**The Effect of a Zinc Deficiency and Alcohol Intake**

**During Gestation in the Rat**

by

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(ABSTRACT)

The effect of alcohol and/or zinc deficiency was evaluated in seven groups of pregnant rats and their pups. Females which had been acclimated to alcohol before breeding were fed liquid alcohol diets with either 14 or 0.1 ppm Zn. Comparisons were made with animals pair fed isocaloric liquid carbohydrate diets with the same Zn levels. Other comparisons were made by pair feeding a high zinc diet to a low zinc diet, and by feeding a high zinc diet ad lib. A reduced food intake and Zn deficiency affected maternal status by decreasing weight gain, liver Zn and plasma Zn concentration. Litter size, litter weight, and fetal liver and brain weight were decreased only in the alcohol zinc deficient group compared to adequately fed controls. The concentration and total quantity of fetal liver Zn were decreased due to a Zn deficiency. The combination of Zn deficiency and alcohol decreased only total Zn in fetal brain. The concentrations of protein, DNA and RNA in fetal liver and brain were similar regardless of dietary treatment. The quantities of protein, DNA and RNA were decreased in fetal liver due to Zn deficiency. In fetal brain, only the combination of alcohol and Zn deficiency decreased total protein and RNA, while DNA was not affected. Although alcohol by itself had no effect on the above variables, its combination with a Zn deficiency did. In addition, there were 58 resorptions and 15 malformations seen in Zn deficient alcoholic dams compared with no more than 15 and 2, respectively, in any of the other groups. Teratogenesis caused by a Zn deficiency was increased with alcohol consumption.

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# 1.0 Introduction

The deleterious effects of alcoholism during pregnancy, termed the fetal alcohol syndrome (FAS), are evidenced as growth retardation, abnormalities of the central nervous system, and anomalies of the face, eyes, heart, and external genitalia. The incidence of FAS in infants born to chronic alcoholics is high (30-50%), and the effects persist postnatally (Hurley, 1980). Alcoholism is commonly accompanied by various nutritional deficiencies, which likely play a role in the teratology. Zinc deficiency, common in alcoholics, results in birth defects similar to those stated above. A possible association between alcohol abuse and an altered zinc status may elucidate the mechanism whereby fetal development is impaired.

Both zinc deficiency and alcohol abuse have been shown to disturb organogenesis, paricularly the development of the central nervous system. The fact that alcohol abuse compromises zinc status suggests that abnormalities associated with the FAS are related to an induced zinc deficiency. Another possibility is that alcohol and low zinc status each act independently, but affect embryogenesis at the same locus or at related points. Evidence points to a critical time when a zinc deficiency affects the developing fetus. If alcohol contributes to a zinc stress, the combinbation of both alcohol intake and a diet deficient in zinc could be greater than either alone.

The purpose of this experiment was to compare the effects of zinc deficiency, alcohol ingestion, and the two combined on gestation in rats. Zinc, nucleic acid, and protein concentrations were measured in fetal brain and liver since the teratology observed often occurs in the central nervous system, and liver is a storage site for zinc in the neonate. Other factors associated with pregnancy outcome, such as dam and fetal weight, litter size, and resorptions were used to indicate the effect of alcohol intake and zinc deprivation.

# 2.0 Review of Literature

## 2.1 Role of Zinc in Gestation

### 2.1.1 Animal Studies

Several animal models have been used to show that zinc has an essential role in prenatal development. A recent review of the effects of a zinc deficit during gestation contains reports of numerous congenital malformations, high resorption rates, and a high incidence of nonviable fetuses. These effects were moreover evident with a transitory zinc deficit, and were not dependent on a reduced food intake (Hurley and Baly, 1982). Pregnant animals suffering from a zinc deficiency typically decrease their food intake and lose weight. Under severe zinc deprivation, an animal may have a discharge around the eyes and vagina and assume a hunched posture.

Pregnant rats subjected to a subtotal zinc deficiency suffered a reduced food intake ($p < 0.01$) and produced offspring with a 20% lower birth weight (Cunnane,1982). Fetal mortalities at birth for animals on a 20 ppm zinc diet were 4.2%, on a 10 ppm were 12.1%, on a 5 ppm were 48.6% ($p < 0.01$), and on a 10 ppm for 15 days followed by a 0.5 ppm to term were 65.1% ($p < 0.001$). Since animals ingesting a 20 ppm zinc diet in amounts equal to this latter group (pair-fed) had the same fetal survival rate as ad lib fed controls, low food intake in the last days of gestation was not the primary cause of fetal mortality.

Herzfeld et al (1985) found that a 10 ppm zinc diet during gestation resulted in a lower fetal zinc concentration ($p < 0.01$) compared to a 50 ppm zinc diet. An experiment (Reinstein et al, 1984) which primarily was designed to show that high dietary levels of copper increased teratogenesis caused by zinc deficiency, also can be used to compare the effects of dietary zinc level. Maternal weight gain and fetal weight were less for animals consuming ad lib diets containing 1 or 4.5 ppm zinc compared to 10 or 100 ppm zinc. Maternal plasma zinc, kidney zinc, liver zinc, intestinal zinc, and whole fetus zinc was greater only with 100 ppm zinc, while there were no differences in fetal liver or brain zinc. Even though the fetuses from rats fed either 1 or 4.5 ppm zinc contained similar zinc concentrations, the frequency of malformations was noteworthy (45%) only at 1 ppm zinc. However, fetal weight increased significantly from 1 to 4.5, and again to 10 ppm zinc.

There is evidence that the period of zinc depletion, as well as the extent of the deficiency, is also a factor in teratogenesis. Although most nutrient deficiencies require a long period of depletion before resulting in congenital malformations, a short zinc deficiency causes plasma zinc to fall rapidly and teratogenesis ensues. In an experiment using 37 pregnant rats, 8.7% of the pups were abnormal when zinc deficiency spanned days 7-12 of gestation, 4.2% for days 10-12, and 2.4% for days 7-9 (Warkany and Petering, 1973). The abnormalities were exencephalies, hydrocephalies, cleft lip and palate, and ocular and skeletal malformations; i.e., primarily defects of the central nervous system. Although there was a general intrauterine growth retardation, in many cases only one of a pair of organs was malformed. According to these researchers, deprivation between days 7 and 12 in a rat corresponds to 2-4 weeks of early pregnancy for a woman.

In another experiment, the consequences of a 1 ppm zinc diet from days 6-14 of gestation were that 35% of the pups were stillborn and 81.8% were malformed at birth, compared with 0% in a pair-fed group receiving 100 ppm zinc (Hurley and Mutch, 1973). Less than 30% survived the first week, compared with 90% of controls. Moreover, adequate zinc nutriture in the latter week

of gestation did not obviate the neonatal abnormalities, suggesting that zinc is essential in the early stages of organogenesis.

Zinc repletion had a significant effect when the dietary level was only suboptimal (Greely, 1984). Rats were fed one of four diets in which zinc levels were either 6 or 40 ppm, and supplemental zinc or magnesium was injected on day 18. Animals on a 6 ppm diet showed increases ($p < 0.01$) in food intake, weight, and plasma zinc (although still depressed) after a zinc injection. The fetuses of this group had higher body weight ($p < 0.05$) and zinc concentration ($p < 0.01$) when compared with animals on a low zinc diet. Magnesium injection had no such effect.

Malformations were observed in another experiment in which the period of zinc deficiency (0.5 ppm) was varied (Hurley et al, 1971). Control animals were fed a 100 ppm zinc diet ad lib. The percent of malformations was 90, 22, 56, and 46 when the days of zinc deficiency were 0-21, 0-10, 0-12, and 6-14 respectively. A zinc deficiency past day 10 greatly increased the incidence of birth defects. If zinc repletion began by day 10, only congenital malformations of the eye remained abnormally high. Even though fetuses were clearly affected, there were no maternal symptoms, including a low weight gain. Since a pair-fed control group, in a follow-up experiment, had 0% malformations compared with 62% in a zinc deficient group, inanition did not cause the malformations. The types of anomalies found during the different periods of zinc deficiency coincided with developmental events occurring at the time. For example, the brain and eye were affected by zinc deprivation before day 10, whereas palate closure was affected later.

Since the offspring are affected to a greater extent by zinc deprivation than is the dam, it appears that this mineral is not easily mobilized from the dam to the fetus. Hurley and Swenerton (1971) tested the ability of maternal liver and bone to transfer zinc to the fetus under a zinc deficiency. The results showed that the dam did not release zinc from these tissues even when the zinc content of fetuses was decreased ($p < 0.01$). Pregnant females do demonstrate an increase in duodenal zinc absorption (Davies and Williams, 1977). The finding that there was no concomitant

increase in lysine uptake indicates that an increased zinc absorption during pregnancy is more than simply a reflection of a general increase in nutrient absorption.

There is evidence that the central nervous system defects associated with a prenatal zinc deficiency may be due to a depression of nucleic acid synthesis. Roughly one hundred zinc-dependent enzymes have been identified, involving numerous physiological functions. DNA and RNA polymerases, transfer RNA synthetase, elongation factor, and thymidine kinase are several zinc-dependent enzymes (Vallee, 1979) which are involved in nucleic acid synthesis. Dreosti and Hurley (1975) found that zinc deficiency for the first twelve days of gestation decreased thymidine kinase activity in rat embryos ($p < 0.01$) compared with a pair-fed control group. The addition of zinc did not restore activity (expressed as pmoles thymidine phosphorylated/mg protein/hr). The authors postulate that there is an impaired synthesis of the enzyme apoprotein, or possibly an increased copper to zinc ratio is involved. In another experiment (Duncan and Hurley, 1978), dams placed on a 0.5 ppm zinc diet were compared to controls on a 100 ppm zinc diet either pair-fed or ad lib fed. Embryos on days 9, 10, 11, and 12 of gestation had a lower activity of thymidine kinase ($p < 0.05$) and DNA polymerase ($p < 0.01$). Similarly, the addition of zinc to the enzyme assay mixture did not restore DNA polymerase activity.

The effect of zinc on DNA metabolism was measured after partial hepatectomy on female rats fed either a zinc deficient diet ( < 0.5 ppm) or a control diet (60 ppm). Duncan and Dreosti (1976) reported that the addition of any combination of the deoxyribonucleotides necessary for DNA synthesis did not increase the incorporation of the $H^3$ -thymidine into the liver DNA of zinc deficient animals. The specific activity of DNA was reported to be significantly reduced ($p < 0.01$) in zinc deficient animals compared with controls (data not shown). Thymidine kinase activity, measured via the formation of $H^3$-thymidine monophosphate, was also reduced in zinc deficiency ($p < 0.01$). Contrary to the results from the previous two experiments, the addition of 1 ppm zinc to the enzyme fractions (nuclear, mitochondrial, post-mitochondrial) forty hours prior to the assay,

did normalize enzyme activity. The authors conclude that the role of zinc in nucleic acid metabolism occurs at least partially after synthesis of DNA precursors.

Fujioka and Lieberman (1964) found that partially hepatectomized rats which were perfused with EDTA, incorporated about one-tenth the amount of $H^3$ -thymidine into the liver as did saline-perfused controls. Moreover, the addition of zinc doubled the lowered rate of DNA synthesis, while magnesium, iron, nickel, cobalt, manganese, or calcium had no effect. The inhibition of DNA synthesis was reversed by zinc addition in yet another experiment involving weanling rats (Sandstead and Rinaldi, 1969). An injection of 100 μg zinc 24 hours prior to analysis increased the incorporation of $H^3$-thymidine into the nuclei of liver parenchymal cells.

A reversal of DNA inhibition with zinc supplementation has also been shown in embryos (Eckhert and Hurley, 1977). Pregnant rats were fed either a 0.4 or a 100 ppm zinc (pair-fed) diet for the first twelve days of gestation. Some zinc deficient animals were injected with $ZnCl_2$ just prior to injection with $H^3$-thymidine. Embryos were divided into head and body regions, and the uptake of $H^3$-thymidine into each was determined. The results were a 67.8% reduction ($p < 0.05$) in $H^3$ -thymidine and a reduced DNA content ($p < 0.01$) with low zinc in only the head region, which did not occur when zinc was injected. The RNA content was lower in both the head and body regions of zinc deficient embryos, but was not increased with supplementation. The particular effect seen on nucleic acid synthesis in the head region could be related to the craniofacial defects seen in zinc deficiency.

There is information on nucleic acid metabolism from studies which also evaluate the effects of zinc deficiency on the dam and fetus. When pregnant rats were fed a 1 ppm zinc diet on days 14-21 of gestation, they weighed less than their pair-fed controls by day 19 (McKenzie et al, 1975). Hematocrit was higher ($p < 0.01$) for zinc deficient dams, and serum zinc concentration was different ($p < 0.01$) between all three groups (including an ad lib control). Serum zinc levels (μg/ml) were 27.8 for zinc deficient, 97.6 for pair-fed, and 108.1 for ad lib fed animals. Although litter size

did not vary, fetal carcass, brain, and liver weights were lower with a zinc deficiency. There was no difference in fetal brain DNA concentration, but total DNA was increased in pair-fed animals. Zinc deficiency resulted in no effect on fetal brain RNA, protein, or zinc. Compared to fetal brain, the liver was severely affected by zinc deficiency. The quantities of DNA, RNA, and protein, but not the concentrations, were decreased. Liver zinc was decreased ($p < 0.01$) in both concentration and amount, as was whole carcass zinc.

Buell et al (1977) studied the postnatal effects of zinc deficiency by feeding nursing dams 1 ppm zinc, while both pair-fed and ad lib fed controls received 25 ppm zinc in the drinking water. Control pups weighed more, and had a lower hematocrit. The cerebella of zinc deficient pups weighed 37%, and that of pair-fed pups weighed 24% less than those of ad lib fed pups. Although the concentrations of cerebellar DNA, RNA, and protein were not different, the total amounts were significantly less for the zinc deficient pups. The fact that zinc deficiency produces less total DNA, but a relatively high concentration, indicates a reduced cell size. Using the same protocol, Fosmire and Sandstead (1977) injected pups with labeled leucine on days 6, 12, and 21 postpartum. DNA concentration was actually increased with zinc deficiency at day 12, while the incorporation of $C^{14}$ -Leucine into protein was decreased in liver.

Duerre et al (1977) used suckling rats to study the effect of zinc deficiency on pup liver and brain, and found no differences in the weight or DNA content of pup cerebrum, cerebellum or liver after 10 days. The rates of incorporation of $H^3$-Lysine into liver proteins and into nonhistone proteins in the cerebrum did not vary by diet. However, there was evidence of an increased histone biosynthesis in the pair-fed controls compared to zinc deficient pups, suggesting that zinc deficiency impairs cell proliferation. Turk (1966) used the young chick to study zinc deficiency. Although zinc deficient diets produced characteristic growth depression and leg deformities in three week old chicks, there were no differences in liver nucleic acids or protein.

