BIOCHEMICAL AND MORPHOLOGICAL EFFECTS OF IRON DEFICIENCY AND/OR CHRONIC ETHANOL CONSUMPTION DURING PREGNANCY ON THE MATERNAL ORGANISM AND FETAL GROWTH AND DEVELOPMENT IN MICE

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THESIS

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In the name of Allah, The Beneficent, The Merciful

Praise be to Allah, Lord of the Worlds

I dedicate this thesis to my family

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CHAPTER I

INTRODUCTION

Alcohol consumption during pregnancy is widely recognized as a serious risk to the fetus. Jones et al. (1973) reported the effects of alcoholism on human fetal development, describing a set of signs and symptoms they termed the "fetal alcohol syndrome (FAS)." The principle features of the syndrome are distinctive and are characterized by growth deficiency; abnormally small heads; facial irregularities; and joint, limb, and genital defects. These anomalies are not always found in the offsprings of all alcoholic women, however, it is possible that exposure to alcohol in lower concentrations might result in functional disturbances of the developing organs and a variety of learning and behavioral handicaps (Jones et al., 1974; Little, 1977; and Shaywitz et al., 1980). Since Jones' original article was published in 1973, many cases of infants with FAS have been described in the literature (Hanson et al., 1978; Jones and Smith, 1975; Mulvihill et al., 1976; and Ouellette et al., 1977).

Although the precise number of alcoholic women in the United States of America is not known, the National Institute of Alcohol Abuse and Alcoholism (1979) estimated that about 3.1 million women are heavy drinkers (drinking more than 1.0 ounce absolute alcohol per day), 9.4 million are moderate drinkers (drinking 0.22-1.0 ounce absolute alcohol per day), and 25.8 million are light drinkers (drinking less than 0.22 ounce absolute alcohol per day). In a recent study of 633 Boston women, Ouellette et al. (1977) found that 19 percent were heavy drinkers. Seventy-one percent of infants born to this group were considered abnormal suggesting a significant relationship between the drinking of alcohol during pregnancy and the outcome

of gestation. Also, extensive animal research over the past few years has demonstrated the teratogenic properties of ethanol in a variety of species (Chernoff, 1977; Kronick, 1976; and Tze and Lee, 1975).

The effects of maternal alcoholism, particularly in relation to incidence of abortion, stillbirth and mortality, prematurity and mental retardation, are complex and controversial problems. Rubin and Lieber (1974) suggested that ethanol has direct toxic effects, and recently Mulvihill and Yeager (1977) pointed out that the risk of a fetus being affected may be directly related to the amount of alcohol to which it is exposed. In addition, it has been suggested that the damage results from nutritional imbalances and deficiencies caused by ethanol (Davidson, 1975). Also, there are indications that the effects of ethanol in the presence of nutritional deficiencies are more drastic than the effects when nutrition is the sole inadequacy (Davidson, 1975). The alcohol-nutrient interaction is confounded by the fact that many female alcoholics have inadequate diets (Ouellette et al., 1977). Roe (1979) indicated that ethanol ingestion alters the status of some nutrients in the body such as folic acid, iron, zinc, thiamin, and vitamin B_6. Herbert et al. (1963) observed that 93 percent of the alcoholic patients they examined had serum folate activity below normal. Disorders of iron metabolism are also associated with consumption of too much alcohol (Sullivan and Herbert, 1964; Charlton et al., 1964; and Celada et al., 1977). Powell (1975), in his review on hemochromatosis, pointed out the frequency of alcoholism in this condition. Hines and Cowan (1970) found that the hematocrit value was reduced to anemic levels during periods of alcohol administration to abstinent individuals, while serum iron was elevated and plasma iron turnover markedly increased.

Available information on the effect of ethanol on the body and its relation to nutrition has been obtained in adult males and less frequently in non-pregnant females. Whether these effects are fully valid when applied to pregnant females remains to be assessed. Significant maternal changes take place during the course of human pregnancy. These maternal changes are likely to modify the physiological disposition of and response to ethanol. The nutritional status of pregnant women is also different. Folic acid and iron deficiencies are more prevalent nutritional disorders in pregnant women than in any other physiological groups (Herbert et al., 1975; and Moore, 1973). Folate deficiency during pregnancy has teratogenic effects (Nelson et al., 1952). Several of these defects are similar to those associated with experimental fetal alcohol syndrome (Cooper et al., 1970).

Although previous findings support the fact that ethanol ingestion during pregnancy influences growth and development of fetuses and the general outcome of gestation, the combined effects of ethanol and nutritional deprivation of iron on the maternal organism and her fetus have not been explored in either human subjects or experimental animals. The present study was undertaken to determine the influence of chronic alcohol ingestion on the maternal organism and the fetus under conditions of nutritional adequacy and the effects of chronic ethanol ingestion combined with iron deficiency on iron and folate metabolism. A mouse model was employed, and morphological and biochemical assessments were performed.

CHAPTER II

REVIEW OF LITERATURE

A. Alcohol metabolism

Alcohol, correctly termed ethanol, is rapidly absorbed through the mucosal membrane of the gastrointestinal tract, especially in the duodenum and jejunum, and enters the portal circulation. It becomes distributed throughout the body in the arterial blood and moves across tissue membranes by process of simple diffusion. Within a few minutes after oral ingestion, it is circulated to every tissue in the body. It is neither blocked by the blood-brain barrier nor the placental barrier (Williams, 1975).

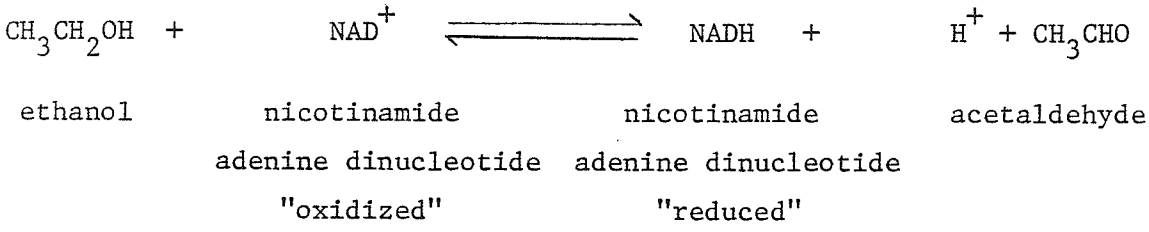
Ethanol is both a rapidly metabolized nutrient that provides 7.1 Kcal per g and a dangerous drug, depending on the amount consumed and the duration of the exposure. Most ethanol elimination takes place by metabolic conversion, but significant quantities, especially at high blood concentrations, can be excreted unchanged through breathing and urination. In man, pulmonary and renal excretion account for 10 to 15 percent of the total body ethanol (Li, 1977).

