E. Staley Mfg. Co., Decatur, ILL) at 3% of the diet in place of xanthan gum.

To prepare an ethanol diet, dextrose was isocalorically replaced with 95% ethanol as exemplified by the following example with reference to table II.2. Since ethanol at 30% of total energy requires a dextrose reduction of 80 g, the diet formulation

TABLE II.1

Composition of the liquid diet

Component	g/l
Casein, micropulverized[1]	69.75
DL-methionine[2]	0.50
Vitamin mixture[3]	12.50
Mineral mixture[4]	10.00
Cellulose powder[1]	10.00
Dextrose[5]	144.00
Corn oil[6]	13.00
Xanthan gum[7]	2.00
Distilled-deionized water	880.00

[1]U.S. Biochemical Corp., Cleveland, Ohio.

[2]J.T. Baker, Phillipsburg, New Jersey.

[3]g/kg mixture: thiamin-HCl, 0.2; riboflavin, 0.2; pyridoxin-HCl, 0.2; calcium pantothenate, 0.4; d-biotin, 0.005; niacin, 0.7; folacin, 0.05; vitamin B_{12} (0.1% in mannitol), 0.5; menadione, 0.01; retinyl palmitate (250,000 U/g), 0.8; ergocalciferol (500,000 U/g), 0.08; d-α-tocopheryl acid succinate (1,210 U/g), 0.8; choline chloride, 30. Made to 1 kg with dextrose.

[4]g/kg mixture: $CaHPO_4$, 580.39; NaCl, 125.44; K_2SO_4, 45.35; K_2CO_3, 74.49; $K_3C_6H_5O_7.H_2O$, 103.73; $MnCl_2.4H_2O$, 4.503; $FeC_6H_5O_7.5H_2O$, 5.991; $MgCO_3$, 57.69; $CuCl_2.2H_2O$, 0.4293; $Na_2SeO_4.10H_2O$, 0.0117; $CrK(SO_4)_2.12H_2O$, 0.4802; KIO_3, 0.0088; $ZnCO_3$, 1.4767.

continued

continued, TABLE II.1

[5]Staleydex 333, A.E. Staley Co., Decatur, Illinois.

[6]Gregg Food Division of A.E. Staley Co., Portland, Oregon.

[7]Keltrol, Kelco Corp., Rahway, New Jersey.

TABLE II.2

Energy content of the liquid diet

Component	kcal/g	kcal/l	
		Control diet	Ethanol diet
Protein	4.27	300	300
Carbohydrate	3.75	585	285
Fat	8.84	115	115
Ethanol	7.10	---	300

described in table II.1 was prepared with 80 g less dextrose so that 181.75 g of the ethanol diet were dispersed as before including the addition of 95% ethanol. The amount of ethanol required to provide 300 kcal/l is 42.25 g or approximately 56.5 ml of 95% ethanol (density of 0.746 g/ml). Similar calculations can be made for lower levels of ethanol.

Animal study. Eight pregnant rats, obtained as previously described (11), were equally divided into two groups and fed liquid diets with and without 30% of kcal from ethanol throughout gestation and lactation. Litter size was reduced to eight one day after parturition. Liquid diet feeding tubes were made by a glass blower but are commercially available (Hazelton Systems, Aberdeen, MD., cat. no. Wahmann LC 273). Statistical comparisons were made by Student's t-test and differences between means were considered to be significant at $P<0.05$ (12).

RESULTS AND DISCUSSION

Adequacy of the liquid diet formulation described in this report for energy intake and growth of dams and their offspring is shown in table II.3. Despite the presence of 30% of kcal from ethanol, liquid diet intake was at least four times higher than that expected of a solid diet (9) which provided adequate kcal for the survival, growth, and development of dams and pups similar to those expected of a conventional diet (9, 13). None of the small differences between groups were significant. Consumption of our liquid diet formulation produced superior maternal weight gain, litter size, and birth weight of offspring compared to a similar study that used the DeCarli-Lieber diet (6).

In terms of diet formulation, we found that spray-dried egg white could not be substituted for casein because of excessive foaming especially in the presence of ethanol. Pre-gelatinized starch produced an effective suspension of diet ingredients, but the diet prepared in this way tended to thicken over 24 hours. Xanthan gum as a suspending and emulsifying agent (14) is highly recommended by this study since it allows dispersion of all dietary ingredients including water insoluble components. Xanthan gum is compatible with ethanol and the liquid diet is stable for at least 48 hours. In addition we were able to increase solids content of the liquid diet by 1.5 times to yield 1.5 kcal/ml during lactation which further exemplifies the suspending and emulsifying properties of

TABLE II.3

Adequacy of the liquid diet formulation for maternal growth and development of offspring with and without 30% of kcal from ethanol[1]

Criteria	% kcal from ethanol	
	0	30
Diet intake, ml/day		
Gestation (1 kcal/ml)	114±11	115±12
Lactation (1.5 kcal/ml)	124±7	120±2
Kcal/100 g body wt/day		
Gestation	35.4±1.3	33.5±1.9
Lactation	52.8±2.4	52.0±1.4
Maternal wt gain through 20 days of gestation, g	141±14	127±11
Litter size[2]	14±3	14±1
Pup wt at day 20 of lactation, g	48±3	45±2
% pups survival to weaning	100	97±6

[1]Mean±SD (n=4).

[2]Litters reduced to 8 one day after parturition.

xanthan gum at 2 g/l. Some foaming was found in the high solids formulation especially in the presence of ethanol, but the foam was arrested by a food grade anti-foaming agent at 0.04% of the diet (Myvacet 9-40, Eastman Chemical Products, Kingsport, TN).

The liquid diet described in this report is easy to prepare and it is possible to incorporate substantial amounts of ethanol into the diet without significantly affecting energy intake, maternal growth or development of offspring.

ACKNOWLEDGMENTS

Thanks are expressed to Dr. Donald R. Buhler for the use of the liquid feeding tubes, the Kelco Corp. for providing xanthan gum, Eastman Chemical Products for providing anti-foaming and dispersing agents, and A.E. Staley Co. for providing a sample of pre-gelatinized starch.

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TEXT FOOTNOTES

1. Taken in part from a thesis submitted by Lee-Chuan C. Yeh to Oregon State University in partial fulfillment of the requirements for a doctorate degree.
2. Oregon Agricultural Experiment Station Technical Paper no. 6951.
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CHAPTER III

INTERACTION BETWEEN ETHANOL AND LOW DIETARY ZINC DURING GESTATION AND LACTATION IN THE RAT[1,2]

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ABSTRACT

Pregnant rats were fed individual liquid diets containing either 2 or 10 μg zinc/ml with or without 30% of kcal from ethanol throughout gestation and lactation. The low zinc diet produced a moderate zinc deficiency in dams as evidenced by decreases in tissue zinc content, serum alkaline phosphatase activity, and urinary zinc concentration. Despite the presence of high zinc content in the diet, ethanol antagonized maternal zinc status to a level typical of that produced by the low zinc diet. The lowest zinc status, however, was found when low dietary zinc and ethanol were combined. The maternal interaction between ethanol and zinc also depressed offspring serum zinc and alkaline phosphatase activity in a similar manner but the magnitude of the interaction was smaller. Lower than normal maternal tissue zinc and decreased maternal urinary excretion of zinc in response to decreased maternal intestinal zinc absorption may suggest a maternal attempt to support the growth and development of offspring. In addition, dams fed either ethanol or low zinc-containing diets had increased urinary hydroxyproline at the end of lactation which suggested maternal bone resorption. The results of this study indicate that a moderate zinc deficiency during a period of high metabolic demand is exaggerated by ethanol in both dams and offspring.

Indexing Key Words: Ethanol, zinc

INTRODUCTION

The antagonistic effect of ethanol (EtOH) on zinc (Zn) metabolism has been reported in humans and laboratory rats. In alcoholics, ethanol appears to have a depressive effect on plasma zinc (1-3) which may result from increased urinary zinc excretion. In rats, ethanol ingestion has been shown to decrease zinc content of plasma, liver, and muscle (4) possibly resulting from increased excretion of urinary and fecal zinc (5). The activities of zinc-dependent enzymes such as serum alkaline phosphatase (ALP) and liver alcohol dehydrogenase (ADH) were found to be significantly reduced in zinc-deficient male rats (6, 7), but they were normal in ethanol-fed rats (8, 9).