Theuer and Hoekstra (1966) studied the effect of zinc deficiency on protein metabolism by injecting $C^{14}$-labeled substrates and monitoring respiratory $CO_2$. The oxidation of leucine and lysine was significantly greater in zinc deficient rats, suggesting a defect in protein synthesis. Since the control animals were fed ad lib, and did weigh more, the deficiency could have caused an energy deficit which led to protein catabolism.

Zinc deficiency has also been related to brain structure and function. Dvergsten et al (1983) evaluated the effect of zinc deficiency on the central nervous system by placing newborn rats on a zinc deficient diet for 21 days. They found a 60% reduction in granule cells (the predominant neural cell) in the cerebellar cortex, suggesting an impaired cell proliferation not adequately accounted for by reduced food intake. In another group of 30 day old rats showing cyclic feeding, hunched posture, and dermatitis commonly associated with a zinc deficiency, there was a reduction in the synthesis of a zinc-binding ligand (metallothionein), but no difference in brain zinc concentration (Ebadi and Wallwork, 1985). In a previous experiment, Wallwork et al (1983) again found no significant differences in the zinc concentration of various brain regions (olfactory lobes, hippocampi, and cerebellum) of animals given a 20% spray-dried egg white diet containing less than 1 ppm zinc. Control animals received 25 ppm zinc in the drinking water.

The effect of a zinc deficiency on synaptic function in the brain was studied by placing electrodes in the origin of the mossy fiber axons of the hippocampus to measure the field potentials evoked by low frequency stimulation (Hesse, 1979). Four week old rats fed a zinc deficient diet for 7-9 weeks were compared with pair-fed and ad lib controls. The synaptic field potentials declined in amplitude with successive stimulation in zinc deficient rats, but increased in control animals. The researchers suggest that zinc deposits in mossy fiber boutons are needed for synaptic transmission.

### 2.1.2 Human Studies

There is epidemiological evidence of a connection between zinc deficiency and congenital malformations in humans (Sever and Emanuel, 1972). In Iran and Egypt, where clinical signs of zinc deficiency are relatively common, there is a high rate of central nervous system malformations compared to other countries. Two factors which contribute to this are a low dietary zinc intake, and a poor bioavailability of zinc related to a diet high in phytates. A 16 month survey was done in an obstetric department in Ankara, Turkey to determine the rate of anencephaly. Ninety women who gave birth to normal babies had a mean serum zinc concentration of 73.4 μg%, while ten women with anencephalic babies had a concentration of 62.1 μg% ($p < 0.001$) (Cavdar et al, 1980a). Serum zinc levels were lower over the 3 trimesters of gestation in poorly nourished pregnant women compared with nonpregnant women controls ($p < 0.05$) or with well-nourished pregnant women ($p < 0.001$) (Cavdar et al, 1980b). However, Prema et al (1979) found that serum zinc levels were similar in high and low income groups in Indian women. (The diet of people on a low income is typically low in meat, and often deficient in zinc.)

Although there is clear evidence of zinc deficiency in Middle Eastern countries related to the high cereal diet, the intake of zinc in the United States has also been shown to be below the RDA. The zinc content of self-selected diets was less than 10 mg/day in one third of a group of high school girls, and in one fifth of a group of college women (White, 1976). Wolf et al (1977) composited six daily diets of 22 subjects aged 14-64, and found the mean daily intake of zinc to be 8.6 $\pm$ 0.5 mg. Pregnant women on self-selected diets without a zinc supplement ingested less than 10 mg zinc per day, and had a serum zinc concentration of 65 $\pm$ 9μg% in the second trimester (Taper et al, 1985). New Zealand women had a mean zinc intake of 11.6 mg/day (Guthrie and Robinson, 1977), while 85% of pregnant women of Mexican descent consumed less than two thirds the RDA (Hunt, 1979).

Soltan and Jenkins (1982) studied maternal and fetal plasma zinc in relation to congenital malformations. Plasma zinc was lower ($p < 0.001$) in women giving birth to babies with birth defects, whether occurring in the past two years or within 24 hours. The zinc level of cord blood was also lower ($p < 0.05$) in abnormal babies. These results suggest that the defects, primarily involving the central nervous system, are related to a persistent zinc deficiency. In other studies, Jameson, (1976) also found lower plasma zinc ($p < 0.001$) in women suffering complications at delivery or bearing immature infants. The woman bearing an infant with a fatal heart defect had the lowest serum zinc (12.1 μmole/l). However, Hunt et al (1985) found no association between serum zinc levels and birth outcome in a group of pregnant teenagers.

Acrodermatitis enteropathica, a genetic disease in which there is an impaired zinc absorption, reveals the effects of a severe zinc deficiency in pregnant women. This condition can now be corrected with a zinc sulfate supplement. Hambidge et al (1975) describe seven pregnancies in which the mothers suffered from this disease. One woman had a first pregnancy abort at twelve weeks, and a second result in an anencephalic stillbirth. A similarly afflicted woman delivered a nonviable offspring with multiple skeletal malformations, but after controlling her disease bore three normal children. A third woman with a mild form of acrodermatitis enteropathica had a normal baby.

A zinc deficiency results in congenital defects in both laboratory animals and humans. In general, a longer period of zinc deficiency increases the extent of malformations. However, a zinc stress extending past the midpoint of gestation has the greatest effect. Enzymes involved in nucleic acid synthesis are affected by zinc status. Thymidine kinase and DNA polymerase activity are decreased by a zinc deficiency. Although the quantity of DNA is frequently decreased, the concentration is not. One explanation is that the total number of cells is reduced due to impaired DNA synthesis. Another possibility is that undernutrition caused by a reduced food intake has reduced tissue mass. While the epidemiological evidence does not clearly demonstrate that birth defects

have been caused by a zinc deficiency, cases in which pregnant women suffered from acrodermatitis enteropathica support the connection.

## 2.2 Effect of Alcohol during Gestation

Hurley (1979) summarizes the features of fetal alcohol syndrome (FAS) as mainly growth and mental retardation, and malformations of the face, heart, joints, and external genitalia. Growth deficiency persists even with good postnatal care. Neurological problems are first evidenced by neonatal irritability, and later as motor dysfunction and developmental delay. Moreover, these abnormalities appear to be linked to a brain dysfunction caused by teratology of the central nervous system. Thirty to fifty percent of chronically alcoholic pregnant women produce babies with FAS, with almost certain symptoms of growth and developmental deficiencies, and often facial abnormalities. Alcohol may exert a direct effect on the embryo via transport across the placenta. Another mechanism is that of an indirect effect through alterations in maternal metabolism. Nutritional deficiencies often accompany alcoholism. Increased levels of acetaldehyde and NADH may affect the fetus. In addition, the mother may suffer from associated risk factors, such as smoking or emotional stress.

Brown et al (1979) used cultures of nine and a half day-old rat fetuses to show that ethanol has a direct effect on the embryo. Days nine and a half to eleven and a half correspond to days 20-30 of human gestation. For twenty-four hours the fetal tissues were subjected to alcohol, either 150 or 300 mg/100 ml, or to control culture media. There was less total DNA and protein ($p < 0.01$) with 300 mg alcohol compared to the other two groups. This group also showed a deficiency in the number of cells compared with controls, so there was also less growth. The ratio of total DNA to protein was not different, indicating no effect on cell size. These researchers suggest that alcohol has a direct action of decreasing cellular proliferation during organogenesis.

Cellular changes induced by an acute dose of ethanol were studied in fetal mice at day 9 of gestation (Brannigan and Burke, 1982). Pregnant mice were given an intraperitoneal injection of 0.03 ml of 25% ethanol/g body weight, and then given tritiated thymidine five hours later. No changes were seen for six hours, after which time the researchers found degenerating cells and necrotic fragments in the neuroepithelium. Many of the degenerating cells were labeled, i.e., were capable of incorporating thymidine, suggesting that DNA synthesis is not the site of toxicity. Abnormal vacuoles found in the cells may be related to a defect in a cellular component. By fifty hours the dead cells and necrotic debris had been phagocytized, but many embryos had neural tube defects remaining. On day 19 living fetuses numbered 8.5 with ethanol and 11.4 with saline. Although mean fetal weights did not differ, 21.3% of the ethanol group had exencephaly.

The effects of alcohol differ depending on whether the insult is acute or chronic, since a physiological adaptation to alcohol occurs (Rider, 1979). Rats which were fed alcohol as 10% of the drinking water for three months prior to pregnancy had better reproductive performance than those started at day 1 of gestation. While 88% of control animals and 100% of adapted animals delivered pups, only 62% of the unadapted animals delivered. The number of pups per litter was 10.5 for controls, 8.8 for adapted animals, and 4.4 for unadapted animals. Caloric intake, measured on days 15-16, was 66% (adapted) or 57% (unadapted) of the control animals' consumption of chow. Alcohol provided 29% of the calories for both groups. There were no differences in weight gain. Another group of pregnant animals offered a 20% v/v drinking solution also decreased their food intake, with both dams and offspring gaining less weight compared with controls (Sigh and Snyder, 1982).

Borges and Lewis (1982) also studied the effects of giving pregnant rats drinking water with 10% alcohol, beginning with mating. The animals ingested 9 ml ethanol/kg body weight/day fairly constantly. There were no differences in maternal weight gain, or fetal body weight and whole brain weight from birth to 21 days postpartum. However, fetal cerebellar weight was significantly reduced

at 21 days with alcohol. At parturition, the ethanol group had a higher total caloric intake ($p < 0.05$), but approximately 28% of these calories were from alcohol. Liquid intake was greater ($p < 0.05$) for controls.

Controlling nutrient, caloric and water intake is difficult in experiments with alcohol. For this reason, DeCarli and Lieber (1967) devised a liquid diet which included the alcohol and provided one Kcal per ml. Control animals could then ingest isocaloric amounts with sucrose substituting for the alcohol calories. Sherwin et al (1980) found that doubling the vitamin and mineral content was necessary to permit offspring to survive and develop normally when dams ingested 30-40% of the calories from a liquid alcohol diet. There was no consistent effect of alcohol in reducing pup weight.

The effect that alcohol has on protein metabolism should be considered in interpreting experimental findings. Alcohol decreases the synthesis of total hepatic protein and glycoproteins, and interferes with the secretion of other hepatic proteins (e.g., albumin and transferin) into the bloodstream (Baraona et al, 1977). Newborn pups dosed with a diet deficient in protein suffered a more severe growth impairment and improved less with refeeding than did those on a low calorie diet (Lau, 1974). Zeman and Stanbrough (1969) found that total DNA was significantly decreased in organs of fetuses and newborns as a result of maternal protein deficiency. To determine the possibility of a protein deficiency accompanying alcohol abuse, Weiner et al (1981) fed pregnant rats a liquid diet containing 29% of the calories as alcohol, and compared them to an isocaloric pair-fed group. The pups had a lower body and brain weight when dietary protein was 16% of the calories, but not 30% of the calories.

Sigh and Snyder (1982) found that pregnant rats ingesting a 36% ethanol diet had pups weighing less than a pair-fed group. These researchers used the original Lieber-DeCarli liquid ethanol diet with 18% of the calories from protein. An alcohol intake of 13 or 20% produced no differences. In another experiment in which the sole source of drinking water was 24% alcohol, the

number of fetuses per dam and fetal weight were less compared with pair-fed controls (Suh and Firek, 1982).

The effect of alcohol on brain nucleic acids has been studied (Woodson and Ritchey, 1979). When dams ingested drinking water containing 15% alcohol, there was a decrease in the number of fetal brain cells compared with pair-fed controls. Although both DNA and RNA/brain was decreased, alcohol caused no significant decrease in mg DNA/g brain tissue. Rats which were acclimated for four weeks to a diet containing 6% w/v alcohol and 18% of the calories from protien gained less weight than pair-fed controls (Henderson and Schenker, 1977). Although alcohol led to no difference in litter size, the mean weight of pups surviving 3 days was significantly lower, and 30% less did survive 3 days ($p < 0.001$). There were no differences in organ weights when expressed as a ratio to body weight, or in protein concentration of organs. Although DNA concentration in brain, heart or kidney was unaffected by alcohol, DNA concentration in liver was 15-19% decreased.

Both chronic and acute alcohol during gestation result in abnormal cellular development in the offspring. Increasing amounts of alcohol cause more severe reductions in weight gain and fetal survival. Although the main effect on nucleic acids is that of a decrease in quantity, fetal liver DNA concentration has been reduced.

## 2.3 Interaction of Zinc Level and Alcohol Intake

As stated previously, alcohol abuse is often accompanied by nutritional deficiencies. Russell (1980) reports that 30-60% of alcoholic patients without liver disease, and 70% with cirrhosis have low serum zinc levels. Drinking alcohol in amounts sufficient to maintain blood

levels of 100 mg/ml is accompanied by an increase in urinary zinc excretion and a decrease in serum zinc. Rats fed a zinc deficient diet for four weeks have higher urinary and fecal zinc losses, and lower tissue zinc levels if also fed alcohol.

Sullivan and Lankford (1965) monitored urine and serum zinc levels in chronic alcoholics without cirrhosis. In 21 out of 30 subjects, serum concentration was more than two standard deviations below the mean of the normal population. Forty-two percent of the subjects had urine zinc levels greater than the mean plus 25 of normal zinc excretion, with zinc excretion being independent of urine volume. Moreover, there was an increase in the renal clearance of zinc in one-third of the subjects having either normal or low serum zinc levels. Another third showed normal clearance although serum zinc levels were depressed. Generally, after one to two weeks with no alcohol plus an adequate diet, urinary zinc excretion returned to normal.

A number of physiological abnormalities observed in cirrhotics are related to zinc metabolism (Russell, 1980). Concurrent with a decreased serum zinc and an increased urinary zinc, is a decreased level of serum albumin. Another factor is that cirrhotics have increased levels of circulaing amino acids due to a decreased metabolism in the liver. This could cause more zinc to be bound to micromolecular ligands (e.g., histidine), resulting in more zinc being filtered at the renal glomerulus.