The liver is the principal organ responsible for the metabolism of ethanol, approximately 75 percent, while the kidney, lung, stomach, intestine, heart, brain, and skeletal muscles all exhibit significant alcohol oxidation ability. However, the contribution of each of the latter organs to the total metabolism of ethanol is small (Lundquist, 1971).

Three principal reactions capable of oxidizing ethanol to acetaldehyde have been described. They are catalyzed by alcohol dehydrogenase (ADH), catalase, and the microsomal ethanol oxidizing system (MEOS).

1. Alcohol dehydrogenase (ADH)

Among the three enzymes mentioned above which catalyze the oxidation of ethanol to acetaldehyde, it is generally believed that alcohol dehydrogenase is the most important quantitatively, especially under normal conditions (Li, 1977). ADH is a cytosol enzyme which catalyzes the following reaction:



It is generally accepted that under normal conditions, this oxidation of ethanol by ADH is the rate-limiting step (Hawkins and Kalant, 1972). The equilibrium of the ADH-catalyzed reaction is unfavorable for alcohol oxidation. In order for the reaction to continue, acetaldehyde must be removed effectively, and there must be rapid reoxidation of NADH that takes place principally in mitochondria via the electron transport chain.

The situation regarding possible changes in ADH activity during chronic alcohol ingestion is controversial. After daily intakes of small amounts of ethanol, ADH was found to be increased not only in the liver, but also in the cerebral cortex (Raskin and Sokoloff, 1970). In another study, Dajani et al. (1963) provided a group of male rats with 20 percent ethanol as the sole drinking fluid for a period of 39 weeks, after a 6 week period of acclimation to 10 percent ethanol. A gradual increase in ADH activity was observed up to 16 weeks, and then the activity diminished until it approached normal or subnormal values by the end of the experimental period. The withdrawal of ethanol from a group of drinking rats for the last 20 weeks of the study was followed by a decrease in ADH level to control values. In

male mice receiving 15 percent ethanol in drinking water, liver ADH activity was increased significantly after 3 and 5 months of continued ingestion, but not after 12 months (Mirone, 1965). An interesting study by Sze et al. (1976) indicated that mice born and nursed by drinking mothers had significantly elevated levels of ADH in their livers.

In contrast to the above findings, Figueroa and Klotz (1962) reported no change in liver ADH in female rats injected intraperitoneally with moderate doses of ethanol for 12 to 16 weeks. Greenberger et al. (1965) failed to note any significant changes in ADH activity of female rats chronically treated with ethanol in moderate doses. However, ethanol in large doses (20 percent ethanol as the sole drinking fluid plus 6 g ethanol/Kg body weight every day by stomach tube for 6 weeks) caused a significant decrease in the enzyme activity. The reasons for these discrepancies have not been elucidated. However, it is well-known that ethanol in large doses produces metabolic changes in the liver, inhibits protein synthesis (Rothschild et al., 1971), and that protein deficiency lowers liver ADH activity (Li, 1977).

2. Catalase

Keilin and Hartree (1936) suggested that catalase has the ability to oxidize ethanol. Catalase, in the presence of peroxide generating systems, would catalyze the following reaction.

$$\underset{\text{ethanol}}{CH_3CH_2OH} + \underset{\substack{\text{hydrogen}\\\text{peroxide}}}{H_2O_2} \rightleftharpoons \underset{\text{acetaldehyde}}{CH_3CHO} + \underset{\text{water}}{2H_2O}$$

In isolated perfused rat livers, it has been shown that ethanol produces a decrease in the concentration of catalase - H_2O_2 complex (Oshino et al., 1973),

providing strong evidence about the role of this system in ethanol oxidation. Studies of inhibitors of catalase activity in isolated rat livers have shown that catalase does not contribute significantly to ethanol oxidation at low blood ethanol concentrations. However, its effect increases progressively as ethanol concentration is increased (Thurmen et al., 1975).

3. Microsomal ethanol-oxidizing system (MEOS)

Orme-Johnson and Ziegler (1965) reported that the mixed function oxidase system in mammalian liver microsomes, which plays a major role in hepatic metabolism of many drugs can also catalyze the NADPH and O_2-dependent oxidation of ethanol to acetaldehyde.

$$\underset{\text{ethanol}}{CH_3CH_2OH} + \underset{\substack{\text{nicotinamide adenine dinucleotide phosphate (reduced)}}}{NADPH} + H^+ + O_2 \rightleftharpoons \underset{\text{acetaldehyde}}{CH_3CHO} + \underset{\substack{\text{nicotinamide adenine dinucleotide phosphate (oxidized)}}}{NADP^+} + H_2O$$

This reaction was designated the microsomal ethanol-oxidizing system (MEOS) by Lieber and DeCarli in 1968. It was reported that MEOS was not inhibited by pyrazole at concentrations which inhibit ADH (Khanna et al., 1970). Furthermore, it was found that ethanol can induce changes in the absorption spectra of microsomal hematoprotein which resemble those induced by phenobarbital (Rubin et al., 1971). These data suggest that ethanol induces the MEOS. However, it also was suggested that MEOS activity represents contamination of microsomal preparations with catalase. The NADPH-dependent drug metabolizing system in liver microsomes includes a linked sequence of reactions. They begin with NADPH oxidase; and in the absence of suitable substrates for the hydroxylation reaction, such

as metabolizable drugs, they end with cytochrome P-450 completing its oxidation-reduction cycle by generating hydrogen peroxide. In the presence of catalase, either as a microsomal constituent or as a contaminant, this system could oxidize ethanol (Hawkins and Kalant, 1972).