During pregnancy ethanol consumption is known to be antagonistic to offspring growth and zinc status in humans and laboratory rats. In humans, low birth weight has been related to low zinc status of women who consumed ethanol during pregnancy (10). In ethanol-fed pregnant rats, zinc content of maternal serum (11) and muscle (12) were decreased. In addition, the decrease in zinc content of placenta and fetal carcass (11, 12) indicated that the availability of zinc to the fetus was decreased by ethanol.

So far, these previous studies were not followed after parturition and information about the possible antagonistic effect of ethanol on zinc is unavailable during lactation. Ethanol can enter the neonates through breast milk (13), which raises the

possibility that maternal ethanol ingestion may affect the postnatal zinc metabolism of pups. Although milk zinc has been reported to be significantly decreased in dams fed zinc-deficient diet (14, 15), milk zinc in ethanol-fed dams has not been reported.

The purpose of this study is to determine the possible interaction between ethanol and low dietary zinc during gestation and lactation in terms of tissue zinc concentrations and potential physiological consequences due to changes in zinc status in dams and offspring. In addition, the experimental design defines the effect of moderate zinc status resulting from ethanol, reduced zinc intake, or a combination of these factors on ethanol metabolism.

MATERIALS AND METHODS

Proven-breeder female outbred Sprague-Dawley albino rats (Charles River Laboratories, Wilmington, MA) were bred overnight with adult male rats of the same species. Presence of spermatozoa in a vaginal smear taken the following morning was considered to be day 0 of gestation. Sixteen confirmed pregnant rats were equally divided among four treatment diets. Liquid diets (16) containing either 2 or 10 μg zinc/ml with or without 30% of kcal from ethanol were fed daily throughout gestation and lactation. Dietary intake for all rats was restricted to that consumed by the group fed the low zinc plus ethanol diet. Body weights were taken at regular time intervals to ensure adequate growth and to maintain similar caloric intakes on a body weight basis. Throughout the entire study, rats were individually housed in polycarbonate cages equipped with stainless steel wire tops. Wood chips were used to absorb wastes.

On day 20 of gestation, rats were transfered to clean cages with enough bedding material for delivery. They were allowed to normally deliver the pups, ingest the placenta and clean the young. Pups were handled with disposable surgical gloves to prevent imparting odors to the young which could upset the dams and induce cannibalism (17).

On day 21 of lactation, dams and five to six weanlings from each litter were lightly anesthetized intraperitoneally with about 3 mg sodium pentobarbital/100 g body weight (Veterinary Laboratories,

Inc., Lenexa, KA) following an overnight fast. Dams were also injected intraperitoneally with 4 USP units of oxytocin (Fort Dodge Laboratories, Fort Dodge, Iowa) to stimulate milk production. Milk samples were collected by gentle hand stripping. Immediately after milking, dams and pups were killed by drawing blood from the abdominal aorta into plastic syringes (Monoject, Sherwood Medical Industries Inc., Deland, FL). Serum samples were collected in silicon-coated tubes (Becton-Dickinson & Co., Rutherford, NJ) and frozen at -20°C until analyzed for minerals, protein (18), ethanol (19) with a commercial kit (Sigma Chem. Co., St. Louis, MO), and activity of alkaline phosphatase (EC 3.1.3.1)(20) with another commercial kit (American Monitor Corp., Indianapolis, IN). Liver, kidney, femur muscle, and tibias free of adhering soft tissue were obtained from both dams and offspring and frozen at -20°C until analyzed for minerals. Pup samples were pooled within litters to ensure adequate sample size for analyses. In addition, a 24 hour urine sample was collected from dams during day 12 of gestation and day 20 of lactation using stainless steel metabolic cages (Hazelton Systems, Aberdeen, MD). Urine specimens were frozen at -20°C until analyzed for minerals, creatinine (21), and hydroxyproline (22).

The activity of the zinc-metalloenzyme, liver alcohol dehydrogenase (EC 1.1.1.1), was measured to determine the effect of zinc status on ethanol metabolism. One gram liver was freshly removed from dams and pups, minced with stainless steel surgical scissors, and then homogenized in 9 ml of cold potassium phosphate

buffer (0.05 M, pH 7.5) with 12 strokes of a motor-driven (Cenco Stirrer, Central Scientific Co., Chicago, ILL) teflon pestle (23). The homogenate was centrifuged at 30,000 X g for 30 minutes at 4°C (Beckman Model J-21C, Beckman Instruments, Inc., Palo Alto, CA). The supernatant was decanted and centrifuged for another 30 minutes to obtain liver extract for determination of liver alcohol dehydrogenase activity (24) and protein (18). Specific activity of liver alcohol dehydrogenase was expressed as μmol NADH/g protein per minute.

For mineral analysis, tibias were extracted sequentially with 95% ethanol and petroleum ether and dry ashed for 24 hours at 590°C in a muffle furnace (Thermolyne Corp., Dubuque, Iowa). Urine, milk, liver, kidney, and muscle were wet ashed with nitric acid followed by 30% hydrogen peroxide. All ashed samples were dissolved in 3 N hydrochloric acid and diluted with distilled water to an appropriate volume for analysis. Serum samples were treated with trichloroacetic acid to remove protein prior to mineral analysis (25). Measurements of zinc, calcium (Ca), and magnesium (Mg) were done by Atomic Absorption Spectrophotometry (Perkin-Elmer Model 403, Norwalk, CT). For calcium determination, the final dilution was made with 0.1% lanthanum solution to reduce interference by phosphorus (Analytical Methods for Atomic Absorption Spectrophotometry, Perkin-Elmer Corp., Norwalk, CT). Phosphorus (P) was measured by the method of Fiske and Subbarow (26).

Precautions to minimize zinc contamination included the

preparation of liquid diets with distilled-deionized water, use of glass feeding tubes fitted with silicon rubber stoppers (Arthur H. Thomas Co., Philadelphia, PA), segregation of feeding tubes by group for cleaning purposes, and exclusive use of reagent grade chemicals for analyses. Laboratory glassware was soaked in 10% nitric acid for four hours, rinsed with re-distilled water, dried and stored in plastic bags prior to use.

The experimental design involved two factors, ethanol and zinc, with two levels of each factor. The data were analyzed based on a 2x2 factorial experiment with 4 observations per treatment. The data presented divide treatment effects into ethanol, zinc, and interaction of these two factors (27). Differences between means were tested by Tukey's honestly significant difference (HSD) and considered to be significant at $P<0.05$.

RESULTS

Caloric intake and growth response. The average dietary intake during gestation and lactation and growth response of both dams and pups are summarized in table III.1. Despite the fact that there was a small reduction in daily intake in the group fed the low zinc plus ethanol diet, caloric intake based on maternal body weight was similar and adequate. The average caloric intake per day was 34 kcal/100 g body weight during gestation and 52 kcal/100 g body weight during lactation. No significant differences were found between groups in terms of maternal weight gain during gestation, litter size, or offspring growth response during lactation, but there was a trend for these parameters to be lowest in the group fed the low zinc plus ethanol diet.

Serum zinc and alkaline phosphatase activity. As shown in figure III.1, maternal and offspring serum zinc were significantly decreased in groups fed either the ethanol or low zinc-containing diets. The greatest reduction in serum zinc however was in the group fed the low zinc plus ethanol diet. As shown in figure III.2, maternal and offspring serum alkaline phosphatase activities were both depressed as a result of ethanol ingestion and low dietary zinc. The greatest reduction was in the group fed the low zinc plus ethanol diet.

Urinary zinc and hydroxyproline. During both gestation and lactation, maternal urinary zinc content was decreased in rats fed

TABLE III.1

Dietary intake and growth response of dams and offspring[1,2]

Dietary zinc status	High		Low	
% kcal from ethanol	0	30	0	30
Dietary intake, ml/day				
Gestation (1 kcal/ml)	114±11	115±2	117±3	122±5
Lactation (1.5 kcal/ml)	125±7	120±2	117±12	119±3
Maternal weight gain, g	141±14	127±11	138±7	134±7
Litter size	14±3	14±1	14±1	13±2
Offspring weight, g				
Lactation day 2	6.3±0.4	6.9±0.4	6.3±0.5	6.5±0.2
7	15±1	15±1	14±1	15±1
14	31±3	30±1	29±2	30±2
20	48±3	45±3	44±2	43±2

[1] Values are mean±SD (n=4).