There is an interaction of zinc, ethanol, and vitamin A (Russell, 1980). Retinol is converted to retinal via alcohol dehydrogenase, a zinc dependent enzyme. Not only is it likely that vitamin A is deficient in alcoholics, but ethanol itself is a competitive inhibitor with retinol for alcohol dehydrogenase. Cirrhotic patients who were low in both vitamin A and zinc were given vitamin A, but there was no improvement in their dark adaptation curves. However, curves were normal after two weeks of zinc treatment (Russell, 1980). Zinc may also be required for mobilization of hepatic vitamin A via the synthesis of retinol binding protein (the specific transport

protein of vitamin A). When children with protein-calorie malnutrition were given zinc supplements, there was an increase in serum vitamin A and retinol binding protein.

In a review of zinc deficiency in the alcoholic, McClain and Su (1983) discuss mechanisms whereby zinc metabolism is impaired in alcoholics. The occurence of zinc deficiency is probable in alcoholics connected with a low zinc intake, impairment of zinc absorption, decreased binding between serum albumin and zinc, or a high urinary loss. Alcohol dehydrogenase is also required for testicular retinal regeneration and thus spermatogenesis. Reports of zinc deficiency in youths, especially from the Middle East, often include hypogonadism as a symptom. Chronic alcoholic men may be both hypogonadal and feminized. There are reports of decreased testosterone levels in alcoholic cirrhotics that improved with zinc supplementation.

This review discusses other dysfunctions seen in both zinc deficiency and alcohol abuse. Skin lesions are symptoms of a severe zinc deficiency, and are evident in laboratory animal experiments and in people suffering from acrodermatitis enteropathica. Chronic alcoholics with skin lesions have been cured with oral zinc supplementation. Similar to the decrease in food intake seen in laboratory animals, chronic alcoholics have a depressed food intake and a disordered taste acuity. Some improvement has been seen with zinc therapy. Carcinomas and an immune dysfunction are also seen in both zinc deficiency and alcohol abuse. A relationship between alcohol and zinc in these instances is theoretical.

Animal models have been used to determine the effect of alcohol on zinc status. At day 20 of gestation, rats were given 4 gm ethanol/kg body weight by nasogastric tube, and then an injection of labeled zinc (Gishan et al, 1982). Although there was a 40% decrease in zinc uptake in the ethanol group compared with controls ($p < 0.001$), there were no differences in maternal or fetal tissue zinc concentration. The effect of alcohol on trace elements in maternal and fetal tissues was determined when rats were fed alcohol as 6% of the water supply for two pregnancies (Mendelson and Huber, 1979). The diets contained either 12 ppm zinc, or 28 ppm zinc as ZnMg, plus

alcohol.There was no effect of alcohol on litter size or average fetal weight. Although the dam had decreased zinc levels in only the femur, alcohol resulted in decreased zinc and increased copper and iron levels in the fetus.

There was an effect on the litter size and weight when pregnant rats were given a 24% alcohol solution in place of drinking water (Suh and Firek, 1982). Control animals were pair fed chow, and the alcohol calories (12-20 % of the total calories) matched with sucrose. Dams that were fed alcohol eight weeks prior to mating and during the first 20 days of gestation gained less weight ($p < 0.05$). Zinc concentration in maternal plasma was the same, but greater in the muscle of controls. The number of fetuses/dam, fetal weight, and zinc concentration in the fetus was less ($p < 0.05$) in alcohol animals. There were ten resorptions in the alcoholic dams, with only one in controls.

In another experiment rats were fed 20% alcohol in the drinking water for four weeks prior to mating, and 30% up to day 20 of gestation (Jones et al, 1981). Weight gain for pair fed and alcohol animals, ingesting only 60% of the caloric intake from diet of ad lib controls, did not differ. There were no differences in maternal plasma zinc concentration among the three groups. As in the previous experiment, alcohol fetuses were significantly smaller than the controls. In this case, though, the reduced intake of both pair fed and alcohol animals reduced litter size. These researchers noted no obvious malformations from alcohol. Fetal resorptions were 2.6% in ad lib animals. 5.6% in pair fed animals, and 7.1% in alcohol fed animals.

The effect of a long period of chronic alcohol intake was evaluated in monkeys (Keen et al, 1985). Monkeys were fed a diet containing 50% of the calories as alcohol for four years. Comparisons were made with a pair fed control group. Monkeys subjected to alcohol had a lower liver zinc concentration ($p < 0.01$).

Contrary to there being no effect of alcohol on plasma zinc concentration, Dreosti and Record (1979) found an increase in zinc deficient dams following ethanol ingestion. Ethanol was in the drinking water from conception till day 20 of gestation. Although a 0.5 ppm zinc diet reduced fetal weight and maternal plasma zinc concentration, the addition of 20% alcohol improved fetal growth 17% and maternal plasma zinc from 0.49 to 0.86 ppm. Plasma zinc concentrations were 1.27, 1.25, and 1.26 ppm for animals ingesting a 100 ppm zinc diet pair fed, ad lib, or in combination with 20% alcohol respectively. These researchers suggest that alcohol caused a release of zinc from maternal stores. At any rate, alcohol did not enhance the nutritional zinc deficiency as it affected fetal growth and plasma zinc concentration.

Alcohol affects zinc status in weanling rats when administered over an extended period of time. Wang and Pierson (1975) determined the distribution of zinc in male rats fed laboratory chow and a 20% alcohol solution for fourteen weeks. Plasma zinc concentration gradually decreased, becoming significantly less than controls at nine weeks. While liver zinc levels were lower at two weeks, muscle zinc was only lower at fourteen weeks. A confounding factor in this experiment was that food intake for alcohol animals was depressed, and their weight was only 62% of that of controls. In addition, alcohol animals drank one-third as much as controls.

Ahmed and Russell (1982) also found a depletion in tissue zinc levels with alcohol. Rats were given a zinc deficient liquid diet with 0.9 μg/ml and 1 kcal/ml, while controls received isocaloric amounts of sucrose. After one week, testes zinc was significantly less in ethanol animals. After four weeks, liver, hair, spleen, kidney, skin, muscle, lung, and heart zinc levels were also less. At this time, body weights of alcohol animals were significantly lower. In addition, urinary and fecal zinc losses were higher with ethanol. Animals on a zinc deficient diet were shown to have a negative zinc balance and a depletion of tissue zinc levels after four weeks. These researchers suggest that ethanol and an accompanying malnutrition eventually induce a tissue depletion of zinc from catabolism, and result in a high urinary zinc loss and low tissue zinc concentration.

Mice were used to determine that a zinc supplement failed to reverse teratogenesis induced by alcohol (Leitch and Rosemond, 1981). Ethanol as 12% of the drinking water was given to mice for six weeks prior to breeding, and until day 17 of gestation. The miscarriage rate was 0/16 for control animals, 1/12 for alcoholic animals with a zinc supplement, and 2/14 for alcohol animals. Mean pup weight did not differ among the three groups. Anomalies found, such as hydrocephalies, were 0 for control animals, 9 for alcohol animals on a zinc supplement, and 7 for alcohol animals.

Collipp et al (1984) studied the effect of zinc deficiency on voluntary alcohol consumption in rats. Animals, made zinc deficient via a diet with less than 2 ppm zinc for six weeks, exhibited the characteristic zinc deficiency symptoms such as reddened scaly paws and eyelids, and sparse hair. Voluntary alcohol consumption was higher ($p < 0.05$) for the zinc deficient animals by the fifth week. A two week zinc repletion period altered this difference in some of the animals.

The effect of zinc deficiency on the elimination rate of alcohol was studied in weanling male rats (Das et al, 1984). Animals fed a liquid diet containing 5% ethanol and less than 1 ppm zinc, and animals fed a diet with less than 1 ppm zinc had a lower liver zinc concentration than animals fed a control diet with or without 5% ethanol. The elimination rate was determined by administering a dose of alcohol and measuring serum alcohol one hour later. Both groups of animals on a zinc deficient diet had lower ($p < 0.01$) rates of ethanol elimination compared with ad lib or pair fed controls. Animals on a control diet with ethanol had higher rates than all other groups. The adaptive increase in ethanol elimination rate seen with chronic alcohol intake was not evident with a zinc deficiency.

Dutta et al (1980) measured the absorption of zinc as a function of ethanol ingestion and zinc nutriture. Data was obtained after administering labeled zinc in the stomach, and recording the counts collected in the animal's urine and stool. The results were that zinc absorption was lowest in ethanol-fed rats (19 ± 3%) compared with control rats (32 ± 6%), zinc deficient rats (74

± 1%), and zinc supplemented rats (22 ± 3%). Ethanol significantly decreased intestinal zinc absorption in the adult rat.

The ability of alcohol to impair zinc transport across the intestine may also be evident in the placenta. From day 4 to 20 of gestation rats were given either a 5% ethanol diet or pair-fed a control diet (Ghishan et al, 1982). Labeled zinc was injected into the femoral vein on day 20. Maternal weight gains were similar in both groups, but fetal weight was significantly lower with ethanol. Fetal zinc uptake was decreased 30% in the ethanol group compared to corresponding values in pair-fed controls ($p < 0.001$). Zinc concentration was significantly less in maternal serum, the placenta, and in the fetus. Maternal femur was not depleted in zinc. Even though fetal zinc was stressed, zinc was not mobilized from maternal bone. In this experiment chronic alcohol ingestion reduced fetal zinc.

In another experiment in which fetal uptake of zinc was measured, the animals were first adapted to alcohol (Jones et al, 1981). Rats were given a 20% alcohol diet four weeks before mating, and a 30% alcohol diet during gestation. The uptake of labeled zinc was determined in the fetus ninety minutes after the dam was injected on day 20. There was no difference in ethanol rats, rats pair-fed the diet with isocaloric substitution of sucrose for the alcohol calories, or rats fed the diet ad lib with no ethanol. There were no fetal malformations with ethanol. When these animals were adapted to alcohol prior to gestation, the impairment of fetal zinc uptake was absent.

Rats were subjected to a moderate alcohol exposure via a liquid diet with 6% alcohol for 30 days prior to mating, and until day 20 of gestation (Henderson et al, 1979). Controls were pair-fed. The weight of the ethanol dams was less ($p < 0.05$), and the resorption rate (expressed as resorptions per 100 implants) was higher ($p < 0.01$). Alcohol caused reductions ($p < 0.05$) in fetal brain, heart, kidney and liver weights. In an accompanying experiment, acute exposure to alcohol for days 11-13 or 14-16 resulted in less total DNA in these organs. However, chronic exposure decreased kidney DNA content only. The effect of chronic alcohol on DNA concentration was an

increase in the aforementioned organs. Tissue protein concentration was also increased with chronic alcohol. Similar to previous results, there was no effect of alcohol on zinc levels of maternal bone or brain; in this case, there was also no effect on fetal bone or muscle.

The interaction of zinc level and ethanol was determined by combining a 2 or 10 ppm zinc liquid diet with or without ethanol as 30% of the calories (Yeh and Cerklewski, 1984). The 2 ppm zinc translates to about 9 ppm zinc dry diet. There were four animals in each of the four diets during gestation, and for 21 days postpartum. There were no differences in food intake, weight gain, or alcohol-dehydrogenase activity in the liver in the dam. The zinc content of maternal liver and kidney decreased due to either low zinc or ethanol, while maternal muscle and offspring kidney and muscle did not differ. Serum zinc concentration and alkaline phosphatase activity was decreased in both dams and offspring when low zinc or ethanol was compared with controls, and also when low zinc plus ethanol was compared with any of the other three groups. These researchers conclude that an interaction between zinc and alcohol intake exists.

Ruth and Goldsmith (1981) also studied an interaction by feeding dams a zinc-deficient diet ( < 2ppm) on days 7-11, and injecting ethanol intraperitoneally on day 10. A control group was injected with saline. Either low zinc or ethanol (2.4 g/kg body weight) resulted in growth retardation, resorption or cranial malformations in one of six litters. When zinc deprivation was combined with 1.9 g/kg alcohol, two of four litters were affected. A combination of 2.4 g/kg alcohol and zinc deprivation caused four of five litters to be affected. There was no effect on maternal weight. The teratogenic effect of either alcohol or zinc deficiency was increased by combining the insults. These researchers suggest that even infrequent intoxication can be harmful when combined with a dietary deficiency.

The Michigan Alcoholic Screening Test was used to identify twenty-five pregnant women who were alcoholic (Flynn et al, 1981). These women had lower plasma zinc ($p < 0.05$) during labor when compared with non-alcoholic pregnant women. There was a significant correlation

($p < 0.05$) between plasma zinc and birth defects in all the subjects. Although fetal cord plasma zinc concentration was lower ($p < 0.05$) in those designated alcoholic, there was no correlation with birth defects. There were more birth defects in infants born of alcoholic mothers (39) than nonalcoholic mothers (22). However, there was no significant correlation between alcohol intake during pregnancy and birth defects. A problem in interpreting these results arises because counseling was initiated at the start of pregnancy for the alcoholic women, and their intake had been reduced. In fact, drinking behavior of the two groups during pregnancy did not differ significantly. Nonetheless, fetal dysmorphogenesis could still be related to zinc status and previous alcohol ingestion.

There is a relationship between zinc status and alcohol. Chronic alcoholics evidence an altered zinc metabolism, i.e., increased urinary zinc excretion and decreased plasma zinc concentration. Animal studies show that tissue zinc levels are depleted by long-term alcohol, whether administered as a drinking solution, or in a liquid diet with pair-fed controls. Although alcohol does not consistently decrease maternal zinc status, the offspring are stressed to a greater extent. Studies report smaller litter size and weight, and reduced zinc content in the fetus in most cases. Contradictory research indicates that alcohol in combination with a zinc deficient diet can actually increase fetal growth and maternal plasma zinc. Moreover, a zinc supplement did not reverse alcohol-induced teratogenesis. An important factor in intrepreting this information is that an adaptation to alcohol moderates its effect on the fetus. It appears that alcohol intake during gestation has a greater effect on zinc status and fetal dysmorphogenesis when accompanied by a zinc deficiency, than either condition alone.

# 3.0 Methodology

## 3.1 Experimental Design

The purpose of this experiment was to compare the effects of alcohol intake and a zinc deficiency during gestation, both singularly and in combination. The four treatments to be compared were a diet low in zinc, a diet low in zinc containing alcohol, a diet containing alcohol, and a control diet. Additional pair fed control groups were included since zinc deficient animals greatly reduce their dietary intake, confounding the effects of a zinc deficiency. Preliminary experiments indicated that alcohol as part of the drinking water was a poor vehicle for ethanol ingestion. The ethanol rats greatly reduced their drinking compared to control animals. Animals offered a liquid ethanol diet ingested more ethanol, and caloric intake was easily manipulated. Animals were acclimated to alcohol before mating since the effects of a chronic, rather than acute, alcohol exposure were determined.