Whatever the system involved in ethanol oxidation, the final products are carbon dioxide and water. Ethanol is first converted to acetaldehyde which in turn is oxidized to acetate in a reaction catalyzed by acetaldehyde dehydrogenase. Acetate is readily utilized as a substrate for most tissues and is further oxidized to carbon dioxide and water via the tricarboxylic acid cycle (Li, 1977).

The main metabolic effects of ethanol result from an increase in the $NADH/NAD^+$ ratio within the cytoplasm and mitochondria of liver cells (Hawkins and Kalant, 1972; and Lundquist, 1975). This, in turn, affects the availability of pyruvate and oxaloacetate, bringing about a wide range of serious disturbances in the mitochondrial oxidation of fatty acid and other substrates, gluconeogenesis, and carbohydrate utilization. In addition, the change in this ratio directly affects many other NAD-dependent reactions involved in the metabolism of amino acids, glycerol, carbohydrates, and porphyrine. Finally, the large amounts of acetate, lactate and lipids, and the smaller amounts of acetaldehyde which leave the liver during ethanol oxidation produce indirect effects on the metabolism of other tissues (Lieber, 1975).

B. Ethanol and internal organs

1. Liver

The liver is the organ primarily responsible for alcohol metabolism, and also it is the organ affected most frequently in alcoholic patients.

Alcoholism in its milder form is characterized by accumulation of excess fat in the liver, the so-called fatty liver. The condition is usually benign and fully reversible (Lieber, 1975). Edmondson et al. (1967), in a liver biopsy study, found that 90 percent of chronic alcoholic men had fatty livers. However, when fatty livers become advanced, more of the hepatic cells die and are replaced by fibrous tissue. The excess fibrous tissue distorts the normal architecture of the liver and alters the liver function. This liver injury, which is called liver cirrhosis, is irreversible (Lieber, 1975). Leevy (1967) reported that 29 percent of 3,000 randomly selected alcoholics had liver cirrhosis, while Lelbach (1975) found that the incidence of liver cirrhosis among 329 alcoholics was 12 percent.

Davidson (1975) considered liver injury in alcoholics to be a result of malnutrition, especially in lipotropic nutrients. However, Rubin and Lieber (1974) succeeded in producing fatty livers and cirrhosis in baboons consuming an adequate diet and alcohol, suggesting that alcohol may have a direct effect on the liver independent of nutritional status. In support of Rubin and Lieber's finding, Oralz et al. (1975) reported that both alcohol ingestion and fasting inhibit protein synthesis in rabbit livers, but either of these stresses can be overcome by excess amino acids, although the combination of fasting and ethanol prevented recovery.

2. Small intestine

Ethanol, besides being absorbed by the intestine, was shown to be metabolized by the intestinal mucosa affecting the capacity of the intestine to absorb a number of nutrients. Mezey (1975) determined the ADH activities in various rat tissues and found that the activity of ADH in the upper intestine was about one-fifth of the activity found in the liver. Baraona et al. (1974) studied the effect of chronic administration of ethanol with

an adequate diet. They found shortening of the intestinal villi, decreases in the number of epithelial cells lining the villi, and decreases in the activities of the villus enzymes lactase, sucrase, and alkaline phosphatase. Halsted et al. (1971) found decreased folate absorption in only a few of their alcoholic patients. Mezey (1975), in his review on intestinal function in chronic alcoholism, suggested that malabsorption of D-xylose, thiamin, folic acid, vitamin B_{12}, and fat in chronic alcoholics occurs principally in alcoholics who have a dietary insufficiency. Lindenbaum and Lieber (1975) reported malabsorption of vitamin B_{12} in 6 of 8 chronically alcoholic volunteers with good nutritional status.

3. Hematopoietic system

Alcoholics often are found to have an abnormal hematological picture, especially macrocytic anemia. Until recently, liver disease and malnutrition were thought to be the primary causes of hematological disorders in alcoholics (Williams, 1975). However, increasing evidence indicates that alcohol alone is capable of producing several types of hematological abnormalities. Hillman (1975) suggested that the impairment of red blood cell production in bone marrow by alcohol is due to a direct toxic effect of stem cell proliferation. He further noted that alcohol may affect the membrane or function of the cell in such a way as to shorten cell life span or interfere with normal cellular activities and distribution. Earlier, Sullivan and Herbert (1964) had already demonstrated a potential inhibitory effect of continual alcohol ingestion on the hematological recovery of patients they studied.

4. Other organs

Chronic alcohol ingestion also affects other organs, particularly the brain, the heart, the pancreas, the endocrine glands, and the skeletal muscles (Williams, 1975; and Roe, 1979).

5. Sex difference

A sex difference for the influence of alcohol in human beings has also been observed. Jones and Jones (1976) reported that women not only react differently to alcohol but also get intoxicated more quickly than men. They attributed this sex difference to hormonal factors and to the low body water content of women. They also reported that women taking oral contraceptives metabolized alcohol more slowly than did those not taking oral contraceptives, and that blood levels of alcohol in women, ingesting equal amonts of alcohol, vary according to the menstrual cycle. In a recent report, Ryback (1977) recorded a number of clinical abnormalities in women who are heavy drinkers, for example, early premenopausal amenorrhea and even ovarian failure. The investigator also noted that menstruation resumed again after 2 to 3 months of complete sobriety.

C. Maternal alcohol consumption and its influence on the fetus

1. Human studies

The effects of maternal alcohol consumption on the fetus were mentioned in old medical literature, but have been generally ignored until recently. Warner and Rosett (1975) documented a historical survey on the effect of drinking on offspring. These researchers reported that observations were made during England's Gin Epidemic (1720-1750), followed by warnings of 19th century medical writers that parents' drinking could damage the fetus. Many concurrent studies were reported in the medical literature from 1865-1920. Then, research interest declined during the period of prohibition, and the authorities later discounted the previous works. Even as late as 1965, as cited by Streissguth (1976), Ashly Montague, in a book entitled Life Before Birth, stated that "it can now be stated categorically, after hundreds of

studies covering many years, that no matter how great the amounts of alcohol taken by the mother or by father, for that matter neither the germ cells nor the development of child will be affected ...."