[2] Litter size was reduced to eight on day 2 of lactation.

Fig. III.1 Effect of ethanol and low dietary zinc on maternal and offspring serum zinc. Values are Mean $\pm$ SD (n=4). Different superscripts in each figure are significantly different ($P<0.05$). Significance levels for the effect of EtOH, Zn, and EtOH + Zn are all $P<0.005$ for dams; $P<0.005$, $P<0.005$, and $P<0.025$ for offspring, respectively.

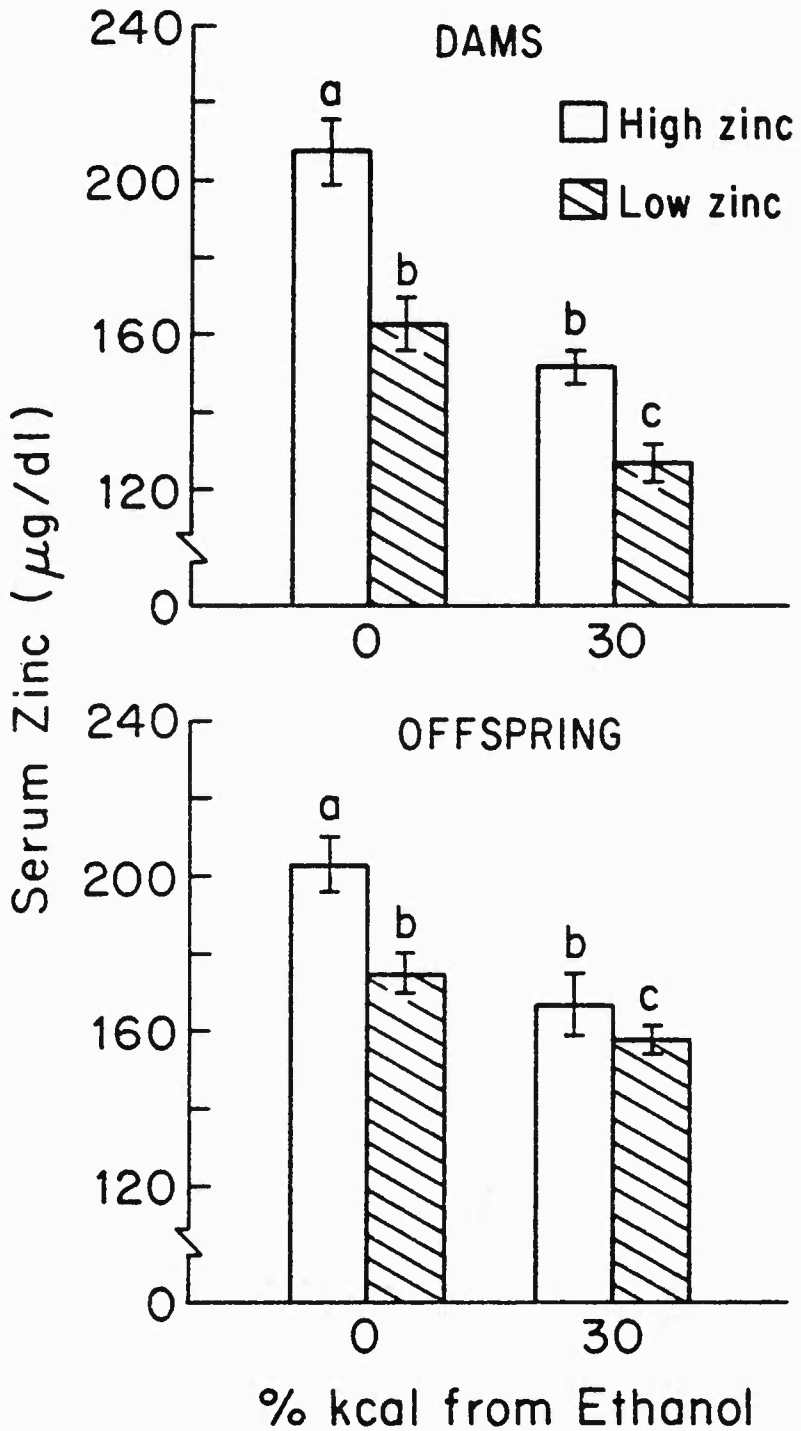
DAMS
High zinc
Low zinc
240
200
160
120
0
a
b
b
c
0
30
OFFSPRING
240
200
160
120
0
a
b
b
c
0
30
Serum Zinc (μg/dl)
% kcal from Ethanol

Fig. III.1

Fig. III.2 Effect of ethanol and low dietary zinc on maternal and offspring serum alkaline phosphatase. Values are Mean ± SD (n=4). Different superscripts in each figure are significantly different ($P<0.05$). Significance levels for the effect of EtOH, Zn, and EtOH + Zn are all $P<0.005$ for dams; $P<0.005$, $P<0.005$, and $P<0.05$ for offspring, respectively.

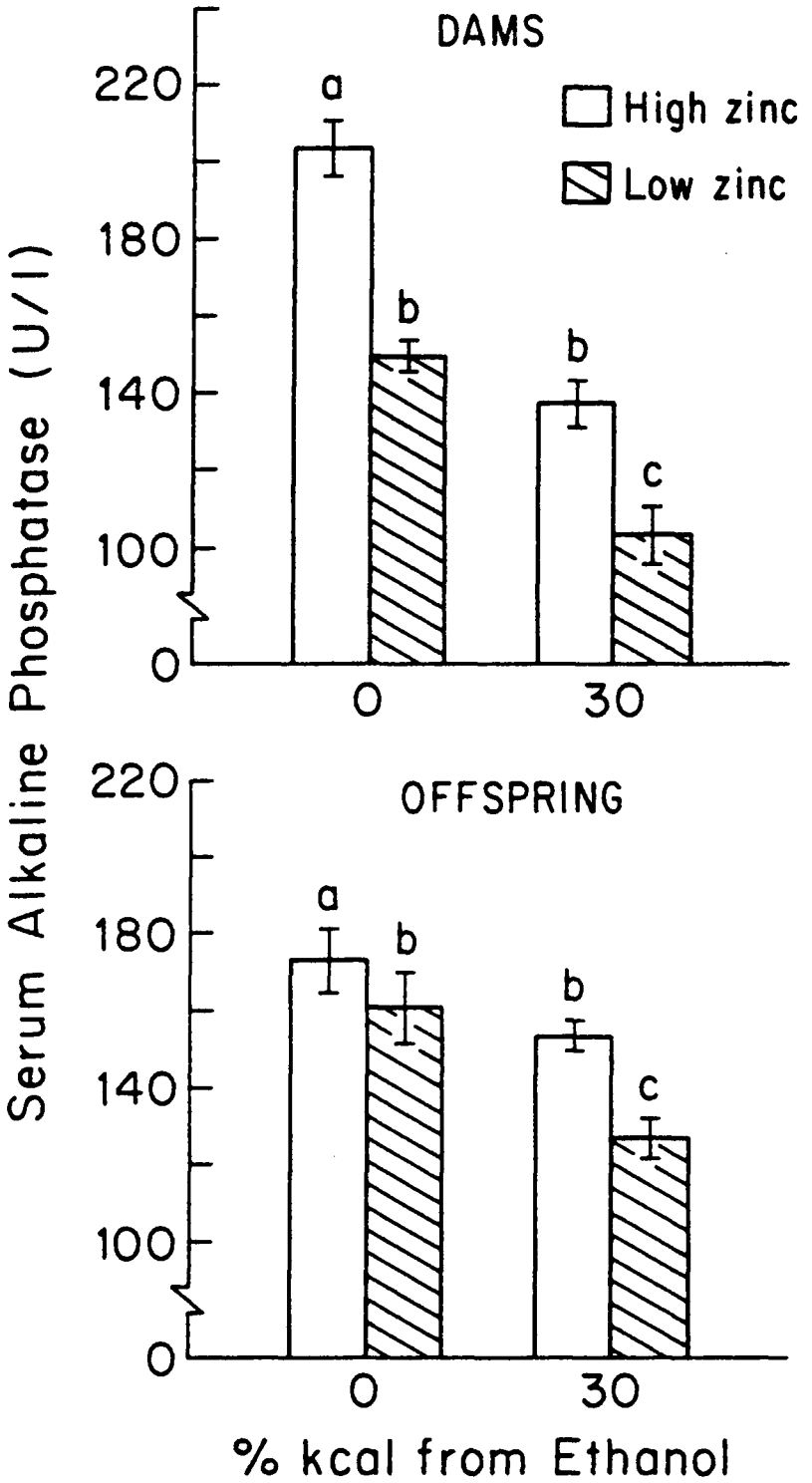
DAMS
High zinc
Low zinc
220
180
140
100
0
a
b
b
c
0
30
OFFSPRING
220
180
140
100
0
a
b
b
c
0
30
Serum Alkaline Phosphatase (U/l)
% kcal from Ethanol