## 3.2 Treatment Groups

The basic experimental design was to feed animals a liquid diet with a zinc level of either 0.1 (-Zn) or 14.3 (+Zn) ppm, with and without alcohol (Alc) as 25% of the calories. The animals with no alcohol received isocaloric amounts of carbohydrate (CHO). These four ad lib fed groups are listed below, along with their pair-fed controls.

1. Alc, -Zn (pair-fed control is) 2. CHO, -Zn
3. Alc, +Zn (pair-fed control is) 4. CHO, +Zn
5. CHO, -Zn (pair-fed control is) 6. CHO, +Zn
7. CHO, +Zn

A comparison between groups 1 and 2 should show any exacerbating effect of alcohol when a zinc deficiency is already present. The differences noted between groups 3 and 4 should be due to the effect of alcohol, and possibly the effect of alcohol on zinc. Groups 5 and 6 were used to evaluate the effects of a zinc deficiency by itself. Group 7 was an ad lib fed control group used to verify that the liquid diet is adequate for gestation.

## 3.3 Animal Protocol

Sprague Dawley virgin female rats, weighing 151-175 g, were obtained from Charles Rivers, Boston, Mass. The experiment was conducted in a limited access barrier facility with constant temperature and humidity. Animals were housed individually in stainless steel cages throughout the experiment, except for the days required for breeding. All were initially fed standard Purina rat chow and given water ad lib.

For three weeks prior to breeding, the females were acclimated to the liquid diet. Since two of the diets contained alcohol, roughly two out of every seven animals were acclimated to alcohol by gradually increasing the dosage. The percent of the calories ingested as alcohol was 9, 15, 21, and 24 for days 3-6, 7-10, 11-14, and 15-21 of acclimitization respectively.

After three weeks of the liquid diet, the females were removed to a breeding cage, and both the male and female ate rat chow and drank deionized water. The presence of a plug was taken to

indicate mating, and the female was returned to a solitary cage and a liquid diet. Animals were placed into dietary groups so that the number in each remained equitable, and also so that the body weights remained comparable. The pair fed control animals were individually offered the identical food intake of an animal in groups 1, 3, or 5 on a given day of gestation.

The food intake of each animal was recorded on a daily basis starting with the acclimation period. A measured volume of liquid diet was poured into a heavy ceramic dish which was clamped into the cage. The dishes were washed daily, and acid-washed for the low zinc diets. Animal weights were obtained at the end of each week of gestation.

## 3.4 Diet Formulation and Analysis

A liquid diet is the best vehicle for meeting the experimental animal's nutritional requirements while allowing accurate measurement of alcohol and caloric intake (Altshuler, 1981; Lieber and DeCarli, 1982 ). Alcohol in the drinking water is often poorly consumed (Borges and Lewis, 1982). The acclimation diets were 20% protein on a dry weight basis, and the liquid diet provided 1 kcal/ml. During gestation, the protein level was increased to 30%. A solution of 5.717 g $ZnSO_4$ /100 ml was added to the liquid diet as 1 ml/liter to provide 13 ppm Zn on a wet weight basis. This translates to 50 ppm Zn on a dry weight basis for the CHO diet. In order to keep the dry diet and alcohol (190 proof) in a suspension, zanthan gum (0.5%) was added. The actual dietary components are listed in Table 1.

The animals designated to eventually receive a 4.4% alcohol (v/v) diet were given gradually increasing alcohol concentrations to maximize food intake (DeCarli and Lieber, 1967) . Alcohol dosages (v/v) were 1.5, 2.5, 3.5, and 4.2 % for acclimation days 3-6, 7-10, 11-14, and 15-21 re-

**Table 1. Formulation of Diets, per Liter of Liquid Diet**

| | Acclimation Period | | | | | Gestation | |
|---|---|---|---|---|---|---|---|
| | Days | | | | | | |
| | 1-21 | 3-6 | 7-10 | 11-14 | 15-21 | Days 0-21 | |
| | CHO | Alc | | | | CHO | Alc |
| Spray-dried Egg White (g) | 51.54 | 51.54 | 51.54 | 51.54 | 51.54 | 74.87 | 74.87 |
| Corn Starch (g) | 85.30 | 85.30 | 63.40 | 54.38 | 48.55 | 73.71 | 37.00 |
| Dextrose (g) | 84.01 | 57.38 | 62.10 | 54.18 | 47.26 | 72.42 | 35.70 |
| Alcohol (ml) | — | 15 | 25 | 35 | 42 | -- | 44 |
| Fat (g) | 12.88 | 12.88 | 12.88 | 12.88 | 12.88 | 12.88 | 12.88 |
| Fiber (g) | 7.73 | 7.73 | 7.73 | 7.73 | 7.73 | 7.73 | 7.73 |
| Choline Chloride (g) | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 |
| Mineral Mix (g)[1] | 12.89 | 12.89 | 12.89 | 12.89 | 12.89 | 12.89 | 12.89 |
| Vitamin Mix (g)[2] | 2.58 | 2.58 | 2.58 | 2.58 | 2.58 | 2.58 | 2.58 |

[1]The composition of the AIN Mineral Mix 76 (Zinc Deficient) in ppm was $1.75 \times 10^4$ $CaH\ PO_4$, $2.59 \times 10^3$ NaCl, $7.7 \times 10^3$ K·Citrate. $H_2O$, $1.82 \times 10^3$ $K_2SO_4$, 840 MgO, 122.5 $MnO_3$ (43-48% Mn), 210 Fe·Citrate (16-17% Fe), 10.5 $CuCO_3$ (53-55% Cu), 0.35 $KIO_3$, 0.35 $Na_2SeO_3 \cdot 5\ H_2O$, 19.25 $CrK\ (SO_4)_2 \cdot 12\ H_2O$, and $4.186 \times 10^3$ sucrose, finely powered.

[2]The composition of the AIN Vitamin Mix 76 in ppm was 600 thiamine HCl, 600 riboflavin, 700 pyridoxine HCl, $3 \times 10^3$ nicotinic acid, $1.6 \times 10^3$ D-calcium pantothenate, 200 folic acid, 200 D-biotin, 1 cyanocabalamin, 800 retinyl palmitate, $2 \times 10^4$ dl-2-tocopheryl acetate, 2.5 cholecalciferol, 5 menaquionine, $9.729 \times 10^5$ sucrose, finely powdered.

spectively. The calories provided as alcohol were subtracted from those supplied by carbohydrate. During gestation, protein was increased from 20 to 30% of the dry diet to avoid the possibility of a protein deficit accompanying alcohol abuse (Weiner et al, 1981; Yeh and Cerklewski, 1984).

The dry diets fed during gestation were analyzed for caloric density via bomb calorimetry, and the value per ml of liquid diet was calculated using the appropriate grams of dry diet per liter of liquid diet. For the alcohol diet, additional calories were added using a value of 7.1 kcal/g alcohol, and a density of 0.8 g alcohol per ml.

The zinc content of the diets was analyzed via atomic absorption spectrophotometry. A sample of each of the four different diets fed during gestation was frozen and later assayed for zinc content. Since each diet was assayed as a five day composite, and gestation for all animals spanned 34 days, seven samples were actually analyzed. Samples of roughly 1.5 grams were wet ashed using nitric:perchloric acids at a ratio of 2:1. The ash was dissolved in 5% hydrochloric acid and analyzed for zinc using a Perkin Elmer flame absorption spectrophotometer model 503.

## 3.5 Tissue Collection and Analysis

On day 21 of gestation animals were anesthesized with carbon dioxide and killed by exsanguination. Blood was collected into mineral-free vacutainers via heart puncture. Capillary tubes were filled for hematocrit determination, and the remainder of the blood was centrifuged. The plasma was frozen for later zinc analysis. The plasma was diluted 1:5 with deionized water and analyzed via atomic absorption spectrophotometry.

The uterus was immediately removed, and any resorption sites noted. Pups were separated, inspected for deformities, weighed as a litter, and the brain and liver immediately removed and kept

on ice. These organs were washed in cold saline, pooled for the litter, and also weighed before freezing.

Pup liver and brain were analyzed for zinc, protein, DNA and RNA concentration. A representative sample from the pooled liver or brain from each litter was homogenized in deionized water and analyzed for zinc as previously described for the diets. In addition, another sample of liver and brain (without homogenizing) were analyzed for zinc. The same homogenate was analyzed for protein and nucleic acids. Protein was determined by Hartree's modification of the Lowry procedure, in which protein in alkaline solution forms a complex with cupric tartrate and the Folin-Ciocalteu reagent (Hartree 1972; Lowry et al, 1951). All analyses were done in duplicate.

The homogenate was treated with cold perchloric acid to remove free nucleotides and to precipitate protein and nucleic acids. DNA and RNA were separated by incubation at 37 degrees centigrade with 0.3 N KOH, treatment with acid, and centrifugation (Munro and Fleck, 1966). The supernatant was analyzed for RNA via UV absorption at 260 nm and using yeast RNA as a standard. The pellet was dissolved in perchloric acid, incubated at 70 degrees centigrade, and centrifuged. DNA in the supernatant was determined by Burton's modification of the diphenylanime method using calf-thymus DNA as the standard (Burton, 1956). In this modification, the color reaction of DNA with diphenylamine in acetic and sulfuric acids is enhanced by the addition of acetaldehyde. A detailed protocol for the determination of zinc, protein, DNA and RNA is found in the Appendix.

## 3.6 Statistics

Analysis of variance (ANOVA) was performed on the seven groups to determine whether differences between groups exceeded differences within groups (Mendenhall, 1971). When a sig-

nificant F value was observed, Duncan's Multiple Range Test at the 0.05 level was used to determine which groups differed. In addition, Least Squares Means Multiple Comparison Tests were used to identify any additional significant differences between any two groups.

# 4.0 Results and Discussion

## 4.1 Diet Formulation

Animals arrived at a weight of 150 - 170 grams, and were fed standard Purina rat chow and distilled water for two days. The largest twenty-eight animals were started on the three-week acclimation diet first. Every four days another group of fourteen animals were started. All animals were offered 100 ml of diet per day, and readily adapted to the liquid diets. Gradually increasing the alcohol content of the diets to 4.2 % kept food intake normal for animals ingesting alcohol, as DeCarli and Lieber (1967) advise. At the start of gestation, all animals, including those consuming alcohol, weighed between 231 and 295 grams. The average animal weight for the groups varied from 261 ± 18 - 274 ± 22 grams (Table 2).

During acclimation, all the diets contained 20% of the calories as protein, and this was adequate for weight gain to be similar with or without alcohol. At the start of gestation, the zinc solution was no longer added to diets 1,2, and 5 and protein was increased to 30% of the calories for all diets (Table 1). Lieber and DeCarli (1982) report that animals fed their ethanol diet, with 18% of the calories as protein, gained less weight than their pair-fed controls. Sigh and Snyder (1982) found that a diet with 36% of the calories as ethanol and 18% of the calories as protein resulted in both dietary intake and weight gain being halved. Yeh and Cerklewski (1984) have shown that pregnant rats on alcohol diets with 30% of the calories as protein have normal weight gain,

**Table 2. Effect of Diet on Dam**

| | 1. Alc-Zn(PF) | 2. CHO-Zn | 3. Alc+Zn(PF) | 4. CHO+Zn | 5. CHO-Zn(PF) | 6. CHO+Zn | 7. CHO+Zn |
|---|---|---|---|---|---|---|---|
| Number in group | 9 | 8 | 8 | 9 | 9 | 10 | 9 |
| Subjects not pregnant | 3 | 2 | 3 | 1 | 2 | 1 | 1 |
| Weight (g) at start of gestation | 264.8 ±13.2[1] | 262.1 ±12.1 | 261.5 ±18.3 | 265.4 ±16.2 | 273.6 ±22.1 | 262.5 ±15.1 | 263.7 ±19.9 |
| Weight gain (g) through gestation (0-21 days) | 26.7[a] ±39.5 | 38.0[a] ±51.5 | 130.8[c] ±31.9 | 132.7[c] ±21.6 | 26.0[a] ±37.4 | 78.7[b] ±34.5 | 126.8[c] ±19.9 |
| Total diet (ml) ingested through gestation | 1614[a] ±281 | 1506[a] ±265 | 2162[c] ±246 | 1968[b,c] ±151 | 1565[a] ±211 | 1494[a] ±208 | 1830[b] ±166 |
| Hematocrit | 44.7[a] ±3.4 | 44.1[a] ±5.2 | 37.5[b] (7)[2] ±5.6 | 37.1[b] ±3.3 | 46.0[a] (8) ±5.4 | 42.5[a] ±3.5 | 37.0[b] ±2.0 |
| Liver weight (g) | 10.378[a] ±2.271 | 9.868[a] ±2.719 | 15.853[b] ±2.020 | 15.761[b] ±1.385 | 10.362[a] ±2.095 | 11.237[a] ±2.471 | 14.258[b] ±1.267 |
| Plasma zinc (μg/ml) | 0.56[a] ±0.23 | 0.54[a] (7) ±0.30 | 1.10[b,c] (7) ±0.23 | 0.97[b,c] (7) ±0.16 | 0.42[a] (5) ±0.12 | 0.88[b] (8) ±0.22 | 1.13[c] (8) ±0.17 |

[a,b,c] Numbers in a row with different superscripts are different ($p < 0.05$).

1 Standard error of the mean.

2 Parentheses indicate the number of determinations.

litter size, and pup weight. A 29% alcohol diet with 17% protein resulted in less weight gain, less pup weight, and less pup brain weight compared with a pair-fed diet, but not when protein was increased to 30% (Weiner et al, 1981). In fact, in a recent update on their liquid ethanol diet, Lieber and DeCarli (1986) advise a diet for pregnant rats which contains 25% of the total energy as protein. In order to avoid the confounding variable of a reduced food intake, even if the result is a possible interaction between alcohol and protein metabolism, protein level during gestation was increased to 30% of the calories.

The zinc deficient diets contained an average of 0.11 ± 0.07 and 0.16 ± 0.12 ppm zinc for Alc-Zn and CHO-Zn respectively, with a maximum value of 0.40 ppm zinc. The Alc + Zn and CHO + Zn diets contained 14.40 ± 0.59 and 14.19 ± 0.58 ppm zinc respectively with a minimum value of 13.09 ppm Zinc. On a dry weight basis, these zinc levels translate to less than 1 ppm zinc for the -Zn diets and 50 ppm for the + Zn diets.

## 4.2 Effect of Diet on the Dam

Although 75 females were judged to be pregnant by the appearance of a copulation plug, at sacrifice 13 showed no sign of pregnancy (Table 2). Of the 13 nonpregnant rats, only 3 were fed the CHO + Zn diet. Seventy-seven percent of the animals that failed to either become pregnant or to support a pregnancy were stressed with either alcohol or a zinc deficiency. Three were stressed with the Alc-Zn diet, three more with the Alc + Zn diet, and four with the CHO-Zn diet.