In France, Lemoine et al. (1967) carried out a study with 127 offspring of alcoholic parents, predominantly female. They described some common signs related to maternal alcoholism which were among the offspring. The offspring showed characteristic facial anomalies, growth retardation, and increased frequency of malformations. In 1972, Ulleland reported observations of 6 infants at the University of Washington's Harborview Medical Center, whose mothers were alcoholic. The infants were born underdeveloped for gestational age, and their postnatal growth and development did not proceed at a normal rate. Because of these results, Ulleland (1972) also did a retrospective study on other low birth weight infants who were delivered at the Center. During an 18 month period, of 1,594 babies delivered, 2.9 percent were small for gestational age. Furthermore, 2.3 percent of these small infants were born to non-alcoholic mothers, whereas 10 of the 12 infants (83.3 percent) born to alcoholic mothers were underdeveloped.

In 1973, Jones et al. from Seattle, Washington published a report describing 8 unrelated children born to alcoholic women. The children had similar patterns of retarded growth and development and craniofacial, limb, and cardiovascular defects. Jones and Smith (1975) summarized the pattern of malformations in their clinical cases as "Fetal Alcohol Syndrome." They classified these malformations into 4 categories: abnormality in performance which includes prenatal growth deficiency, postnatal growth deficiency, and developmental delay; craniofacial anomalies as microcephaly, short palpebral fissures, epicanthal fold, maxillary hypoplasia, cleft palate, and micrognathia; such limb anomalies as joint anomalies and altered palmar crease patterns;

and, other anomalies including cardiac anomalies, anomalcus external genitalia, capillary hemangiomata, and fine-motor dysfunction. By 1976, the Seattle group (Hanson et al., 1976) reported a total of 41 cases of FAS.

In 1977, a prospective study was done at Boston City Hospital by Ouellette et al. to evaluate the risk to offsprings of heavy drinking during pregnancy. A total of 633 mostly low, socioeconomic status women participated. These women were divided into 3 groups: Group 1, abstinent or rare drinkers representing 52 percent of the total, who drank less than once a month; Group 2, moderate drinkers, i.e., women representing 39 percent of total, who consumed liquor more than once a month, but did not meet the criteria of heavy drinkers; Group 3, heavy drinkers, i.e., women representing 9 percent of the total, who drank on the average 45 ml of absolute alcohol a day. The nutritional status was evaluated on the basis of replies to the questions, "What did you eat yesterday? Are yesterday's meals typical?" The nutritional status did not differ significantly among the three groups, although heavy drinking was associated with heavy smoking. Of 42 infants born to heavy drinkers, 71 percent were considered abnormal as they displayed congenital anomalies, and growth or neurological abnormalities. Thirty-five percent and 36 percent of infants born to Groups 1 and 2, respectively, were considered abnormal. Another prospective study which was designed by Little (1977) to investigate the relation between moderate maternal alcohol use and decreased birth weight showed that maternal alcohol use in the 6 months prior to pregnancy and in the fifth through the eighth month of pregnancy was associated with a significant decrease in birth weight of offspring. After correcting for maternal age, height, and smoking, as well as age and sex of offspring, this investigator found that daily intake

of 1 ounce of absolute alcohol in the prepregnancy period was associated with an average decrease of 91 g in the offspring weight. This amount consumed in late pregnancy was associated with a 160 g decrease in birth weight. Similar conclusions on the effects of moderate alcohol consumption during pregnancy on fetal growth were reported by Hanson et al. (1978). They also found morphological anomalies in the offspring born to moderately alcoholic mothers. In a very recent study, Little et al. (1980) compared the corrected birth weights of 59 infants born to women who reported total abstinence during pregnancy, but who had a history of alcoholism prior to conception, with weights of 59 infants born to heavy drinkers who continued drinking during pregnancy, and weights of 59 infants of non-alcoholics. The authors reported significant decreases of 258 g and 493 g from the weights of control infants for offspring of abstinent alcoholics and drinking alcoholics, respectively.

Jones et al. (1976) reported that children at 7 years of age who had chronically alcoholic mothers, when compared to carefully matched controls, had a significantly lower intelligence. In a recent report, Shaywitz et al. (1980) studied the behavior and learning ability of 15 children of normal intelligence who were born to alcoholic mothers. The children ranged in age from 6.5 to 18.5 years. They found that all growth measurements were affected. The children had a continuum of dysmorphic features of FAS. All of the children exhibited persistent academic failure and had problems of activity and attention regulation.

2. Animal studies

Many investigations of the toxic effects of ethanol on prenatal development in animals have been performed. However, the results are

somewhat contradictory. Sandor and Elias (1968) studied the effects of ethanol on chick embryos. Early maldevelopment and mortality occurred in a considerable portion of the embryos. Tze and Lee (1975) studied female rats whose drinking water contained ethanol at 30 g per 100 ml as their only available fluid. They found that only 50 percent of the ethanol-fed mothers known to have compulated delivered litters; also, the average litter size was lower than were the litters from females drinking ordinary water. Furthermore, some offsprings of ethanol-fed mothers exhibited microcephaly and cracked, dry, and loose skin. Woodson and Ritchey (1979) designed a study in rats to determine the effects of maternal alcohol on brain development during the fetal period. One week prior to mating, the experimental group of female rats was placed on 15 percent ethanol as the only fluid; this procedure continued until day 18 of gestation. The investigators found that the average number of fetuses, the weights of fetuses, and the weights of fetal brains were lower in the ethanol-fed group than in the control group. They further found that deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) contents and the number of fetal brain cells were significantly reduced in the ethanol-fed group.

Using mice as experimental animals, Kronick (1976) injected pregnant dams interperitoneally with a 25 percent solution of ethanol in saline at a dosage of 0.03 ml per g body weight during different periods of organogenesis. Fetal mortality rate was substantially increased during gestation days 9 through 12 following this alcohol treatment. The incidence of fetal anomalies was significantly increased following alcohol treatment, but only on days 8, 9, and 10. Fissure of the iris, coloboma, was most frequently observed in animals injected on days 8 and 9; absence of the forepaw was seen only in offspring of mothers injected on day 10. Critical periods for other anomalies were not apparent.