Fig. III.2

either the ethanol or low zinc-containing diets as shown in figure III.3. Urinary hydroxyproline decreased in rats fed either ethanol or low zinc-containing diets during day 12 of gestation, compared to rats fed the high zinc-containing diet as shown in figure III.4. During day 20 of lactation, however, urinary hydroxyproline increased significantly in rats fed either the ethanol or low zinc-containing diets.

Maternal and offspring mineral analyses. As summarized in table III.2, maternal liver and kidney zinc content was significantly decreased in groups fed either the ethanol or low zinc-containing diets. The greatest reduction in zinc content of these tissues, however, was in the group fed the low zinc plus ethanol diet. Zinc concentrations in milk and maternal tibia were significantly depressed as a result of low dietary zinc regardless of ethanol level. In offspring, zinc concentrations in tibia and liver were also significantly decreased in the group fed the low zinc-containig diets as shown in table III.3.

Significant differences could not be demonstrated between groups for zinc concentration of maternal muscle, offspring muscle, or offspring kidney. The average values for calcium, phosphorus, and magnesium of maternal serum (10.2, 7.9, and 2.5 mg/dl), maternal tibia (362, 175, and 6.7 mg/g ash), offspring serum (10.3, 8.2, and 2.4 mg/dl), and offspring tibia (341, 181, and 9.1 mg/g ash) were also respectively determined but they were essentially the same between groups.

Fig. III.3 Effect of ethanol and low dietary zinc on maternal urinary zinc. Values are Mean $\pm$ SD (n=4). Different superscripts in each figure are significantly different ($P<0.05$). Significance levels for the effect of EtOH, Zn, and EtOH + Zn are all $P<0.005$ for both day 12 of gestation and day 20 of lactation.

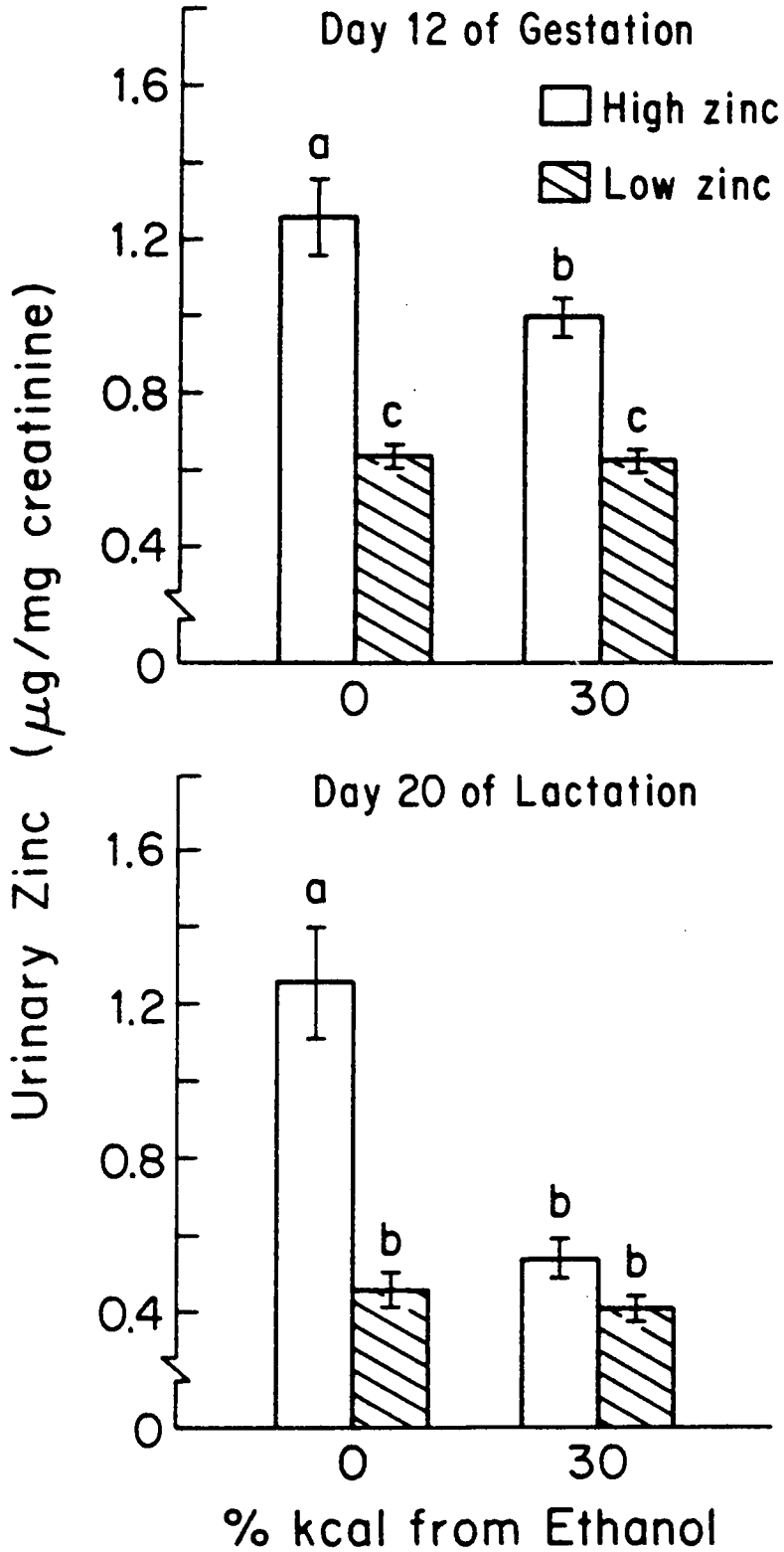
Day 12 of Gestation
High zinc
Low zinc
1.6
1.2
0.8
0.4
0
a
b
c
c
0
30
Day 20 of Lactation
1.6
1.2
0.8
0.4
0
a
b
b
b
0
30
Urinary Zinc (μg/mg creatinine)
% kcal from Ethanol

Fig. III.3

Fig. III.4 Effect of ethanol and low dietary zinc on maternal urinary hydroxyproline. Values are Mean ± SD (n=4). Different superscripts in each figure are significantly different ($P<0.05$). Significance levels for the effect of EtOH, Zn, and EtOH + Zn are $P<0.005$, $P<0.005$, and $P<0.05$ for day 12 of gestation; $P<0.005$, $P<0.025$, and $P<0.005$ for day 20 of lactation, respectively.