Other studies indicate that alcohol affects the ability to sustain a pregnancy. Female rats which were fed 11% alcohol in the drinking water and rat chow had 63% completed pregnancies vs 100 and 88% in rats adapted to alcohol and rats fed a control diet respectively (Rider, 1979). In another experiment, rats were acclimated to 30% alcohol in the drinking water for five weeks

prior to mating. Only 50% of the alcohol animals showing positive vaginal smears were pregnant, compared with 90% of pair-fed and ad lib controls (Tse and Lee, 1975).

The dams suffering a zinc deficiency (groups 1,2, and 5) gained less weight during gestation ($p < 0.01$) compared with all the other dams (Table 2). Although dams fed a control diet (group 6) gained more weight than dams ingesting a zinc deficient diet (group 5) in equivalent amounts, they also weighed less than animals fed more of a high zinc diet (groups 3,4,and 7). A zinc deficiency clearly prevented normal weight gain during pregnancy, at a level greater than the reduced weight gain caused by a reduced food intake.

Alcohol had no deleterious effect on maternal weight gain or dietary intake in this study, contrary to other reports using diets with more alcohol and less protein (Lieber and DeCarli, 1982; Weiner et al, 1981; Henderson and Schenker, 1977). In fact, dams consuming an Alc + Zn diet ate more than dams on the ad lib control diet, although weight gain between these two groups (3 and 7) did not differ (Table 2 ). In this study, alcohol was 4.4% (v/v) of the diet. Dams ingesting alcohol as 24% of the drinking water and rat chow also gained less weight (Suh and Firek, 1982). Other studies report no decrease in weight gain when dams are fed alcohol as 10% of the drinking water, even when not adapted to alcohol prior to mating (Rider, 1979; Borges and Lewis, 1982). Sherwin et al (1980) found that dams acclimated to a 30% protein and 40% alcohol diet gained weight normally. Weight gain was also normal for acclimated dams fed a diet with 27% of the calories as protein and 25% as alcohol (Gordon et al, 1985). In an experiment designed to compare the effects of protein level (17 or 30% of the calories) in a 29% alcohol diet, maternal weight gain was decreased only with the low protein diet (Weiner et al, 1981).

The literature consistently contains reports of decreased maternal weight gain from either a severe or marginal zinc deficiency. With a 25% albumin diet containing 1 ppm zinc, maternal food intake and weight gain were decreased compared with a 10 ppm zinc diet (Reinstein et al, 1984). Hurley, et al (1971) report a weight loss in dams fed a diet with less than 1 ppm zinc com-

pared with a 76 gram weight gain in controls. Dams consuming a marginally deficient diet (10 ppm zinc) also suffered a decreased weight gain (100 g) compared with controls fed a 50 ppm zinc diet (127 g) (Herzfeld et al, 1985).

A relationship between weight gain and diet consumption can be seen for groups 1 and 2, and groups 3 and 4 (Table 2). In both these cases the dietary zinc level influenced the amount of diet consumed and the weight gain of the dams. When the diet was zinc deficient, both these variables were decreased. Dams on a zinc deficient diet (group 5) ate the same quantity as those pair-fed a control diet (group 6), but gained less. Since pair-fed controls consuming a high zinc diet were intermediate in weight gain, maternal status was compromised by both calories consumed and dietary zinc level. The cyclic feeding pattern observed in zinc deficient animals leads to a breakdown of tissue resulting in availability of zinc from the cells (Wallwork et al, 1981). The anorexia associated with a severe zinc deficiency is a mechanism which mobilizes available zinc, but also further stresses the nutritional status of the dam.

Dietary zinc level was also reflected in plasma zinc concentration of the dams at sacrifice (Table 2). Animals on a low zinc diet (groups 1,2, and 5) had a plasma zinc level of between $0.42 \pm 0.10$ and $0.56 \pm 0.08$ ppm, which was about half that of the other groups. Since pair-fed animals on a control diet (group 6) also had lower plasma zinc compared with animals ingesting a high zinc diet in greater quantities (groups 2,3,and 7), a reduced food intake also decreases plasma zinc concentration. Numerous studies report decreased plasma zinc with either severe or marginal zinc deficiency (Herzfeld et al, 1985; Reinstein et al, 1984; Hurley and Mutch, 1973).

Alcohol had no effect on plasma zinc concentration, regardless of dietary zinc level (Table 2). Dams suffering a zinc deficiency had no lower plasma zinc concentration when alcohol was added to the diet (groups 1 and 2). There are numerous reports of low plasma zinc in alcoholics (McClain and Su, 1983; Prasad, 1979). However, the results from animal studies in which malnutrition is easier to avoid, are not consistent. Weanling male rats fed the Lieber-DeCarli diet

with 5% alcohol had decreased serum zinc (0.75 ppm) compared with controls (1.1 ppm). In the same study, zinc deficient animals suffered no additional decrease from alcohol (0.63 vs 0.65 ppm) (Das et al, 1984). When pregnant rats ingested chow and 12-20% of the calories as alcohol in drinking water, plasma zinc concentration was unaffected (Suh et al, 1982). Dreosti and Record (1979) actually found an increase in plasma zinc, from 0.5 to 0.8 ppm, in dams fed a 0.5 ppm zinc diet and either 10 or 20% alcohol in the drinking water. The authors postulate the increase to be due to a redistribution of zinc. A variable which could be affecting plasma zinc is caloric intake from alcohol and subsequent nutrient deficiencies, which could lead to tissue breakdown and an increase in circulating zinc. Another problem could be a reduced water intake with the addition of alcohol to the drinking water, resulting in dehydration.

While plasma zinc concentration primarily reflected the dietary zinc level, and secondarily dietary intake level, hematocrit was highest for all the groups consuming less (Table 2). Hematocrits were higher ($p < 0.05$) for groups 1,2,5, and 6 compared with groups 3,4, and 7. Pregnant rats that are severely zinc deficient often appear dehydrated and decrease their water consumption (Wallwork et al, 1981). The mesentery surrounding the abdominal cavilty often appears thin and dry. A reduced liquid diet intake in this study would most certainly cause a comparatively low water intake, leading to an even greater elevation of hematocrit with zinc deficiency.

The fact that zinc deficient animals had a high hematocrit, and alcohol had no effect on hematocrit, is consistent with the literature. Reinstein et al (1984) found that dams on a 1 or 4.5 ppm zinc diet had higher hematocrits (39-43) compared with those on a 10, 100 or 1000 ppm zinc diet. Dams which were fed alcohol as 6% of the drinking water through two pregnancies, had an hematocrit (40) similar to that of their pair-fed controls (38) (Mendelson and Huber, 1980). In another group of dams acclimated to 24% alcohol in the drinking water, hematocrit levels were similar to that of the pair-fed control group ( 43.5 and 47.9 respectively) (Gordon et al, 1985).

The hematocrit data are supportive of the conclusion that the low plasma zinc concentrations in groups 1,2, and 5 are not confounded by the increased blood volume normally seen in pregnancy. During normal geatation, the plasma concentrations of some minerals, notably iron and zinc, are lower than in the nonpregnant state due to an increased quantity of plasma. The higher hematocrit seen in these zinc deficient dams indicates that the blood volume is decreased. A normal blood volume would decrease plasma zinc even further in the zinc deficient animals.

Liver weight of the dam was also affected by amount of diet consumed (Table 2). Both zinc deficient animals and their pair-fed controls had roughly two-thirds the quantity of liver as those animals experiencing a normal weight gain during pregnancy. The dams consuming alcohol (groups 1 and 3) did not differ from their pair-fed controls in liver weight. Das et al (1984) found no difference in liver zinc concentration in zinc deficient dams with or without alcohol. Similarly, a dietary zinc level of less than 1 ppm again did not decrease maternal liver zinc concentration (Masters et al, 1983). In another study maternal liver zinc concentration did not change at 1, 4.5 or 10 ppm, but did increase at 100 ppm in the diet (Reinstein et al, 1984).

## 4.3 Effect of Diet on Pregnancy Outcome

Litter size did not vary greatly (Table 3). There were fewer pups ($p < 0.05$) from dams ingesting an Alc-Zn diet (group 1) compared to those ingesting either Alc + Zn or their pair-fed controls (groups 3 and 4). In addition, the results of a Least Squares Means Comparison Test showed that there were fewer pups ($p < 0.05$) in the Alc-Zn dams compared to groups 2,3,4,6, and 7 (Table 4). A zinc deficient diet (compare groups 5 and 6), alcohol ingestion (compare groups 1 and 2, and 3 and 4), or food restriction (compare group 6 with 4 or 7) alone did not affect litter size.

**Table 3. Effect of Diet on Litter**

| | 1.Alc-Zn(PF) | 2.CHO-Zn | 3. Alc+Zn(PF) | 4.CHO+Zn | 5.CHO-Zn(PF) | 6.CHO+Zn | 7. CHO+Zn |
|---|---|---|---|---|---|---|---|
| Number of dams | 9 | 8 | 8 | 9 | 9 | 10 | 9 |
| Litter weight (g) | $32.87^{a}$ ±20.98 | $47.88^{a,b,c}$ ±13.57 | $65.69^{c}$ ±16.46 | $63.76^{c}$ ±18.03 | $44.75^{a,b}$ ±18.11 | $56.52^{b,c}$ ±20.33 | $63.68^{c}$ ±12.96 |
| Litter size | $8.9^{a}$ ±4.8 | $12.9^{a,b}$ ±3.0 | $13.9^{c}$ ±3.5 | $13.4^{c}$ ±3.8 | $11.8^{a,b}$ ±5.2 | $12.9^{a,b}$ ±4.8 | $13.1^{a,b}$ ±2.8 |
| Number of resportions | 58 | 15 | 2 | 7 | 7 | 1 | 2 |
| Percent resportions[2] | 42 | 13 | 2 | 5 | 7 | 1 | 2 |
| Number of dams with resorptions | 6 | 4 | 2 | 2 | 1 | 1 | 2 |
| Number of deformed pups | 15 | 1 | 0 | 0 | 2 | 0 | 0 |
| Number of dams with deformed pups | 5 | 1 | 0 | 0 | 1 | 0 | 0 |

$^{a,b,c}$Numbers in a row with different superscripts are different ($p < 0.05$).

1 Standard error of the mean.

2 [(Number of resorptions) ÷ (Number of resorptions plus number of pups)] times 100.

**Table 4. Least Squares Means for Litter Size**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .0498 | .0151 | .0217 | .1003 | .0372 | .0327 |
| 2. | CHO - Zn | .0498 | -- | .6267 | .7755 | .7021 | .9898 | .9058 |
| 3. | Alc + Zn | .0151 | .6267 | -- | .8298 | .3785 | .6172 | .7021 |
| 4. | CHO + Zn | .0217 | .7755 | .8292 | — | .4920 | .7731 | .8633 |
| 5. | CHO - Zn | .1003 | .7021 | .3785 | .4920 | -- | .6762 | .6060 |
| 6. | CHO + Zn | .0372 | .9898 | .6172 | .7731 | .6762 | -- | .9109 |
| 7. | CHO + Zn | .0327 | .9058 | .7021 | .8633 | .6060 | .9109 | -- |

In fact, zinc deficient dams ingesting less diet did not have fewer pups than ad lib fed controls. The combined stresses of alcohol intake and zinc deficiency decreased the number of pups supported by the dam.

Dams given a marginal zinc diet (10 ppm) did not have smaller litters (Herzfeld et al, 1985), nor did dams whose dietary zinc level was further decreased to 0.5 ppm for the last seven days of pregnancy (Cunnane, 1982). However, Hurley et al (1971) reported a drop in litter size from 11 pups/litter for ad lib controls to 6.7 pups/litter for dams fed a diet with less than 1 ppm zinc.

Alcohol seems to have a less consistent effect in reducing litter size. When the drinking water contained 24 (Suh and Firek, 1982) or 30% alcohol (Tse and Lee, 1975), litter size was smaller. However, dams whose drinking water contained 6 (Mendelson and Huber, 1981) or 11% (Rider, 1979) alcohol had the same number of pups as their controls. Two other studies produced results in which litter size did not differ between pair-fed control dams and alcoholic dams. A 40% alcohol diet produced no fewer fetuses, but the diet also contained 20-25% protein as total calories (Sherwin et al, 1981). Litter size was also the same with a 27% alcohol and 25% protein diet (Gordon et al, 1985). A protein level of less than 20% of the calories and alcohol together may prove especially deleterious for the litter size. The Lieber-DeCarli '82 Formula, which is roughly 16-18% protein calories, has been shown to be deficient during gestation (Derr et al, 1987; Yeh and Cerklewski, 1984).

As would be expected from the small litter size of the Alc-Zn dams, the total litter weight is the lowest, and is less than the total pup weights found in groups 3,4,6, and 7 (Table 3). Total litter weight is smaller for zinc deficient dams in groups 1 and 5 compared with groups 3,4, and 7 when the data is analyzed by Least Squares Means Comparison Test (Table 5). While zinc deficiency was not a consistent factor in reducing the quantity of fetal tissue supported through gestation, alcohol had more of an effect, in combination with a zinc deficiency. Again, this evidence

**Table 5. Least Squares Means for Total Litter Weight**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .0840 | .0003 | .0005 | .1573 | .0049 | .0005 |
| 2. | CHO - Zn | .0840 | -- | .0478 | .0686 | .7138 | .3055 | .0700 |
| 3. | Alc + Zn | .0003 | .0478 | -- | .8225 | .0174 | .2764 | .8150 |
| 4. | CHO + Zn | .0005 | .0686 | .8225 | -- | .0256 | .3738 | .9921 |
| 5. | CHO - Zn | .1573 | .7138 | .0174 | .0256 | -- | .1506 | .0262 |
| 6. | CHO + Zn | .0049 | .3055 | .2764 | .3738 | .1506 | -- | .3793 |
| 7. | CHO + Zn | .0005 | .0700 | .8150 | .9921 | .0262 | .3793 | -- |

also is suggestive of an additive stress caused by the combination of zinc deficiency and alcohol. Although the deleterious effect of a zinc deficiency on support of the fetus is increased by alcohol, alcohol alone appears to have no such effect.

The literature contains evidence that a zinc deficiency alone does not reduce litter weight. Although ad lib fed control dams supported more fetal tissue weight than did dams fed a 30% protein diet with less than 1 ppm zinc, there was no difference with pair fed controls (Hurley et al, 1971). Cunnane (1981) also reports no difference in litter weight when the ppm zinc in the diet was 20, 10, 5, or further reduced to 0.5 for the last five days of gestation.