In another study, Chernoff (1977) succeeded in inducing in mice pathological and morphological changes similar to those observed in the human fetal alcohol syndrome. He maintained CBA/J and C3H pregnant mice on liquid diets consisting of Metracal (Mead Johnson, Evansville, Indiana) with ethanol for the experimental groups and Metracal without ethanol for the controls. The diets were fed to the mice for 30 days prior to and throughout gestation. In both strains of mice, the fetal resorption rate increased with an increasing intake of ethanol-derived kilocalories from 15 percent to 35 percent. Maldevelopment also increased with the level of ethanol intake.

Similar results were obtained by Randall et al. (1977) who administered ethanol on days 5 to 19 of gestation to C57BL/6J mice. Ethanol was given as a part of a nutritionally balanced liquid diet and provided 25 percent of the kilocalories. Twice as many fetuses were resorbed in the ethanol-fed group as in the control groups. The control groups were maintained on a solid diet or basic liquid diet with added sucrose so that it was isocaloric with the ethanol-containing diet. The ethanol-fed group had more malformed fetuses and significantly lower fetal weights than the controls. In 1979, Boggan et al. published results of two experiments of the same design. The experimental C57BL/6 mice were fed ethanol-containing liquid diets, 30 percent ethanol-derived kilocalories from day 5 of gestation to day 11; there were two control groups, one maintained on a liquid diet containing sucrose that was isocaloric to the ethanol diet for the same period of gestation and the other control group maintained on a solid diet. All three groups were maintained on solid diets after day 11 of gestation. One experiment was planned to study the effects of prenatal exposure to

ethanol on renal anomalies in pups sacrificed at 14, 21, 28, and 35 days of age. The investigators found a high incidence of hydronephorsis, distention of the kidney, only in prenatal ethanol-exposed pups. The other experiment was done to study effects of prenatal ethanol exposure on sexual maturity. They found that prenatal exposure of mice to ethanol resulted in delayed sexual maturation as measured by the time of vaginal opening. In a recent study, Diaz and Samson (1980) fed young rat pups through intragastric cannulas from postnatal day 4 through day 18. They reported a 19 percent reduction in total brain weight when ethanol was included in the pups' milk formula on days 4 through 7. The ethanol-exposed pups were hypoactive during the period of ethanol administration.

D. Interrelationship among alcohol ingestion, maternal iron, and folic acid deficiencies

The etiological role of alcohol consumption in fetal anomalies is confounded by the fact that most alcoholic women also have inadequate diets. This fact, coupled with other environmental factors, probably results in cumulative effects on the fetus. Jones and Smith (1975) evaluated the nutritional status of one chronically alcoholic mother shortly after delivery of her fetal alcohol syndrome infant. Serious iron deficiency was the only abnormality detected in the mother. From another study, Ouellette et al. (1977) found that only 7 percent of 633 Boston women who they classified as abstinent, moderate, and heavy drinkers met the Recommended Dietary Allowance of the National Research Council for iron. Though there is no available literature on iron and folate status of the alcoholic women during pregnancy, it is well-known that alcohol consumption affects iron and folate metabolism. However, iron deficiency, folic acid deficiency or a combined deficiency of iron and folic acid is a common phenomenon in pregnancy (Prasad, 1978).

G. Maternal folic acid deficiency

Folic acid is an essential nutrient. The reduced forms of folate, involved in the transfer of single carbon units, are required for the cell metabolism as well as the synthesis of purine and pyrimidine which are components of DNA and RNA. Therefore, when the total number of cells in the body or the rate of cell synthesis increases, the requirements of folate increase. Rodriguez (1978) reported that in the last third of pregnancy and in fetuses, the number of new cells being synthesized is very large. At the same time, folate stores in the body decreased rapidly during folate deprivation. More cases of folic acid deficiency have been reported in pregnant women that in any other population group. The World Health Organization, in 1968, suggested that up to a third of all the pregnant women in various countries outside the United States have a folate deficiency. Herbert et al. (1975) estimated that 16 percent of 110 pregnant women from low income families in New York, at the time of their first prenatal visit to the clinic, were deficient in folic acid, as indicated by red cell folate.

Folic acid deficiency has been regarded as a major factor in a variety of obstetric complications. Rothman (1970), in his review, reported the possible relationship between folate deficiency in pregnancy and fetal malformations or other abnormalities of pregnancy. However, the exact relationship, if any, is still a subject of controversy. Mackenzie and Abbott (1960) found that 6 percent of patients with megaloblastic anemia also had premature detachment of placenta (abruptio placenta), compared with an incidence of 3 percent in normoblastic population. Using elevated urinary excretion of formiminoglutamic acid (FIGLU) following a histamine-load as a sign of folate deficiency, Hibbard (1964) found that 64 percent

of patients with abruptio placenta also had folate deficiency. In contrast, Gatenby and Lillie (1960) and Giles (1966) were unable to find any association between abruptio placenta and megaloblastic anemia.

Other complications have also been noted. Hibbard (1964) found that the incidence of folate deficiency in patients who had abortions was four times that in a control group of women with normal pregnancies. Martin et al. (1965), after having assayed the serum folic acid activity in 150 cases with recurrent abortion, reported that 53 percent of the patients had low serum folic acid activity. In addition, they found that 34 pregnant women who had histories of recurrent abortion delivered normally after they were treated with folic acid from early pregnancy. Giles (1966) found the rate of stillbirths among patients with megaloblastic anemia was more than twice that of all women confined in the hospital. However, he did not find any relation between folic acid deficiency and fetal malformation.

Some complications were found in the offspring. Fraser and Walt (1964) reported that 5 of 17 women with megaloblastic anemia gave birth to infants with serious birth defects. Hibbard and Smithells (1965) found folate deficiency in 62 percent of 98 mothers of malformed infants as compared with 17 percent of mothers of normal infants. Hibbard (1975), in his prospective study of erythocyte folate levels in 805 women in early pregnancy, noted that low folate levels were found in 61 percent of mothers when the fetus was malformed, 18 percent of preterm infants, and 49 percent in mothers of small for dates infants.