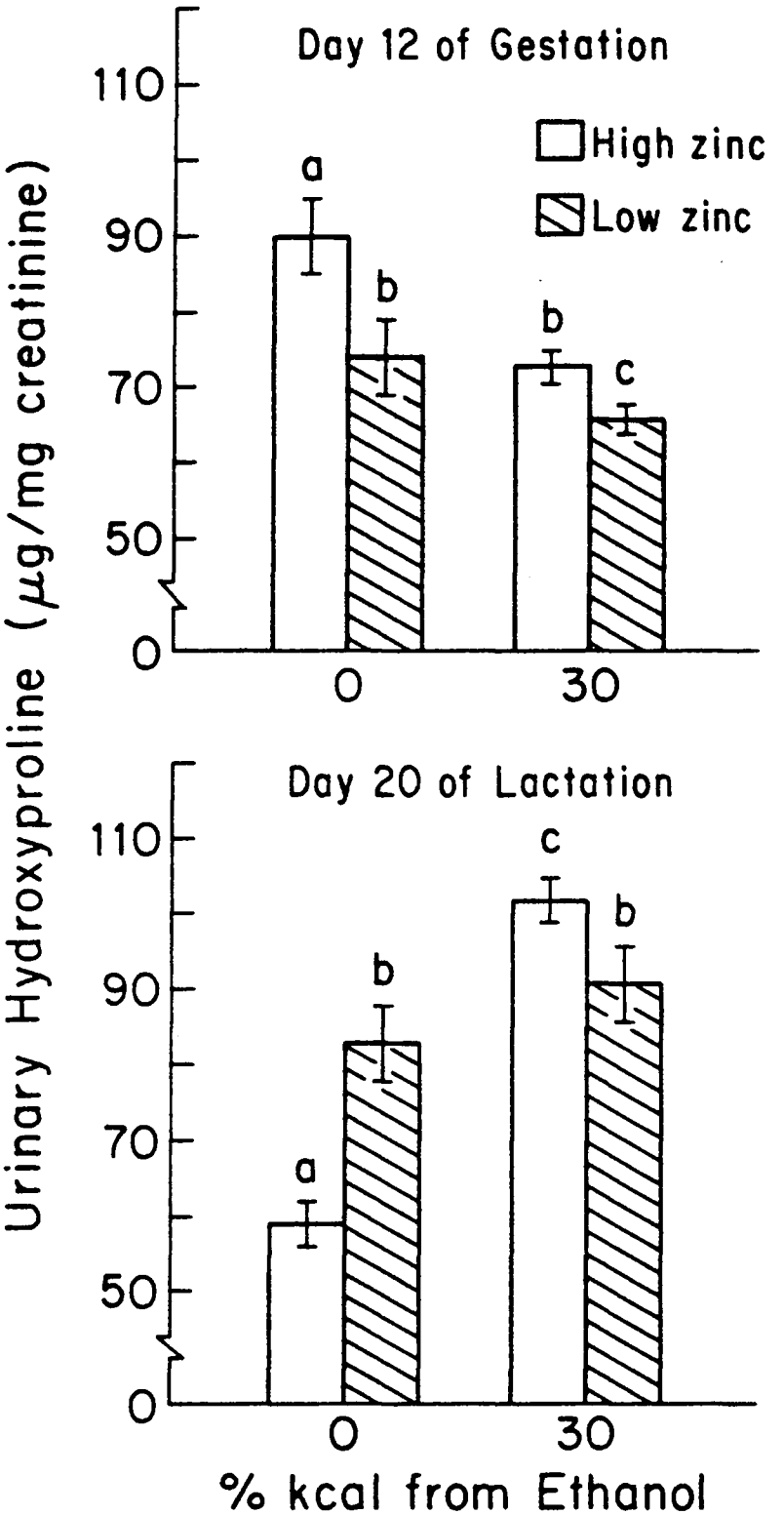
Day 12 of Gestation
High zinc
Low zinc
Urinary Hydroxyproline (μg/mg creatinine)
110
90
70
50
0
a
b
b
c
0
30
Day 20 of Lactation
110
90
70
50
0
a
b
c
b
0
30
% kcal from Ethanol

Fig. III.4

TABLE III.2

Zinc content of maternal tissues[1,2]

Dietary zinc status	High		Low		Significance levels		
% kcal from ethanol	0	30	0	30	EtOH	Zn	EtOH+Zn
Liver, µg/g[3]	30.5±0.8[a]	28.7±0.6[b]	28.9±0.8[b]	26.5±0.6[c]	P<0.005	P<0.005	NS[4]
Kidney, µg/g	27.0±0.8[a]	23.3±0.6[b]	23.6±1.5[b]	20.8±1.1[c]	P<0.005	P<0.005	NS
Muscle, µg/g	10.3±0.5	11.2±0.5	11.4±0.4	11.9±1.7	NS	NS	NS
Milk, µg/g	10.4±1.5[a]	10.0±0.6[a]	8.2±0.6[b]	9.2±0.8[b]	NS	P<0.01	NS
Tibia, µg/g ash	338±8[a]	328±9[a]	320±16[b]	319±6[b]	NS	P<0.05	NS

[1]Values are mean±SD (n=4).

[2]Values in the same row with different superscript are significantly different (P<0.05) from each other.

[3]Units represent microgram zinc per gram wet weight.

[4]Not significant.

TABLE III.3

Zinc content of offspring tissues[1,2]

Dietary zinc status	High		Low		Significance levels		
% kcal from ethanol	0	30	0	30	EtOH	Zn	EtOH+Zn
Liver, μg/g[3]	51.0±5.9	55.5±2.7	48.9±1.6	45.8±2.5	NS[4]	P<0.01	NS
Kidney, μg/g	25.6±1.7	25.5±0.8	24.3±0.6	24.7±1.0	NS	NS	NS
Muscle, μg/g	10.6±0.2	10.9±0.3	10.8±0.5	10.8±0.4	NS	NS	NS
Tibia, μg/g ash	478±21[a]	453±20[a]	387±42[b]	383±32[b]	NS	P<0.005	NS

[1]Values are mean±SD (n=4).

[2]Values in the same row with different superscript are significantly different (P<0.05) from each other.

[3]Units represent microgram zinc per gram wet weight.

[4]Not significant.

Other analysis. Liver alcohol dehydrogenase activity was reduced in both dams (from 8.6 to 7.6 μmol/g protein per minute) and pups (from 7.5 to 7.2 μmol/g protein per minute) as a result of either ethanol ingestion or low dietary zinc. The reductions, however, were insignificant. Serum ethanol levels were obtained in fasted dams and pups (2.8 and 2.6 mg/dl, respectively) as a result of ethanol ingestion regardless of the level of dietary zinc. Protein contents of maternal and offspring liver (92 and 79 mg/g), maternal and offspring serum (5.6 and 4.5 g/dl), and maternal milk (151 mg/ml) were similar between groups.

DISCUSSION

The results of this study indicate that an interaction exists between ethanol and low dietary zinc during gestation and lactation in both dams and offspring. The low zinc diet which provided about two-thirds of the rat's minimum requirement for zinc (28) successfully produced a moderate zinc deficiency in dams as evidenced by decreases in tissue zinc content, serum alkaline phosphatase activity, and urinary zinc concentration. Despite the fact that the high zinc diet provided more than three times the minimum requirement for zinc, ethanol antagonized maternal zinc status to a level typical of the moderate zinc deficiency produced by the low zinc diet. The combination of ethanol and low dietary zinc resulted in the greatest depression of both maternal and offspring zinc status despite the fact that maternal caloric intake, gestational weight gain, and growth of offspring were adequate when compared to rats fed high zinc-containing diet without ethanol.

The possible mechanism for the depressive effect of ethanol on zinc status in both dams and offspring may be explained by depressed food intake (29), decreased maternal intestinal absorption of zinc (30), impaired placental transport of zinc (11), or increased urinary excretion of zinc (5). Depressed food intake is not a likely mechanism in this study because caloric intake was kept constant for all rats. Placental transport of zinc does not seem to be impaired in this study since offspring zinc status was less

affected by maternal ethanol ingestion compared to dams. Depressed maternal tissue zinc content in the present study is consistant with another report (14) and with the possiblity of decreased intestinal absorption of zinc (30). Lower than normal maternal tissue zinc suggests an attempt was made to meet the demand of offspring at the expense of maternal tissue zinc (31). Although an increase in urinary zinc concentration has been reported in ethanol-fed non-pregnant rats (5), a decrease in maternal urinary zinc concentration was found in our pregnant rats at day 12 of gestation and day 20 of lactation which is consistant with a maternal attempt to conserve zinc during gestation and lactation (32). Therefore, the interaction between ethanol and zinc in our study is best explained by the maintenance of a lower than normal maternal tissue zinc and decreased maternal urinary excretion of zinc in response to decreased maternal intestinal zinc absorption to support the growth and development of offspring.