On the other hand, studies in which the dam was stressed with alcohol do show evidence of a decreased litter weight (Suh and Firek, 1982; Ghishan et al, 1982; Tse and Lee, 1975; Singh et al, 1987). These studies employed diets with less than 20% of the calories from protein. However, in other experiments in which the protein level was also low, litter weight was the same for pair fed controls and dams ingesting alcohol (Mendelson and Huber, 1979; Rider et al, 1979). When alcohol was 40% and protein was greater than 20% of the calories, again litter weight was unaffected (Sherwin et al, 1981).

The same combined effect of alcohol and zinc stress is reflected in the number of resorptions in each group (Table 3). There were 58 resorption sites counted in the Alc-Zn dams (group 1) compared to a maximum of 15 in any of the other groups. If the percent resorption is calculated as (the number of resorptions) divided by (the number of pups plus resorptions) times 100, then there are 42% resorptions in the Alc-Zn dams, and 1-13% in the other groups. The number of deformed pups, chiefly involving gross craniofacial malformations, was 15 in this same group, compared to a total of 3 in the other two zinc deficient groups, and none in the other alcohol fed dams. Since a litter effect may magnify these differences, it was also noted that the number of dams with either resorptions or deformed pups was greatest when alcohol intake and a zinc deficiency were combined.

In two experiments in which the diet was less than 1 ppm zinc, there were (1) fewer live fetuses per implantation site compared with ad lib fed controls (Masters et al, 1985), and (2) 41% of implantation sites were resorbed compared with 4.3% for ad lib fed controls (Hurley et al, 1971). Resorptions caused by a zinc deficiency were complicated by a reduced food intake, since pair fed controls were not different from the zinc deficient group. Herzfeld et al (1985) showed no increase in malformations with a 10 ppm zinc diet, but a 1 ppm zinc diet resulted in 45% of the fetuses malformed compared with 0% in pair fed dams ingesting 4.5 or more ppm zinc (Reinstein et al, 1984).

In an experiment in which dams were fed 24% alcohol in the drinking water and rat chow, there were fewer resorptions in pair fed controls (Suh and Firek, 1982). When the diet was high in protein with 40% of the calories from alcohol, the percentage of dead pups did not differ in pair fed controls, but was less in ad lib fed controls (Sherwin et al. 1981). Similarly, increasing protein from 17 to 30% of the calories in a 29% alcohol diet eliminated an increase in resorptions (Weiner et al, 1981). Although both a zinc deficiency and alcohol intake have been reported to increase the frequency of resorptions and malformations, a reduced food intake or a relatively low protein intake may confound the results.

In the present experiment, with a high dietary protein level, a zinc deficiency resulted in more resorptions whereas alcohol as 25% of the calories did not. The combination of the two abuses had more than twice the effect. However, only the combined abuses served to increase the number of malformations. Ruth and Goldsmith (1981) studied the interaction of ethanol and zinc level by feeding dams a zinc deficient diet (less than 2 ppm) for days 7-11 of gestation, and giving an intraperitoneal injection of ethanol on day 10. Although either of these stresses alone slightly increased deformities, the two together greatly increased both resorptions and deformities.

## 4.4 Effect of Diet on Fetal Liver

The weight of fetal liver (pooled for each litter) was lower ($p < 0.05$) for the zinc deficient animals ingesting alcohol (group 1) compared with animals on a high zinc diet (groups 3,4,6, and 7) (Table 6). Fetal liver weight did not differ between groups on a zinc deficient diet and their pair-fed controls (groups 5 and 6). As previously seen in this experiment, alcohol by itself had no effect (compare groups 3 and 4). The dams in groups 3,4, and 7 consumed significantly more calories, which most certainly contributed to a greater quantity of fetal tissue. However, the dams in group 6 consumed no more than those in group 1, yet had a higher fetal liver weight ($p < 0.05$). Groups 3,4, and 7, all of which consumed more diet, had a greater pooled fetal liver weight when compared with all three groups on a zinc deficient diet. Again, caloric intake is not the only factor, since group 6 was food-restricted and fell within both ranges of fetal liver weight. The combination of zinc deficiency with either alcohol or a reduced food intake decreased the quantity of fetal liver tissue.

When the fetal liver weight is expressed as weight per pup, the lowest tissue weight occurs with dams consuming less diet (groups 1,2,5, and 6) (Table 6). Since the litter size was smaller for dams on an Alc-Zn diet (group 1), liver weight per pup was not lower as was pooled liver weight. Even though groups 5 and 6 both consumed less diet, only the litters from the zinc deficient dams (group 5) had a lower liver weight per pup ($p < 0.01$) compared with those from dams on a high zinc alcohol diet (groups 3) by Least Squares Means Comparison Tests (Table 7). When fetal liver weight was corrected for the number of pups in the litter, no effect of alcohol, even in combination with a zinc deficiency, was seen. The significant differences were linked to diet consumed and zinc level.

**Table 6. Effect of Diet on Fetal Liver Weight and Zinc Content**

| | 1.Alc-Zn(PF) | 2.CHO-Zn | 3.Alc+Zn(PF) | 4.CHO+Zn | 5.CHO-Zn(PF) | 6.CHO+Zn | 7.CHO+ZN |
|---|---|---|---|---|---|---|---|
| Number of dams | 9 | 8 | 8 | 9 | 9 | 10 | 9 |
| Average litter size | $8.9^{a}$ | $12.9^{a,b}$ | $13.9^{b}$ | $13.4^{b}$ | $12.1^{a,b}$ | $12.9^{a,b}$ | $13.1^{a,b}$ |
| Liver weight (g) | $2.051^{a}$ ±0.367[1] | $2.800^{a,b}$ ±0.389 | $4.253^{c}$ ±0.389 | $4.258^{c}$ ±0.367 | $2.574^{a,b}$ ±0.367 | $3.244^{b,c}$ ±0.348 | $4.232^{c}$ ±0.367 |
| Average liver weight per pup (mg) | $244^{a}$ ±20 | $220^{a}$ ±22 | $313^{b,c}$ ±22 | $320^{b,c}$ ±20 | $236^{a}$ ±20 | $259^{a,b}$ ±19 | $325^{c}$ ±20 |
| Liver zinc (μg/g) | $45.9^{a}$ ±4.8 | $47.7^{a}$ ±5.1 | $90.1^{b}$ ±5.1 | $78.8^{b}$ ±4.8 | $37.9^{a}$ ±4.8 | $87.5^{b}$ ±4.6 | $87.9^{b}$ ±4.8 |
| Total liver zinc (μg) | $102.3^{a}$ ±35.6 | $143.7^{a}$ ±37.8 | $390.1^{b}$ ±37.8 | $345.9^{b}$ ±35.6 | $96.0^{a}$ ±35.6 | $290.8^{b}$ ±33.8 | $376.3^{b}$ ±35.6 |

$^{a,b,c}$Numbers in a row with different superscripts are different ($p < 0.05$).

[1] Standard error of the mean.

**Table 7. Least Squares Means for Fetal Liver Weight per Pup**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .4327 | .0234 | .0106 | .7941 | .5922 | .0066 |
| 2. | CHO - Zn | .4327 | -- | .0037 | .0014 | .5941 | .1885 | .0009 |
| 3. | Alc + Zn | .0234 | .0037 | -- | .8156 | .0124 | .0671 | .6860 |
| 4. | CHO + Zn | .0106 | .0014 | .8156 | -- | .0052 | .0339 | .8598 |
| 5. | CHO - Zn | .7941 | .5941 | .0124 | .0052 | -- | .4227 | .0032 |
| 6. | CHO + Zn | .5922 | .1885 | .0671 | .0332 | .4227 | -- | .0220 |
| 7. | CHO + Zn | .0066 | .0009 | .6860 | .8598 | .0032 | .0220 | -- |

Both the concentration of zinc and the total quantity of zinc in fetal liver was affected by dietary zinc level only (Table 6) . Low zinc concentrations were found in the fetal liver of groups suffering a zinc deficiency (groups 1,2, and 5) compared to all other groups. Dietary zinc level affected the total amount of zinc accumulated in the fetal liver in the same way. Neither alcohol nor a reduced food intake had an effect on fetal liver zinc concentration or on total fetal liver zinc.

There is evidence in the literature that the effect of both zinc deficiency and alcohol on fetal liver can be modified. Although Masters et al (1983) found a lower zinc concentration in the fetal livers when dams were fed a diet with less than 1 ppm zinc and compared with ad lib fed controls, Reinstein et al (1984) found no differences at several dietary zinc levels between 1 and 100 ppm zinc. The dams in this latter experiment were consuming a diet with a relatively high protein content - roughly 25% of the calories from spray-dried egg white.

Fetal liver weight was less when dams were fed a 1 ppm zinc diet with 20% egg white in comparison with their pair fed controls (McKenzie et al, 1975). In an experiment in which dams were fed a diet with 30% of the calories from alcohol and 25% of the calories from protein, pup liver weight at Day 20 of gestation was the same as in pair-fed controls (Gordon et al, 1985). Pups which were subjected to alcohol as 30% of the calories in utero had a significantly lower liver weight up to 21 days of age when compared with either pair-fed or ad lib-fed controls (Singh et al, 1987). These researchers used the old Lieber-DeCarli formula with about 18% of the calories from protein. In another experiment (Henderson and Schenker, 1977) in which dams were fed a diet with 6% alcohol (w/v) (roughly 35% of the calories) and 18% of the calories as protein, the livers of three-day old pups subjected to alcohol weighed less ($p < 0.02$). A subsequent experiment (Henderson et al, 1979) similarly reported a 21% reduction in pup liver weight with alcohol. Their diet contained only 3 μg Zn/g liquid diet, or roughly 9 ppm zinc dry diet. Mendelson and Huber (1980) found that both alcohol ingestion (as 6% of the drinking water) and pair feeding decreased fetal zinc levels compared with ad lib fed controls comparably. However, supplementation of the alcohol diet

with zinc sulfate to roughly double the amount, negated this decrease. This diet had roughly 11% of the calories from protein.

## 4.5 Effect of Diet on Fetal Brain

While the differences in fetal liver weight were due to a zinc deficiency in combination with either alcohol or a reduced food intake, fetal brain weight was decreased by only all three of these factors (Table 8). Dams on an Alc-Zn diet produced pups with less fetal brain tissue in comparison with dams fed adequate amounts of a high zinc diet. Dams on a low zinc diet fell intermediate between the highest and lowest amounts, with no significant differences. Dietary intake had less of an effect on brain tissue, since pair-fed controls fell within only the high range. Using ANOVA, the weight of brain tissue per pup was less ($p < 0.05$) for dams on an Alc-Zn diet (group 1) than for dams on either high or low zinc diets (groups 4,5,6, and 7) without alcohol. Another statistical difference ($p < 0.05$) emerged when individual groups were compared via Least Squares Means Comparisons Tests (Table 9); i.e., dams on a control diet ad lib produced pups with more brain tissue than did dams on one of the CHO-Zn diets (group 2), though not on the other (group 5).

Although neither alcohol nor zinc deficiency by themselves caused a significant reduction in fetal brain tissue (compare groups 3 and 4, and 5 and 6), their combined effect was significant (Table 8) in comparison with the control diet. Dams consuming alcohol in combination with a low zinc diet, did not produce less brain tissue per pup than those on a Alc + Zn diet, as occurred with fetal liver. Since there are fewer differences between groups seen in brain than in liver, it is more likely that zinc level has less of an effect on fetal brain than it does on fetal liver. This is supported by the fact that there were no differences in fetal brain zinc concentration due to zinc level, alcohol intake, or dietary consumption. Since the differences in total fetal brain zinc exactly paralleled those

**Table 8. Effect of Diet on Fetal Brain Weight and Zinc Content per Litter**

| | 1. Alc-Zn(PF) | 2. CHO-Zn | 3. Alc+Zn(PF) | 4. CHO+Zn | 5. CHO-Zn(PF) | 6. CHO+Zn | 7. CHO+Zn |
|---|---|---|---|---|---|---|---|
| Number of dams | 9 | 8 | 8 | 9 | 9 | 10 | 9 |
| Average litter size | $8.9^{a}$ | $12.9^{a,b}$ | $13.9^{b}$ | $13.4^{b}$ | $12.1^{a,b}$ | $12.9^{a,b}$ | $13.1^{a,b}$ |
| Total brain weight (g) | $1.241^{a}$ ±0.206[1] | $1.827^{a,b}$ ±0.218 | $2.094^{b}$ ±0.218 | $2.112^{b}$ ±0.206 | $1.715^{a,b}$ ±0.206 | $1.990^{b}$ ±0.195 | $2.113^{b}$ ±0.206 |
| Average brain weight per pup (mg) | $133^{a}$ ±6 | $142^{a,b}$ ±7 | $151^{a,b}$ ±7 | $157^{b}$ ±6 | $153^{b}$ ±6 | $156^{b}$ ±6 | $161^{b}$ ±6 |
| Brain zinc (μg/g) | 8.6 ±0.3 | 8.1 ±0.3 | 7.9 ±0.3 | 8.2 ±0.3 | 7.9 ±0.3 | 8.1 ±0.3 | 7.7 ±0.3 |
| Total brain zinc (μg) | $9.8^{a}$ ±2.0 | $14.8^{a,b}$ ±1.7 | $16.5^{b}$ ±1.7 | $17.4^{b}$ ±1.7 | $14.6^{a,b}$ ±1.8 | $17.6^{b}$ ±1.6 | $17.1^{b}$ ±1.8 |

$^{a,b,c}$Numbers in a row with different superscripts are different ($p < 0.05$).

[1] Standard error of the mean.

**Table 9. Least Squares Means for Brain Weight per Pup in a Litter**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .3092 | .0525 | .0098 | .0292 | .0090 | .0023 |
| 2. | CHO + Zn | .3092 | -- | .3570 | .1218 | .2570 | .1216 | .0430 |
| 3. | Alc + Zn | .0525 | .3570 | -- | .5407 | .8502 | .5554 | .2693 |
| 4. | CHO + Zn | .0098 | .1218 | .5407 | -- | .6624 | .9694 | .6081 |
| 5. | CHO - Zn | .0292 | .2570 | .8502 | .6624 | -- | .6820 | .3439 |
| 6. | CHO + Zn | .0090 | .1216 | .5554 | .9694 | .6820 | -- | .5725 |
| 7. | CHO + Zn | .0023 | .0430 | .2693 | .6081 | .3439 | .5725 | -- |

of total brain weight, there appear to by no real differences in fetal brain zinc due to the aforementioned factors.