H. Teratogenic effects of folate deficiency in laboratory animals

Folate deficiency in pregnant laboratory animals during the period of embryogenesis has been associated with multiple malformations as well as

early fetal death and resorption. Nelson et al. (1952) determined the teratogenic effects of folic acid deficiency in rats. These investigators fed a folic acid-deficient diet, containing folic acid antagonists, to different groups of female rats starting on days 7, 9, 10, 11, 12, 13, and 15 of gestation and continued feeding this diet until they were sacrificed on day 21 of gestation. Instituting the deficiency of folic acid as late as 9 days after breeding resulted in total fetal resorption, whereas delaying the deficiency to 11 days after breeding resulted in 95 percent of the animals having litters of fetuses with multiple congenital abnormalities. These abnormalities were marked edema and anemia, multiple morphological abnormalities such as cleft palate, cataracts of the eyes, and retarded development of the viscera. In 1963, Johnson, Nelson, and Monie studied effects of transitory folic acid deficiency between days 8 and 10 of gestation on fetal and placental development in rats. To determine the rate of fetal death due to folic acid deficiency, they sacrificed a group of rats daily between day 11 and 15 of gestation. Eighteen percent of fetal death occurred by day 11, 65 percent by day 12, and 100 percent by day 13 of pregnancy. Although neural anomalies were recorded in these fetuses, no changes were recorded in the placentas due to folic acid deficiency.

In another study, Monie and Nelson (1963) reported anomalies in pulmonary blood vessels of rat fetuses due to maternal folic acid deficiency. Stempak (1965) maintained a group of female rats on folic acid-free diets for only 48 hours between days 8 and 19 of gestation; then, this period was terminated by ingestion of 500 µg folic acid. Hydrocephalus was the only defect observed in the treated animals' offspring.

Schreiber et al. (1973) placed two strains of pregnant mice on folic acid-deficient diets at varying times from 7 to 0 days prior to parturition

and then sacrificed pups on the seventh postnatal day. The maternal folic acid-deficient diet was found to reduce the liver and brain weights of the pups. Furthermore, folate store in the livers of the pups was also low as compared to that in the controls.

Recently, Thener (1979) studied the effects of maternal folate deficiency on rat fetuses. She maintained female rats on folate-deficient diets at age 21 days through breeding until sacrificed on day 21 of pregnancy. Folic acid deficiency had no significant effects on maternal body and liver weights, nor on the mean numbers of living fetuses in each litter. However, low hematocrit values were recorded in folic acid deficiency. In addition, maternal folic acid deficiency significantly reduced placenta and fetal body and liver weights, and had a marked effect on reducing fetal folate storage.

I. Folic acid deficiency in alcoholics

Varying degrees of folate deficiency are commonly encountered in chronic alcoholics. Herbert et al. (1963) observed that 93 percent of alcoholic patients they examined had serum folate activity below normal. Eichner and Hillman (1973) gave alcohol orally and intravenously to 17 normal and chronically alcoholic volunteers to study alcohol effects on folate metabolism. The investigators reported that alcohol induced decreases in serum folate levels and that when ethanol was stopped, the serum folate levels returned rapidly to normal. After an oral dose of labeled pteroyl-glutamic acid (^{3}H-PGA), Halsted et al. (1967) demonstrated that the plasma levels of radioactivity in 23 patients with chronic alcoholism were below normal. On the other hand, in alcoholics who had abstained from alcohol for at least a week, levels of radioactivity were normal. They suggested

that sustained alcohol ingestion results in folic acid malabsorption. However, Lane et al. (1973) gave a different explanation for low serum folate and megaloblastic anemia in chronic alcoholism. They suggested that the suppression of serum folate level in chronically alcoholic subjects was not due to interference with uptake, storage, and conversion of pteroylglutamic acid to N-5 methyl tetrahydrofolate, but rather to a block in the release of N-5 methyltetrahydrofolate from storage pools, particularly the liver.

Tigner and Roe (1978) found a significant positive correlation between dietary intake of folic acid and maternal plasma, whole blood, liver folate, and fetal liver folate in rats. Lin et al. (1978) explained the anomalies produced in mice fetuses as a result of ethanol ingestion, possibly because ethanol may have inhibited active transport of folate through the placenta.

J. Ethanol and dihydrofolate reductase activity

Dihydrofolate reductase is an essential enzyme in folate metabolism. It catalyzes the reduction of oxidized form of folic acid to tetrahydrofolic acid. Dihydrofolate reductase is also involved in DNA synthesis via its effect on thymidine triphosphate (TTP) synthesis, the end product of the dihydrofolate reductase dependent de novo pathway (Wilmanns, 1971). Another study by Evans (1978) indicated that the teratogenic effects of some anti-convulsants in mice were due to interference in folate metabolism through inhibition of dihydrofolate metabolism through inhibition of dihydrofolate reductase. The precise role of alcohol-folate interaction as a teratogen is not known, and there is no available literature on the effect of ethanol ingestion on dihydrofolate reductase activity.

In summary, it is clear that alcohol ingestion during pregnancy can induce teratogenic effects both in the human being and in experimental animals.

These effects have distinctive features such as growth and mental retardation. Most of the studies which have been done have been descriptive. Even with the amount of knowledge already available concerning the effects of ethanol intake on internal organs and metabolism, the role of ethanol as a teratogenic agent is still not clear and is even controversial.

Although nutritional problems, like iron and folate deficiency, are common during pregnancy, there are no available data that link maternal alcohol ingestion and nutritional status.

Much more research is needed to clarify the effects of maternal alcohol ingestion with nutritional adequacy or nutritional deficiency on reproductive performance and fetal growth and development.

CHAPTER III

MATERIALS AND METHODS

A. Experimental design

The purpose of this investigation was to examine the effect of chronic ethanol consumption alone (Experiment 1) and combined with iron deficiency (Experiment 2) on reproductive performance, maternal characteristics, and fetal growth in mice. Experiment 1 was designed to determine the level of ethanol-derived calories (EDC) in the diet which most effectively produced anomalies observed in the human fetal alcohol syndrome and to determine the impact of ethanol withdrawal after conception.

Based on results of Experiment 1, Experiment 2 was conducted to examine the combined effects of ethanol consumption and iron deficiency on selected maternal and fetal indicators. A 2 x 2 factorial design (Figure 3) was employed, with ethanol-derived calories and iron as independent variables.