The physiological consequences of ethanol-antagonized zinc status were determined by serum alkaline phosphatase activity, liver alcohol dehydrogenase activity, and urinary excretion of hydroxyproline. Alkaline phosphatase which is involved in bone formation (31) was measured in serum to detect the response to zinc deficiency. The significant reduction of serum alkaline phosphatase activity may have influenced bone remodeling (33). No attempt however was made to measure the alkaline phosphatase activity in other tissues. Activity of the zinc metalloenzyme, liver alcohol

dehydrogenase, was measured to detect the possibility that reduced zinc status would impair ethanol metabolism (7) but it was not influenced by zinc deficiency in this study. Rats however were fasted overnight prior to the termination of the study for the purpose of collecting serum which limited our ability to detect an effect of zinc deficiency on ethanol metabolism (34). Although both serum alkaline phosphatase and liver alcohol dehydrogenase are zinc-dependent enzymes, they responded differently to decreased zinc status. Differences in the sensitivity of these zinc-dependent enzymes may be partly explained by differences in the zinc-ligand affinity of the zinc metalloenzymes and in their turnover rates in the affected tissues (35). Urinary hydroxyproline was measured to detect impaired collagen synthesis (36). Maternal urinary hydroxyproline was increased during day 20 of lactation in rats fed either ethanol or low zinc-containing diets compared to those fed the high zinc-containing diet which is in agreement with previous findings in male rats (33, 37). Since collagen is the major protein in bone, increased urinary excretion of hydroxyproline in the present study may suggest the possibility of maternal bone resorption. Maternal urinary hydroxyproline, however, was decreased during day 12 of gestation in rats fed either ethanol and low zinc-containing diets compared to those fed the high zinc-containing diet. One possible explanation is that high circulating levels of anabolic hormones such as estrogen and progesterone typical of gestation (38) depressed bone resorption (39) thereby counteracting

any effects of low zinc status or ethanol on bone remodeling at this time. At initiation of lactation, estrogen and progestrone rapidly decline in maternal circulation (38) which may have made it possible to observe increased maternal urinary excretion of hydroxyproline at the end of lactation.

Streissguth et al. (40) have suggested that there is a need to investigate the effects of poor nutrition on the metabolism of ethanol without confounding effects of general malnutrition. We believe that our study of the interaction between ethanol and zinc during gestation and lactation in the rat has accomplished this goal.

ACKNOWLEDGEMENTS

Thanks are expressed to Dr. Te-May Ching for her technical assistance in setting up the analysis for liver alcohol dehydrogenase activity.

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TEXT FOOTNOTES

1. Taken in part from a thesis submitted by Lee-Chuan C. Yeh to Oregon State University in partial fulfillment of the requirements for a doctorate degree.
2. Oregon Agricultural Experiment Station Technical Paper no. 7047.
3. To whom reprint requests should be sent.

CHAPTER IV

DEVELOPMENTAL INFLUENCE OF MATERNAL ETHANOL INGESTION ON MOLAR COMPOSITION AND DENTAL CARIES OF RAT OFFSPRING[1,2]

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ABSTRACT

The purpose of this study was to determine the effect of maternal ethanol ingestion on the development of offspring teeth. Pregnant rats were fed liquid diets containing either 2 or 10 μg zinc/ml with or without 30% of kcal from ethanol throughout gestation and lactation. Two to three male rats from each group were then separated from the litter, orally inoculated with the cariogenic bacteria Streptococcus mutans, and fed a caries-promoting diet for six weeks. Maternal ethanol ingestion coinciding with offspring molar development negatively influenced offspring dental health as evidenced by decreased molar enamel and dentin zinc content, increased dental caries score, and decreased cross-linking in enamel ultrastructure as visualized by scanning electron microscopy. The negative influence of maternal ethanol ingestion on offspring dental health appears to be specifically associated with an antagonism of zinc during a critical period of molar development.

Indexing Key Words: Ethanol, zinc, caries

INTRODUCTION

We have previously reported that maternal ethanol ingestion during gestation and lactation antagonizes the zinc status of both dams and offspring and that there is a significant interaction between ethanol and dietary zinc level (Chapter III). This study also provided indirect evidence that the ethanol and zinc interaction affected skeletal health as evidenced by measurements of serum alkaline phosphatase and urinary hydroxyproline. In fact impaired skeletal health is a known consequence of the fetal alcohol syndrome in humans (1). Present knowledge however does not define the possible effect of ethanol ingestion on the teeth.

The fact that ethanol ingestion reduces zinc status in rats led us to hypothesize that dental health would also be impaired by ethanol ingestion since zinc deficiency increases dental caries in both weanling rats (2) and in rat offspring originating from zinc-deficient dams (3, 4). The antagonistic effect of maternal ethanol ingestion on zinc status of offspring is especially interesting since rat gestation and lactation coincides with a critical period of molar development (5).

The purpose of the present study was therefore to determine the influence of maternal ethanol ingestion during gestation and lactation on offspring molar composition and dental caries. In addition we report the effect of maternal ethanol consumption on the crystalline structure of rat mandibular molars as visualized by

scanning electron microscopy.

MATERIALS AND METHODS

Pregnant rats were fed individual liquid diets (6) containing either 2 or 10 μg zinc/ml with or without 30% of kcal from ethanol throughout gestation and lactation as previously described (Chapter III). At day 20 of lactation, two to three male weanling rats were selected from each litter, group-housed in polycarbonate cages, and fed a caries-promoting diet (4) for six weeks. At the start of the caries test period, each rat had three drops of a 24-hour culture of a cariogenic bacteria, Streptococcus mutans 6715-15 (American Type Culture Collection, Rockville, MD), placed into their mouths. A one hundred fold dilution of this culture was also placed in the drinking water for two successive days as suggested by Larson et al. (7).

At the end of the six-week caries test period, rats were sacrificed by guillotine under carbon dioxide anesthesia. Heads were steam-autoclaved for 5 min to soften the connective tissues and allow removal of both maxillary and mandibular molars. Maxillary first and second molars were pooled from each litter and dried in an oven for three days at 100°C. The dried molars were pulverized using an amalgamator capsule and stainless steel ball (4) until the powder passed through a 200 mesh polyester sieve (Sargent-Welch Scientific Co., Anaheim, CA). Enamel and dentin were separated by floatation in a bromoform-acetone (91:9) mixture by the method of Gilda (8). Enamel and dentin were wet ashed with reagent grade

nitric acid and then dissolved in 3 N hydrochloric acid. Further dilutions were made with distilled water to an appropriate volume for mineral analysis.

Mandibular molars were removed and stained with murexide (9) for caries score determination on the right and left buccal surface of first and second molars (10). Lesions were evaluated by one of us (FLC) without knowledge of the true sample identity.

After caries scores were recorded, right mandibular molars were ground to the midline from the lingual side with a separating disc (S.S. White, Philadelphia, PA). The disc was held in a straight handpiece (Doriot type, Schein, Inc., Port Washington, NY) which was driven by a bench-type dental engine (Teledyne Emesco Model no. 90N, Englewood, NJ). Teeth were then acid-etched for ten seconds with 1.5 N hydrochloric acid, rinsed alternately with distilled water and 0.1 M sodium hydroxide several times, and dried with acetone prior to sample preparation for scanning electron microscopy.

The etched molar teeth were mounted on aluminum planchets and rotary coated with approximately 100 A° of 60:40 gold/palladium in a Varian VE-10 vacuum evaporator (Varian, Palo Alto, CA). Second mandibular molar enamel was examined with an Amray scanning electron microscope (Model no. 1000-A, Bedford, MA) operating at 20 Kv accelerating voltage. The site of examination in each case is shown in figure IV.1. Both site and instrument parameters were kept constant for all samples. Images were recorded on Polaroid type 55 film.

Fig. IV.1 Site selection for scanning electron microscopic examination of offspring second mandibular molar enamel. The site is indicated by the small black square. X40.