A review of the literature shows that the effect of a low zinc intake on fetal brain tissue is inconsistent. Again, Reinstein et al (1984) found no difference in fetal brain zinc when dietary zinc level ranged from 1 - 1000 ppm zinc, using a diet with roughly 25% of the calories from egg albumin and ad lib-fed controls. However, McKenzie et al (1975) found a reduced fetal brain weight when the diet was only 20% egg white. Henderson et al (1979) found a 16% reduction in fetal brain weight ($p < 0.05$) in animals acclimated to 6% alcohol (roughly 35% of the calories), but the liquid diet again only contained 3 ppm zinc. In an earlier experiment, Henderson and Schenker (1977) found no difference in fetal brain weight when dams were fed a 6% w/v alcohol diet with 18% of the calories from protein. Other experiments also reporting no difference in brain weight used pair-fed controls and a high protein alcohol diet (Gordon, 1985), a rat chow diet and 10% alcohol in the drinking water (Borges and Lewis, 1982), and a 5% alcohol (w/v) diet with either 16 or 30% of the calories from protein (Weiner et al, 1981). Pups subjected to a liquid diet with 30% of the calories as alcohol in utero and suckled by foster dams on a control diet, had a lower brain weight at 7 and 21 days of age, but not thereafter. This diet had about 18% of the calories from protein.

## 4.6 Effect of Diet on Protein and Nucleic Acids

When expressed as concentration and analyzed by ANOVA, there were no differences in protein in both fetal liver and brain (Tables 10 and 11). The only significant difference for protein concentration was found via a Least Squares Means Comparison Test, in which the protein concentration of fetal brain was higher ($p < 0.05$) in the litter from dams fed Alc + Zn (group 3) com-

pared with dams fed a control diet that was calorically deficient (group 6) (Table 12). Although combining alcohol with a higher caloric intake is not known to increase protein concentration in the fetal brain, there is evidence that alcohol interferes with the transport of proteins out of the liver rather than the rate of protein synthesis (Baraona et al, 1980).

There were statistical differences in total protein content of fetal liver and brain (Tables 10 and 11). Using ANOVA, total protein was reduced ($p < 0.05$) in the fetal livers of groups on a low zinc diet (groups 1,2,and 5) compared to the adequately fed controls (groups 3,4, and 7). The quantity of fetal liver protein from dams on a control diet that was restricted (group 6) fell between these two protein levels. The results from Least Squares Means Comparison Tests were that animals consuming restricted amounts of a control diet (group 6) had (1) more total fetal liver protein than animals stressed with a zinc deficiency and alcohol, and (2) less total fetal liver protein than animals fed a control diet with alcohol, and their pair fed controls (groups 3 and 4) (Table 13). There were no differences due to either zinc deficiency (groups 5, 6) or alcohol intake (groups 1 and 2, or 3 and 4). The differences in total protein almost exactly parallel the differences in both liver weight and dietary intake. An important factor in these differences is undoubtedly the quantity of tissue. Total brain and fetal protein may be reflecting the quantity of diet ingested more than the diet itself. The differences in total protein in fetal brain tissue even more closely parallels the pattern of brain weight. Only the dams ingesting both alcohol and a zinc deficient diet (group 1) produced less fetal brain protein ($p < 0.05$) than did dams fed a control diet in unrestricted amounts (groups 3,4, and 7).

Similarly, there were no differences in the protein concentration of fetal liver or brain when dams were given a low zinc (less than 1 ppm) diet and 20% egg white and compared with pair fed controls (McKenzie et al, 1975). Although both fetal liver and brain weighed significantly less with a zinc deficiency, only the liver had less total protein, probably due to reduced tissue mass. In a slightly different protocol, newborn pups were suckled to dams on a 1 ppm zinc diet for ten days (Duerre et al, 1977). There were no differences in protein synthesis in fetal liver, but histone protein

**Table 10. Effect of Diet on Nucleic Acids and Protein in Fetal Liver per Litter**

| | 1.Alc-Zn(PF) | 2.CHO-Zn | 3.Alc+ZN(PF) | 4.CHO+Zn | 5.CHO-Zn(PF) | 6.CHO+Zn | 7.CHO+Zn |
|---|---|---|---|---|---|---|---|
| Number of dams | 9 | 8 | 8 | 9 | 9 | 10 | 9 |
| Protein (mg/g) | 109.0 ±2.0[1] | 107.7 ±2.2 | 110.4 ±2.2 | 107.9 ±2.0 | 109.5 ±2.0 | 104.7 ±1.9 | 106.6 ±2.0 |
| Total protein (mg) | 226.0$^{a}$ ±41.1 | 299.2$^{a}$ ±43.5 | 468.3$^{b}$ ±43.6 | 462.4$^{b}$ ±41.1 | 282.6$^{a}$ ±41.1 | 343.4$^{a,b}$ ±39.0 | 451.5$^{b}$ ±41.1 |
| DNA (mg/g) | 5.06 ±0.24 | 4.72 ±0.26 | 5.14 ±0.26 | 4.80 ±0.24 | 4.55 ±0.24 | 4.54 ±0.23 | 4.49 ±0.24 |
| Total DNA (mg) | 10.03$^{a}$ ±1.83 | 13.20$^{a}$ ±1.44 | 21.62$^{c}$ ±1.94 | 20.08$^{c}$ ±1.83 | 11.90$^{a}$ ±1.83 | 14.51$^{a,b}$ ±1.73 | 19.37$^{b,c}$ ±1.83 |
| RNA (mg/g) | 12.74 ±0.42 | 12.92 ±0.45 | 13.28 ±0.45 | 12.92 ±0.42 | 13.27 ±0.42 | 12.84 ±0.40 | 12.58 ±0.42 |
| Total RNA (mg) | 25.53$^{a}$ ±4.82 | 36.19$^{a,b}$ ±5.11 | 56.27$^{c}$ ±5.11 | 54.36$^{c}$ ±4.82 | 33.84$^{a,b}$ ±4.82 | 41.46$^{b,c}$ ±4.57 | 53.57$^{c}$ ±4.82 |
| Liver weight (g) | 2.051$^{a}$ | 2.800$^{a,b}$ | 4.253$^{c}$ | 4.258$^{c}$ | 2.574$^{a,b}$ | 3.244$^{b,c}$ | 4.232$^{c}$ |

$^{a,b,c}$Numbers in a row with different superscripts are different ($p < 0.05$).

[1] Standard error of the mean.

**Table 11. Effect of Diet on Nucleic Acids and Protein in Fetal Brain per Litter**

| | 1.Alc-Zn(PF) | 2.CHO-Zn | 3.Alc+Zn(PF) | 4.CHO+Zn | 5.CHO-Zn(PF) | 6.CHO+Zn | 7.CHO+Zn |
|---|---|---|---|---|---|---|---|
| Number of dams | 9 | 8 | 8 | 9 | 9 | 10 | 9 |
| Protein (mg/g) | 62.62 ±1.02[1] | 63.52 ±1.09 | 64.23 ±1.02 | 62.33 ±1.02 | 61.73 ±0.96 | 61.19 ±0.91 | 62.64 ±0.96 |
| Total Protein (mg) | $86.8^{a}$ ±13.7 | $112.8^{a,b}$ ±14.7 | $134.2^{b}$ ±13.7 | $137.1^{b}$ ±13.7 | $108.1^{a,b}$ ±12.9 | $122.7^{a,b}$ ±12.3 | $132.3^{b}$ ±12.9 |
| DNA (mg/g) | 4.72 ±0.14 | 4.63 ±0.13 | 4.49 ±0.13 | 4.43 ±0.13 | 4.45 ±0.13 | 4.38 ±0.12 | 4.37 ±0.13 |
| Total DNA (mg) | 6.50 ±1.60 | 8.46 ±0.99 | 9.33 ±0.99 | 9.34 ±0.93 | 7.52 ±0.99 | 8.88 ±0.87 | 9.25 ±0.94 |
| RNA (mg/g) | $4.79^{a}$ ±0.06 | $4.72^{a,b}$ ±0.05 | $4.74^{a,b}$ ±0.06 | $4.69^{a,b}$ ±0.05 | $4.57^{b}$ ±0.05 | $4.69^{a,b}$ ±0.05 | $4.64^{a,b}$ ±0.05 |
| Total RNA (mg) | $6.68^{a}$ ±1.05 | $8.62^{a,b}$ ±0.98 | $10.71^{b}$ ±1.05 | $9.90^{b}$ ±0.93 | $7.96^{a,b}$ ±0.93 | $9.32^{a,b}$ ±0.88 | $9.80^{b}$ ±0.93 |
| Brain weight (g) | $1.241^{a}$ | $1.827^{a,b}$ | $2.094^{b}$ | $2.112^{b}$ | $1.715^{a,b}$ | $1.990^{b}$ | $2.113^{b}$ |

$^{a,b,c}$Numbers in a row with different superscripts are different ($p < 0.05$).

[1] Standard error of the mean.

**Table 12. Least Squares Means for Fetal Brain Protein Concentration**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .5518 | .2693 | .8393 | .5276 | .2991 | .9927 |
| 2. | CHO - Zn | .5518 | -- | .6335 | .4297 | .2249 | .1073 | .5470 |
| 3. | Alc + Zn | .2693 | .6335 | -- | .1925 | .0801 | .0304 | .2597 |
| 4. | CHO + Zn | .8393 | .4297 | .1925 | -- | .6718 | .4081 | .8275 |
| 5. | CHO - Zn | .5276 | .2249 | .0801 | .6718 | -- | .6832 | .5090 |
| 6. | CHO + Zn | .2991 | .1073 | .0304 | .4081 | .6832 | -- | .2796 |
| 7. | CHO + Zn | .9927 | .5470 | .2597 | .8275 | .5090 | .2796 | -- |

**Table 13. Least Squares Means for Total Fetal Liver Protein**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .2264 | .0002 | .0002 | .3343 | .0428 | .0003 |
| 2. | CHO - Zn | .2264 | -- | .0081 | .0086 | .7818 | .4531 | .0138 |
| 3. | Alc + Zn | .0002 | .0081 | -- | .9216 | .0030 | .0369 | .7796 |
| 4. | CHO + Zn | .0002 | .0086 | .9216 | -- | .0031 | .0400 | .8516 |
| 5. | CHO - Zn | .3343 | .7818 | .0030 | .0031 | -- | .2873 | .0052 |
| 6. | CHO + Zn | .0428 | .4531 | .0369 | .0400 | .2873 | -- | .0613 |
| 7. | CHO + Zn | .0003 | .0138 | .7796 | .8516 | .0052 | .0613 | -- |

synthesis was reduced in the cerebrum and nonhistone protein synthesis was reduced in the cerebellum of zinc deficient pups compared with pair fed controls.

Previous studies also report no differences in fetal liver or brain protein from alcohol ingestion. When pups from dams given a diet with 30% of the calories from alcohol during gestation were suckled to foster mothers for seven days, there was no decrease in either liver or brain protein content, although brain and liver weights were less than in pair fed controls (Singh et al, 1987). Henderson and Schenker (1977) fed dams a 6% (w/v) alcohol (roughly 35% of the calories) diet using the original Lieber-DeCarli formula up to gestation day 20, and then a control diet till the pups were three days old. Pair fed controls had a similar fetal liver protein concentration (155.1 vs 140 mg/g), but actually a lower ($p < 0.05$) fetal brain protein concentration (63.3 vs 72.0 mg/g). The acclimation period was long in this experiment - an average of 21 weeks. The protein content of placenta from ethanol fed dams has also been greater compared with pair fed controls (Gordon et al, 1985).

The concentration of RNA in fetal liver also showed no effect of alcohol intake, zinc deficiency, or caloric intake (Table 10). However, there was a significantly larger RNA concentration in the fetal brain (Table 11) from dams fed an Alc-Zn diet (group 1) compared to dams fed a CHO-Zn diet (group 5) using ANOVA. These groups did not differ in food intake or dietary zinc level, but only in alcohol intake. The results of Least Squares Means Tests were that two other groups (Alc + Zn and another CHO-Zn) also were higher in fetal brain RNA concentration than was group 5 (Table 14). It is unclear why groups 2 and 5 were different, since diet and food intake were the same.

Total RNA content of fetal liver (Table 10) was less in dams fed Alc-Zn (group 1) than in dams fed a high zinc diet (groups 3,4,6, and 7). A zinc deficiency by itself (groups 2 and 5) also resulted in less liver RNA when compared with dams fed adequate amounts of a high zinc diet (groups 3,4, and 7). The results of Least Squares Means Comparison Tests showed that a food re-

**Table 14. Least Squares Means for Fetal Brain RNA Concentration**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .4074 | .5158 | .1704 | .0056 | .1976 | .0554 |
| 2. | CHO - Zn | .4074 | -- | .8739 | .5833 | .0400 | .6586 | .2583 |
| 3. | Alc + Zn | .5158 | .8739 | -- | .4895 | .0326 | .5542 | .2112 |
| 4. | CHO + Zn | .1704 | .5833 | .4895 | -- | .1150 | .9010 | .5451 |
| 5. | CHO - Zn | .0056 | .0400 | .0326 | .1150 | -- | .0827 | .3249 |
| 6. | CHO + Zn | .1976 | .6586 | .5542 | .9010 | .0825 | -- | .4567 |
| 7. | CHO + Zn | .0554 | .2583 | .2112 | .5451 | .3249 | .4567 | -- |

stricted group (6) had less total fetal liver RNA than a group on a high zinc diet with alcohol and its pair fed control (groups 3 and 4) (Table 15). RNA was not compromised by a zinc deficiency unless accompanied by a low food intake or alcohol. Only the combined abuses of a zinc deficiency and alcohol decreased total RNA in fetal liver in excess of that caused by a reduced food intake. A low dietary intake by itself did not decrease total liver RNA, since group 6 did not differ statistically from groups 4 or 7. Similarly, alcohol by itself (groups 3 and 4) did not compromise fetal liver RNA. Moreover, there was more RNA in the fetal liver from dams fed adequate amounts of a high zinc diet with alcohol (group 3) compared to those ingesting inadequate amounts of a control diet (group 6).

The total quantity of RNA in fetal brain (Table 11) was lowest ($p < 0.05$) for dams on an Alc-Zn diet (group 1) compared with dams that ingested unrestricted amounts of a high zinc diet (groups 3,4, and 7). No other statistical differences were found. In the fetal liver, differences in RNA content exactly paralelled those seen in liver weight. In the fetal brain, alcohol and a zinc deficiency (group 1) reduced brain weight but not total brain RNA when compared with a restricted control group (6). In fetal brain, RNA content was affected by a combination of three factors - a reduced food intake, alcohol, and a zinc deficiency.