B. General procedure

1. Experiment 1

Sixty day old CBA/J female mice* were randomly assigned to one of six experimental groups (16 animals per group). The animals were housed individually in polypropylene "shoe-box" cages with Sanical bedding** in a temperature-controlled room (20°C) with a 12 hour light-dark cycle. The design of this experiment was a randomized complete design (Figure 1). Groups A and B were both treated as controls and were fed a stock diet and liquid diet without ethanol (0% EDC), respectively. Groups C, D, and F were fed liquid diets containing 10, 20, and 30 percent EDC, respectively.

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*Jackson Laboratory, Bar Harbor, Maine.

**Paxton Processing Company, Inc., Paxton, Illinois.

Group (A)[1]	Group (B)[2]	Group (C)[3]	Group (D)[4]	Group (E)[5]	Group (F)[6]
Stock diet	0% EDC*	10% EDC	20% EDC continued	20% EDC until conception, then 0% EDC during pregnancy	30% EDC

Figure 1. Experimental design of Experiment 1.

[1]Group (A). Maintained on a stock diet throughout the experiment.

[2]Group (B). Received a liquid diet providing 0% of the calories from ethanol.

[3]Group (C). Received a liquid diet providing 10% of the calories from ethanol.

[4]Group (D). Maintained on a liquid diet providing 20% of the calories from ethanol.

[5]Group (E). Maintained on a liquid diet providing 20% of the calories from ethanol until conception, then received a 0% EDC diet during pregnancy.

[6]Group (F). Received a liquid diet providing 30% of the calories from ethanol.

*(EDC) is ethanol-derived calories.

Group E received a liquid diet containing 20 percent EDC until mating, after which it was maintained on a liquid diet containing 0 percent EDC until the day of sacrifice.

The stock diet* contained 17 percent crude protein, 11 percent crude fat, 3 percent fiber, and 6.5 percent ash according to the label declaration of the manufacturer.

The nutrient content of Sustacal** which formed the basis of the liquid diets is shown in Table 1. Varying levels of ethanol and sucrose were added to the base liquid to formulate isocaloric diets providing 0, 10, 20, and 30 percent EDC (Table 2). All diets provided recommended levels of nutrients for the pregnant mouse (NAS/NRC, 1972).

The feeding schedule shown in Table 3 was followed to acclimate animals to dietary ethanol. The liquid diets were prepared fresh daily and were the only source of nutrients for the animals. Animals received the liquid diets ad libitum from inverted 50 ml plastic syringes through stainless steel drinking tubes (Figure 2) as described by Chernoff (1977). Daily liquid diet consumption was determined in ml, and caloric intakes were calculated from measurements made each day between 5 and 6 p.m. Feed intake for animals fed a stock diet was determined weekly by weighing the feed, and daily caloric intakes were calculated.

Female mice were weighed weekly throughout the experimental periods. Mating procedures and maternal and fetal analyses were the same for both experiments and will be discussed in a later section.

Male mice were housed five per cage and received the stock diet and tap water ad libitum throughout the experimental period.

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*Purina Mouse Chow, St. Louis, Missouri.

**Mead-Johnson, Evansville, Indiana.

Table 1. Nutrient constituents of Sustacal liquid diet[1]

Nutrients	per 354.88 ml
Protein, g	21.7
Fat, g	8.3
Carbohydrates, g	49.6
Calories	360.0
Vitamin A, I.U.	1670.0
Vitamin D, I.U.	133.0
Vitamin E, I.U.	10.0
Vitamin C, mg	10.0
Folic acid, mg	0.133
Thiamin, mg	0.5
Riboflavin, mg	0.6
Niacin, mg	7.0
Vitamin B_6, mg	0.7
Vitamin B_{12}, µg	2.0
Biotin, mg	0.1
Pantothenic acid, mg	3.5
Calcium, g	0.36
Phosphorus, g	0.33
Iodine, µg	50.0
Iron[2], mg	6.0
Magnesium, mg	135.0
Copper, mg	0.7
Zinc, mg	5.0
Manganese, mg	333.0
Potassium, mg	740.0

[1]Values as reported by the manufacturer (Mead Johnson Laboratories, Evansville, Indiana).

[2]Sustacal prepared for Experiment 2 was prepared without added iron and contained 2 ppm as determined by direct analysis.

Table 2. Composition of liquid diets[1]

Ingredient (%)	Diets			
	0% EDC	10% EDC	20% EDC	30% EDC
Liquid base[2]	89.1	89.1	89.1	89.1
Ethanol[3]	0	3.64	7.26	10.9
Sucrose[4]	10.9	7.26	3.64	-

[1]The four liquid diets were isocaloric containing 127.25 kcal/100 ml.

[2]Sustacal liquid diet (Mead Johnson, Evansville, Indiana) containing 1 kcal/ml.

[3]63.3% v/v ethyl alcohol (3.5 kcal/ml).

[4]87% w/v sucrose (3.5 kcal/ml).

Table 3. Schematic representation of feeding schedule to acclimate mice to ethanol prior to mating

Weeks	Groups					
	A	B	C	D	E	F
1	Stock diet	0% EDC diet	0% EDC diet	0% EDC diet	0% EDC diet	0% EDC diet
2	↓	↓	10% EDC diet	10% EDC diet	10% EDC diet	10% EDC diet
3	↓	↓	↓	20% EDC diet	20% EDC diet	20% EDC diet
4	↓	↓	↓	↓	↓	30% EDC diet
5	↓	↓	↓	↓	↓	↓
6	↓	↓	↓	↓	↓	↓
7	↓	↓	↓	↓	↓	↓
8[1]	↓	↓	↓	↓	0% EDC diet	↓
	↓	↓	↓	↓	↓	↓
	End of experiment					

[1]Week mating was initiated.