Fig. IV.1

Tibias were cleaned of adhering tissues, extracted with 95% ethanol and petroleum ether in a Soxhlet apparatus, and ashed in a muffle furnace (Thermolyne Corp., Dubuque, Iowa) overnight at 590°C. The ashed tibia were pooled within litters and dissolved in .3 N hydrochloric acid. Further dilutions were made with distilled water to an appropriate volume prior to mineral analysis.

Enamel, dentin, and tibia were analyzed for zinc, calcium (Ca), and magnesium (Mg) by Atomic Absorption Spectrophotometry (Perkin-Elmer Model 403, Norwalk, CT). For calcium determination, the final dilution was made with 0.1% lanthanum solution to reduce interference by phosphorus (Analytical Methods for Atomic Absorption Spectrophotometry, Perkin-Elmer Corp., Norwalk, CT). Phosphorus (P) was measured in the samples by the method of Fiske and Subbarow (11).

The experimental design involved two factors, ethanol and zinc, with two levels of each factor. The data were analyzed based on a 2x2 factorial experiment with 4 observations per treatment. The data presented divide treatment effects into ethanol, zinc, and interaction of these two factors (12). Differences between means were tested by Tukey's honestly significant difference (HSD) and considered to be significant at $P<0.05$.

RESULTS

Offspring growth. During the caries-test period, the average food intake was 20 g/day for each rat. The diet was found to contain 20 ppm zinc by analysis. Average weaning weight was 45±3 g, mean±SD (n=4), at the start of the test period increasing to 282±15 g, mean± SD (n=4), at the end of six-week caries test period.

Molar zinc and dental caries score. The effects of ethanol and low dietary zinc on enamel and dentin zinc content of first and second maxillary molars are shown in table IV.1. Molar enamel zinc content was significantly decreased in offspring nursed by dams fed the ethanol-containing diet regardless of the level of dietary zinc. Molar dentin zinc content, however, was significantly decreased in offspring nursed by dams fed either ethanol or low zinc-containing diets. Table IV.1 also summarizes the fact that dental caries scores on the buccal surface of molars for E (enamel) and Ds (dentin slight) were increased in offspring nursed by dams fed either ethanol or low zinc-containing diets. Although the interaction of ethanol and zinc failed to achieve significance, there was a trend for molar zinc content to be lowest and dental caries score to be highest in offspring nursed by dams fed the low zinc plus ethanol diet.

Scanning Electron Micrographs. Scanning electron micrographs of offspring second mandibular molar enamel are shown in figure IV.2. Each micrograph represents a visual average of a treatment group.

TABLE IV.1

Molar zinc content and dental caries score of offspring[1,2]

Dietary zinc status	High		Low		Significance levels		
% kcal from ethanol	0	30	0	30	EtOH	Zn	EtOH+Zn
Enamel Zn, µg/g dry wt							
1st molar	53.0±1.4[a]	45.2±2.0[b]	50.6±3.6[a]	43.5±1.6[b]	P<0.005	NS[3]	NS
2nd molar	65.7±0.8[a]	57.2±6.2[b]	60.5±4.6[a]	55.7±5.7[b]	P<0.025	NS	NS
Dentin Zn, µg/g dry wt							
1st molar	228±1[a]	204±8[c]	216±3[b]	202±4[c]	P<0.005	P<0.01	P<0.01
2nd molar	222±4[a]	204±4[c]	211±6[b]	197±2[c]	P<0.005	P<0.005	NS
Dental Caries Score							
Enamel	6.8±1.0[a]	11.0±2.6[b]	8.5±1.9[a]	11.3±2.6[b]	P<0.01	NS	NS
Dentin slight	4.8±0.5[a]	8.8±1.0[b]	7.0±0.8[b]	10.5±1.7[c]	P<0.005	P<0.005	NS

[1]Values are mean±SD (n=4).

[2]Values in the same row with different superscript are significantly different (P<0.05) from each other.

[3]Not significant.

Fig. IV.2 Scanning electron micrographs of offspring second mandibular molar enamel. Each micrograph represents a visual average of the crystal structure of mandibular second molar enamel from rat offspring nursed by dams fed diets containing high zinc without ethanol (A), high zinc with ethanol (B), low zinc without ethanol (C), and low zinc plus ethanol diet (D). X3000. The solid bar in the lower right-hand corner denotes 10 μm.

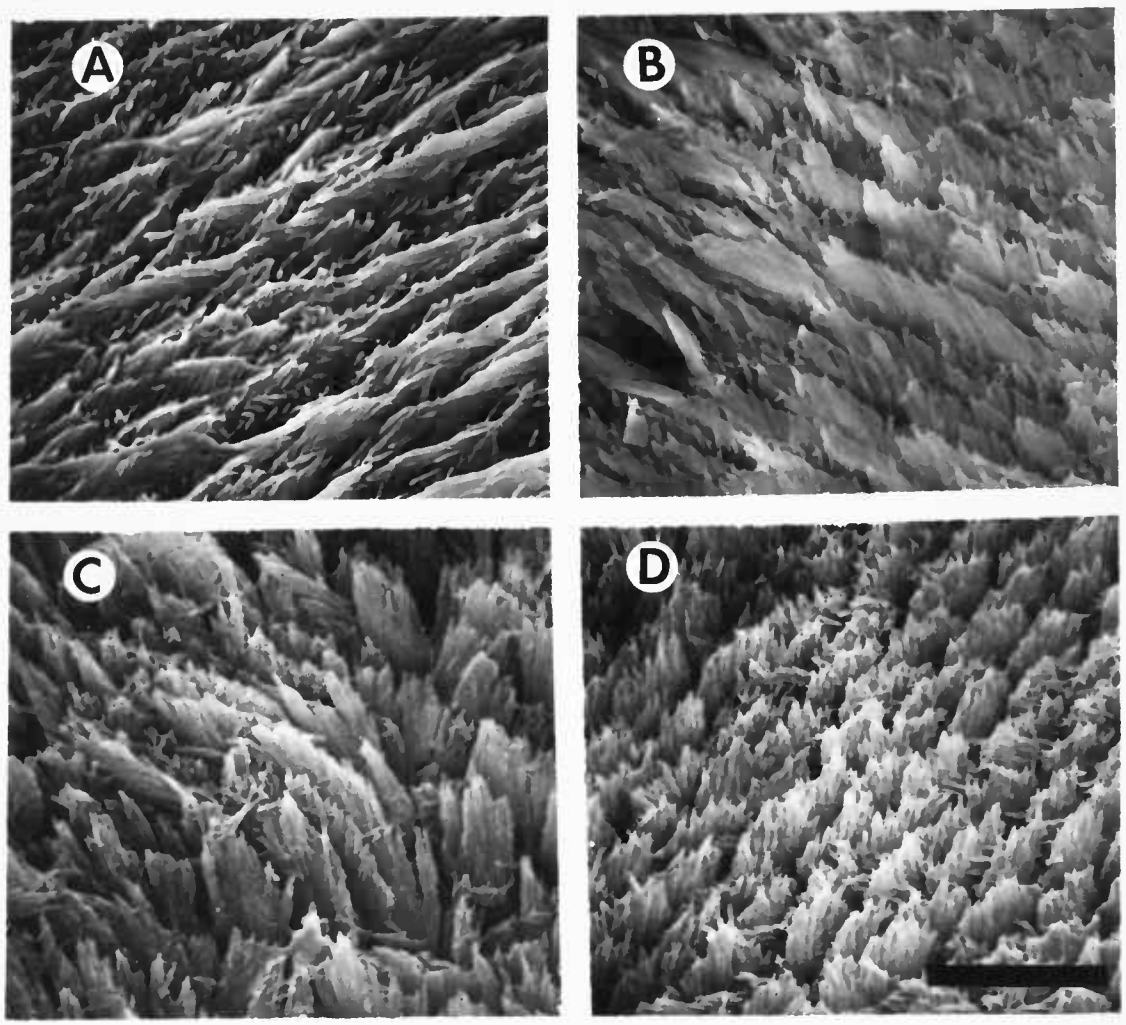

Fig. IV.2

A typical cross-linking pattern of enamel rods was observed in the group nursed by dams fed the high zinc-containing diet without ethanol. Slight reduction in cross-linking of enamel rods was seen in the group fed either ethanol or low zinc-containing diets. Obvious reduction in cross-linking and less organization in pattern of enamel rods were seen in the group nursed by dams fed the low zinc plus ethanol diet.