The concentration of DNA in both fetal liver and brain was not affected by zinc deficiency, alcohol intake, or dietary intake (Tables 10 and 11). However, dams consuming alcohol generally produced litters with the highest liver DNA concentration. The data leave no doubt that neither alcohol nor zinc deficiency reduced DNA concentration.

Total DNA in fetal liver (Table 10) was less for the zinc deficient groups (1,2, and 5) compared with dams fed adequate amounts of a control diet (groups 3,4, and 7). Animals on a restricted control diet (group 6) fell between these values, and had less total fetal liver DNA than animals ingesting a high zinc diet with alcohol (group 3), and its pair fed control group (4). Although zinc deficiency (compare groups 1 and 2, and 5 and 6) or a reduced food intake (compare

**Table 15. Least Squares Means for Total Liver RNA**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .1348 | .0001 | .0001 | .2277 | .0198 | .0001 |
| 2. | CHO - Zn | .1348 | -- | .0074 | .0123 | .7394 | .4447 | .0164 |
| 3. | Alc + Zn | .0001 | .0074 | -- | .7863 | .0023 | .0351 | .7018 |
| 4. | CHO + Zn | .0001 | .0123 | .7863 | -- | .0039 | .0572 | .9081 |
| 5. | CHO - Zn | .2277 | .7394 | .0023 | .0039 | -- | .2559 | .0054 |
| 6. | CHO + Zn | .0198 | .4447 | .0351 | .0572 | .2559 | -- | .0737 |
| 7. | CHO + Zn | .0001 | .0164 | .7018 | .9081 | .0054 | .0737 | -- |

group 6 with 4 or 7) by themselves did not significantly lower the quantity of DNA in fetal liver, the combination of both factors did. These differences in fetal liver DNA content parallel the differences in fetal liver weight, suggesting that a reduced tissue mass is a factor. There were no differences from an ANOVA in fetal brain DNA content. The results from Least Squares Means Comparison Tests were that total DNA was less in fetal brain from dams ingesting an Alc-Zn diet (group 1) compared with those ingesting adequate amounts of a CHO + Zn diet (group 3), and were probably also influenced by quantity of brain tissue (Table 16).

A review of the literature on the effect of dietary zinc level on nucleic acids also indicates that DNA content rather than concentration is compromised. Duerre et al (1977) found that newborn pups suckled to zinc deficient dams had more liver and brain DNA when compared with ad lib fed controls, but not with pair fed controls. DNA concentration did not differ between the three groups. Similarly, zinc deficiency during gestation days 14-21 did not affect the concentrations of DNA or RNA in pup liver or brain (McKenzie et al, 1975). However, there was less total DNA in the brain, and less total DNA and RNA in the liver compared to pair fed controls. The quantity of liver and brain was also less. Eckhert and Hurley (1977) report less total DNA in the head region, and less total RNA in both the head and body of fetuses when dams were fed a zinc deficient diet till gestation day 12. A zinc injection on day 11 increased ($p < 0.05$) total head DNA above that of the uninjected zinc deficient fetus.

Reports on the effect of alcohol on nucleic acids have not been consistent. Although feeding dams alcohol as 15% of the drinking water did not change brain weight, total RNA and DNA and RNA concentration was reduced compared with pair fed controls (Woodsen and Ritchey, 1979). Henderson and Schenker (1977), using the original Lieber-DeCarli diet with 6% (w/v) alcohol (roughly 35% of the calories) and 18% protein, found that DNA concentration was decreased 15-19% ($p < 0.05$) only in the liver of the ethanol group. Total RNA and DNA were decreased with ethanol in both liver and brain. In a subsequent experiment (Henderson and Schenker, 1979), rats were acclimated to a 6% alcohol diet containing only 3 ppm zinc, which is

**Table 16. Least Squares Means for Total Fetal Brain DNA**

| | | 1. | 2. | 3. | 4. | 5. | 6. | 7. |
|---|---|---|---|---|---|---|---|---|
| 1. | Alc - Zn | -- | .1835 | .0567 | .0493 | .4840 | .0912 | .0571 |
| 2. | CHO - Zn | .1835 | -- | .5369 | .5171 | .5086 | .7524 | .5629 |
| 3. | Alc + Zn | .0567 | .5369 | -- | .9899 | .2037 | .7366 | .9544 |
| 4. | CHO + Zn | .0493 | .5171 | .9899 | -- | .1869 | .7184 | .9426 |
| 5. | CHO - Zn | .4840 | .5086 | .2037 | .1869 | -- | .3130 | .2107 |
| 6. | CHO + Zn | .0912 | .7524 | .7366 | .7184 | .3130 | -- | .7742 |
| 7. | CHO + Zn | .0571 | .5629 | .9544 | .9426 | .2107 | .7742 | -- |

about 10 ppm zinc on a dry weight basis. The control diet also was low in zinc. RNA concentration in the pup liver and brain did not differ when compared with a pair fed control group, but DNA concentration actually increased ($p < 0.05$) with ethanol. Liver and brain weights were reduced with ethanol, and net DNA was the same for ethanol and pair fed groups. Singh et al (1987) also found that a 30% ethanol diet did not affect DNA content of pup brain and liver.

# 5.0 Conclusions

Although some of the dietary treatments in this study included alcohol as 25% of the calories, the factors which most often affected gestation were zinc deficiency and a reduced food intake. None of the parameters measured was affected by the addition of alcohol to a high zinc diet. However, the addition of alcohol to a zinc deficient diet often was the greatest stress imposed on the dam and fetus.

Weight gain of the dams ingesting a liquid diet with less than 1 ppm zinc was 30% of that seen in adequately fed controls, and insufficient during gestation. This stress was not just due to a low food intake, since pair fed control dams gained 60% of that gained by adequately fed controls. A diet with 25% of the calories from alcohol had no effect on weight gain during pregnancy. Maternal liver weight was consistently reduced by a low food intake, and unaffected by either alcohol or zinc deficiency. Maternal plasma zinc concentration again reflected both zinc deficiency and a reduced food intake, but not alcohol intake. Plasma zinc was lowest for the dams on a zinc deficient diet compared to pair fed controls, which were also lower compared to adequately fed controls. Hematocrit in the dam was lower when the quantity of liquid diet consumed was reduced.

The combination of alcohol with a zinc deficiency compromised the ability to sustain a pregnancy, and to produce a fetus that was not resorbed or malformed. Zinc deficiency coupled with alcohol resulted in more resorptions and malformations involving more dams, than did either zinc deficiency or alcohol alone. As expected, the number of pups in a litter and total litter weight were lowest with alcohol and zinc deficiency.

Although the quantity of fetal liver per litter was less when the dams ate less, and even more reduced with a zinc deficiency, only the combination of alcohol with a zinc deficiency was significant. Alcohol by itself had no effect, whether fetal liver was expressed for the entire litter or per pup. Since there were fewer pups with alcohol and zinc deficiency, liver weight per pup was reduced comparably for all dams suffering a zinc deficiency or a reduced food intake. Both the concentration and total quantity of zinc in the fetal liver were reduced only in dams suffering a zinc deficiency. Alcohol did not affect fetal liver zinc.

The quantity of fetal brain tissue per litter followed the same pattern as did fetal liver tissue. Although both zinc deficiency and a reduced food intake appeared to decrease fetal brain weight, only the combination of zinc deficiency and alcohol was significant. Alcohol by itself had no effect. There were less differences when fetal brain tissue was expressed per pup, but again the combination of zinc deficiency and alcohol produced less brain tissue. Unlike fetal liver, fetal brain zinc concentration did not reflect zinc status, alcohol intake, or a reduced food intake. Total brain zinc was lowest in dams suffering a zinc deficiency and ingesting alcohol, following the pattern of fetal brain weight.

The effect on litter size, resorptions, and malformations indicates that teratogenesis is caused by a combination of alcohol and zinc deficiency more than by either alone. The results of this experiment do not show a relationship between teratogensis and nucleic acid concentrations in fetal liver or brain. The concentrations of protein and DNA in these tissues are not compromised by alcohol, zinc deficiency, a reduced food intake, or any combined effect. The only change in RNA concentration was an increase when the zinc deficient alcoholic pups are compared with one of the control groups.

However, total protein in the fetal liver was less with zinc deficiency and a reduced food intake. In the fetal brain, total protein was decreased only with a combination of zinc deficiency and alcohol intake. The quantity of DNA in the fetal liver was decreased by zinc deficiency the

most, but also by a reduced food intake. Although low in dams consuming alcohol with a zinc deficient diet, total DNA in fetal brain did not differ statistically among the seven groups. Total RNA in fetal liver likewise was decreased with zinc deficiency, especially in combination with alcohol, and also decreased with inadequate food intake. Total fetal brain RNA was only decreased with both zinc deficiency and alcohol ingestion.

Since protein and nucleic acid concentrations were comparable, and organ weights matched the differences in total protein, DNA and RNA, these differences appear to reflect the quantity of tissue. It's also possible that tissue quantity is reduced due to a lack of protein, DNA or RNA. If one entertains the theory that the quality of the diet influenced protein, DNA and RNA metabolism, and thus the quantity of tissue, the factors of zinc deficiency and a reduced food intake appear to be primarily involved. However, the total quantity of DNA is even more reduced when zinc deficiency and alcohol are combined. The fact that there is less total DNA points to a defect in cell proliferation. Moreover, the decrease in total RNA, also associated with a zinc deficient alcohol diet, suggests a defect in gene expression. As previously cited, zinc deficiency inhibits synthesis of histones. The interaction of histones and DNA are necessary for the induction of enzyme activities required for replication and expression. A decrease in tissue mass could result from a defect in these associations.

There is little support from this study that alcohol is related to zinc status during gestation. Alcohol did not change zinc concentration in maternal plasma, fetal liver, or fetal brain. Alcohol by itself had no effect on protein, DNA or RNA in these tissues. The high protein level of these gestational diets may have affected the results, since other studies have shown that alcohol intake increases the protein requirement. Perhaps the reports that alcohol intake causes a zinc deficiency were actually linked to the effect of alcohol on protein status. However, alcohol did exacerbate the reduction in these parameters seen with a zinc deficiency. Combining a zinc deficiency with alcohol during gestation particularly increased resorptions and deformities, and reduced litter weight, litter size, total brain and liver weights of the litter, and total fetal brain zinc, protein and RNA content.

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# Appendix A. Protocol for Determination of Zinc, Protein, DNA and RNA

1. A respresentative sample of the pooled liver or brain (approximately one gram) was weighed and homogenized using a polytron in 19 volumes of cold deionized water. The sample was homogenized three times for 10 seconds each, with a one minute period of incubation on ice in-between.

2. In duplicate, 2.5 ml of 0.6 N perchloric acid (PCA) was added to 5 ml of homogenate. The contents were stirred periodically with a glass rod and held on ice for ten minutes.

3. Tubes were centrifuged at 18,000 x G for 20 minutes at 4 degrees centigrade.The supernatant was discarded. The precipitate was resuspended in 2 ml of 0.2 N PCA and recentrifuged for two washes.

3. The pellet was first dissolved in 1 ml of deionized water , to which was added 3 ml of 0.4 N KOH. Tubes were mixed, capped, and incubated at 37 degrees centigrade for one hour.

4. Samples were cooled in ice water before the addition of 4 ml of 1.2 N PCA. After 10 minutes, samples were centrifuged for 30 minutes at 18,000 x G at 4 degrees centigrade. The supernatant was collected into 50 ml volumetric flasks, as were two washes, each with 4 ml of 0.2 N PCA.

5. The RNA fraction was diluted to 50 ml, and a further 1:4 dilution made with 0.02 N PCA. A standard of yeast RNA was exposed to the same extraction procedure. A concentration

of 5 mg/ml was diluted with KOH, treated as in step 3., and brought up to volume in a 25 ml flask. The resulting working standards were 8, 16, and 24 μg/ml.

6. RNA was read on a Bausch and Lomb spectrophotometer for the standards and samples zeroed against 0.05 N PCA.

7. The pellet from step 4. was dissolved in 2 ml of 1 N PCA, mixed, and incubated in a 70 degrees centigrade water bath for 15 minutes. After centrifuging at 18,000 x G for 15 minutes at 4 degrees centigrade, the supernate plus one wash was collected into a 25 ml volumetric flask.

8. A stock solution of DNA standard was made by dissolving 40 mg calf thynus DNA in 100 ml of 4 mM NaOH. For each assay, 2 ml of stock standard and 2 ml of 1 N PCA were heated at 70 degrees centigrade for 30 minutes, and appropriate dilutions made for working standards.

9. 1.5 g of diphenylamine was dissolved in 100 ml of glacial acetic acid, and 1.5 ml of conc. sulfuric acid was added. On the day of assay, a 2% acetaldehyde solution was made and added to the dephenylamine reagent at a ratio of 1 to 200.

10. 1 ml of the working standard and the sample were vortexed with 2 ml of the reagent, covered with foil, and incubated at room temperature for 16-20 hours. Absorbance was read at 600 nm.

11. Protein was assayed using the homogenate from step 1. A 1 ml sample of the homogenated was pipetted into a tared beaker, and the weight brought up to 80 gm.

12. Protein standard was made usine bovine serum albumin. Working standards of 10 - 200 μg/ml were made up to a total volume of 1 ml.

13. The reagents were made as follows. Two grams of NaK tartrate and 100 g $Na_2CO_3$ are dissolved in 500 ml of 1 N NaOH and diluted to 1 liter with water (solution A). Two grams of NaK tartrate and 1 g $Cu_2SO_4 \cdot 5H_2O$ are dissolved in approximately 90 ml water, 10 ml of 1 N NaOH added, and the volume brought up to 100 ml (solution B). One volume of Folin-Ciocalteu reagent is diluted with 15 volumes of water, and prepared daily (solution C).

14. 0.9 ml of solution A was added to 1 ml of sample or standard, and the tubes were placed in a water bath at 50 degrees centigrade for 10 minutes.

15. After cooling, 0.1 ml of solution B was added, and the samples left at room temperature for 10 minutes.

16. Solution C was added rapidly, the tubes vortexed, and heated at 50 degrees centigrade for another 10 minutes.

17. After cooling, tubes were read at 650 nm.

18. For zinc analysis, approximately 0.15 g of homogenate (step 1.) was weighed into an acid-washed beaker in duplicate , and wet-ashed as previously described for the diets. Samples from the original pooled tissue were also wet-ashed in duplicate, and the 4 values averaged.

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