Other mineral analyses. Calcium, phosphorus, and magnesium contents of offspring maxillary molars and tibia were unaffected by maternal ethanol ingestion and the values were similar to those previously reported (13). Zinc content of offspring tibia averaged 410 μg/g ash for all treatment groups. Measurements of molar calcium and phosphorus confirmed that enamel and dentin fractions were successfully separated since calcium and phosphorus contents of each fraction were in agreement with a standard reference (14).

DISCUSSION

The results of this study indicate that maternal ethanol ingestion during gestation and lactation negatively influences offspring dental health as evidenced by decreased enamel and dentin zinc content of first and second molars and increased susceptibility to dental caries. Scanning electron microscopy suggests that increased caries scores are related to a disruption of normal enamel crystal structure.

Possible mechanisms to explain the antagonistic effect of ethanol on offspring dental health include disruption of organic matrix synthesis which would impair subsequent mineralization or a direct effect upon mineralization of the matrix. Although this study does not clarify which mechanism is most important, we do know that ethanol is capable of inducing skeletal anomalies in offspring of rats given ethanol during a critical period of organogenesis as represented by gestation (15, 16). Since rat enamel and dentin were developing during the time of maternal ethanol ingestion (5), it is possible that enamel and dentin development were impaired at least as visualized by scanning electron microscopy.

The fact that maternal ethanol ingestion specifically reduced offspring molar enamel and dentin zinc suggests that zinc may play a role in the development of crystal structure as it does in bone formation (17). Previous studies from our laboratory (4) and others (2, 3) have shown that zinc deficiency is associated with increased

dental caries. To our knowledge this is the first indication that maternal ethanol ingestion is associated with impaired offspring dental health.

ACKNOWLEDGEMENTS

Thanks are expressed to Mr. Harvey T. Holmes for his assistance in preparing 24-hour cultures of *Streptococcus mutans* and Mr. Alfred Soeldner, Director of the OSU Electron Microscope Facility, for the preparation of scanning electron micrographs.

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TEXT FOOTNOTES

1. Taken in part from a thesis submitted by Lee-Chuan C. Yeh to Oregon State University in partial fulfillment of the requirements for a doctorate degree.
2. Oregon Agricultural Experiment Station Technical Paper no. Partial support was also provided by NIH grant no. DE05628.
3. To whom reprint requests should be sent.

CHAPTER V

CONCLUSIONS

Although the relationship between ethanol and zinc is still questioned by several investigators, the results obtained in the present study clearly indicate that there is an interaction between chronic ethanol ingestion and low dietary zinc during gestation and lactation. Although the direction of interaction was predominately an effect of ethanol on zinc rather than the effect of zinc on ethanol, this study indicates that zinc deficiency is an important consequence of maternal ethanol ingestion.

The antagonistic effects of ethanol on zinc status are exaggerated when ethanol is combined with a low zinc diet. Since zinc metalloenzymes are involved in metabolism of carbohydrate, fat, protein, and nucleic acids, it is reasonable to suggest that the ethanol-antagonized zinc status during gestation and lactation accounts for a large part of the metabolic effects of ethanol on dams and their offspring.

The present study provides the first comprehensive information on both perinatal and postweaning effects of the interaction between maternal ethanol ingestion and low dietary zinc on dams and offspring. The maintenance of a lower than normal maternal tissue zinc and decreased maternal urinary excretion of zinc in response to a hypothesized decreased maternal intestinal zinc absorption is

consistent with a maternal attempt to support the growth and development of offspring. Physiological consequences of ethanol-antagonized zinc status were high lighted by impaired maternal bone health and offspring dental health.

The present study also provides practical information on the use of a nutritionally adequate liquid diet to investigate other possible single nutrient deficiency in ethanol fed rats during gestation and lactation without confounding effects of general malnutrition.

Additional studies are needed to confirm the impairment in intestinal zinc absorption and placental zinc uptake by ethanol ingestion which may contribute significantly to the development of a zinc deficiency state. Supplementation of the diet with zinc during gestation and lactation may be required to correct ethanol-antagonized zinc status and to reduce the occurrence of ethanol-related health hazards. This last aspect however requires further study.

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APPENDIX

TABLE A.1

Mineral analyses of maternal and offspring serum and tibia[1]

Dietary zinc status	High		Low	
% kcal from ethanol	0	30	0	30
Maternal				
Serum Ca, mg/dl	10.2±0.5	10.2±0.3	10.1±0.3	10.1±1.1
P, mg/dl	7.9±0.2	7.9±0.2	7.9±0.2	7.9±0.2
Mg, mg/dl	2.5±0.1	2.5±0.1	2.5±0.2	2.5±0.2
Tibia Ca, mg/g ash	362±12	360±4	363±11	361±7
P, mg/g ash	176±2	175±1	175±1	174±1
Mg, mg/g ash	6.9±0.2	6.6±0.4	6.7±0.3	6.7±0.2
Offspring				
Serum Ca, mg/dl	10.3±0.5	10.3±0.3	10.3±0.3	10.3±0.5
P, mg/dl	8.2±0.2	8.1±0.1	8.1±0.3	8.1±0.2
Mg, mg/dl	2.4±0.2	2.3±0.2	2.4±0.2	2.4±0.1
Tibia Ca, mg/g ash	341±8	340±10	340±4	343±2
P, mg/g ash	181±8	181±6	181±3	182±2
Mg, mg/g ash	9.2±0.2	9.0±0.3	9.2±0.3	9.1±0.1

[1] Values are mean±SD (n=4).

TABLE A.2

Other analyses of maternal and offspring tissues[1]

Dietary zinc status	High		Low	
% kcal from ethanol	0	30	0	30
Maternal				
Liver ADH, μmol/g protein per min	8.6±1.0	7.5±0.7	7.6±0.8	7.6±0.5
Liver protein, mg/g	94±6	91±5	93±5	90±2
Milk protein, mg/ml	151±5	151±7	149±8	152±7
Serum protein, g/dl	5.6±0.2	5.6±0.6	5.6±0.3	5.5±0.5
Serum ethanol, mg/dl	0	2.8±0.3	0	2.8±0.3
Offspring				
Liver ADH, μmol/g protein per min	7.5±0.6	7.3±0.6	7.2±1.0	7.0±1.2
Liver protein, mg/g	80±4	80±5	78±5	77±4
Serum protein, g/dl	4.8±0.4	4.6±0.1	4.4±0.1	4.2±0.2
Serum ethanol, mg/dl	0	2.6±0.3	0	2.6±0.3

[1]Values are mean±SD (n=4).

TABLE A.3

Mineral analyses of molars and tibia of offspring[1]

Dietary zinc status		High		Low	
% kcal from ethanol		0	30	0	30
Enamel Ca, mg/g[2]	M_1[3]	338±10	336±11	336±11	337±7
	M_2	330±10	327±12	329±5	328±10
P, mg/g	M_1	170±4	169±5	169±5	169±3
	M_2	167±4	166±6	165±2	165±4
Mg, mg/g	M_1	1.3±0.1	1.3±0.1	1.3±0.1	1.3±0.1
	M_2	1.1±0.1	1.1±0.1	1.1±0.1	1.1±0.1
Dentin Ca, mg/g	M_1	282±6	281±7	282±2	281±4
	M_2	271±5	268±4	271±5	269±3
P, mg/g	M_1	141±3	141±3	141±2	141±2
	M_2	140±2	139±2	139±3	139±2
Mg, mg/g	M_1	3.0±0.1	3.0±0.2	3.0±0.3	3.0±0.2
	M_2	2.4±0.1	2.4±0.1	2.4±0.1	2.4±0.1
Tibia Ca, mg/g ash		310±5	310±5	309±2	307±2
P, mg/g ash		178±1	177±2	176±2	176±1
Mg, mg/g ash		6.6±0.2	6.6±0.2	6.6±0.2	6.6±0.2

[1]Values are mean±SD (n=4).

[2]Values are expressed as mg/g dry weight.

[3]M_1 and M_2 represent first and second molar, respectively.