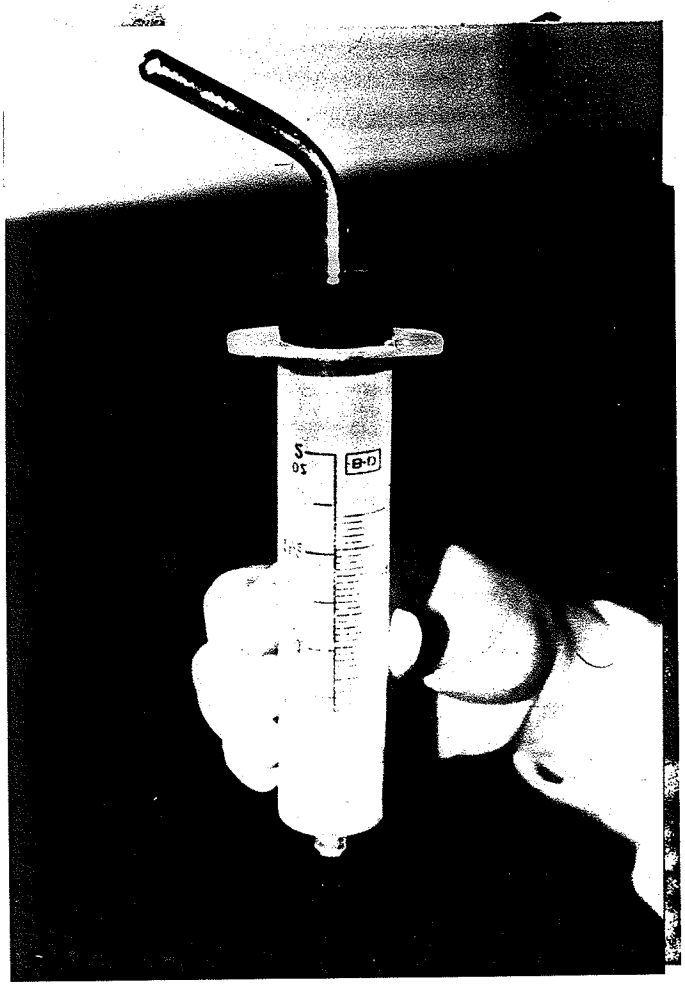

Figure 2. Design of feeding tube for administration of liquid diets.

2. Experiment 2

Sixty day old CBA/J female mice were randomly assigned to one of four groups (26 animals per group) according to the experimental design shown in Figure 3. The control group (CA) was maintained on an iron-adequate (30 ppm) liquid diet containing 0 percent EDC. Group CD received an iron-deficient (2 ppm) liquid diet that provided 0 percent of ethanol-derived calories. Group EA was fed an iron-adequate liquid diet providing 20 percent EDC. Group ED was maintained on a 20 percent EDC liquid diet deficient in iron. As in Experiment 1, the diets were isocaloric. For this experiment, Sustacal was specially prepared by the manufacturer to be low in iron; ferrous sulfate was added in our laboratory to formulate iron-adequate diets. The actual iron content of the liquid diets was determined by atomic absorption spectroscopy* (AOAC, 1970). In all other respects, the method of diet preparation was the same as in Experiment 1.

The system of feeding, acclimation to ethanol and weighing schedule were the same as earlier described. After the groups had been maintained on the respective diets for 4 weeks, but before the initiation of mating, five females were randomly selected from each group and were sacrificed to measure the effects of the experimental treatment on non-pregnant mice.

C. Specific procedures

1. Mating procedure

Mating was initiated in both experiments after females had received their experimental diets for at least 4 weeks and was restricted to a 3 hour period, starting at 8 a.m. During that time, the mating couple was deprived of food and water. Detection of a copulation plug signaled the

*Jarrell-Ash Model JA 82-50.

Iron Status

Ethanol derived calories	Adequate	Deficient
0%	CA 30 ppm Fe 0% EDC[1]	CD 2 ppm Fe 0% EDC
20%	EA 30 ppm Fe 20% EDC	ED 2 ppm Fe 20% EDC

Figure 3. Experimental design of Experiment 2.

[1]EDC, ethanol-derived calories.

first day of pregnancy. Another mating trial was conducted with the females who had a vaginal plug after first mating, but were not pregnant or with those without vaginal plugs after first mating. The second mating was initiated two weeks after initial failure.

2. Animal analysis and preparation of samples

On day 18 of pregnancy, the females were weighed and killed by cervical dislocation (The Universities Federation for Animal Welfare, 1976). Blood samples were collected directly from the heart for subsequent analyses. The uteri were removed and opened, and the number and location of living fetuses were examined for external malformations, blotted dry, and weighed. One or 2 fetuses from each litter were randomly selected for skeleton staining. The remainder of the fetuses were fixed in 10 percent formalin buffer solution, pH 7.0, for examination of internal malformations. Maternal livers were removed, blotted dry, and weighed. Half of the liver was fixed in 10 percent formalin buffer, pH 7.0, for histological staining and the other half was used for chemical analyses. In Experiment 2, the above procedures were also followed with the females who were sacrificed before mating.

D. Fetal examination

1. Skeleton staining

The selected fetuses were placed in a solution of 70 percent ethanol for four hours, removed, eviscerated, and returned to 70 percent ethanol for at least 24 hours. The fetuses were then stained using the alizarin red S stain method (Tipton and Burt, 1977). After staining, each fetus was placed in a separate vial containing a clearing solution consisting of equal parts of glycerol and 70 percent ethanol until it was examined.

Skeleton examination for anomalies was conducted under the dissecting microscope. The stained skeletons were compared to the normal one, as described by Rugh (1968) and shown in Figure 4, to determine missing bones.

2. Internal malformations

The fetuses selected for examination of internal anomalies were kept in the 10 percent formalin buffer fixative for a minimum of two weeks. The fetuses were then sectioned by freehand razor. Several transverse sections were made through the heads, trunks, and internal organs and the sections were examined for anomalies using the technique of Barrow and Taylor (1969).

E. Maternal examination

Hemoglobin, hematocrit, blood ethanol levels, liver alcohol dehydrogenase (ADH), and liver lipids were determined in both experiments. In addition, in Experiment 2, serum iron, total iron binding capacity (TIBC), percent saturation of transferrin, serum folate, red cell folate, and liver dihydrofolate reductase were determined.

F. Blood analyses

1. Hemoglobin

The cyanmethemoglobin procedure for determining hemoglobin content of whole blood was followed (Drabkin, 1949). Five ml of hemoglobin reagent* were placed into a test tube, 20 µl of whole blood were added, the resulting solution was mixed and the hemoglobin concentration was measured against known standards at 540 nm in a Gilford Spectrophotometer, Model Staser II**.

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*Hycel, Inc., Houston, Texas.

**Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

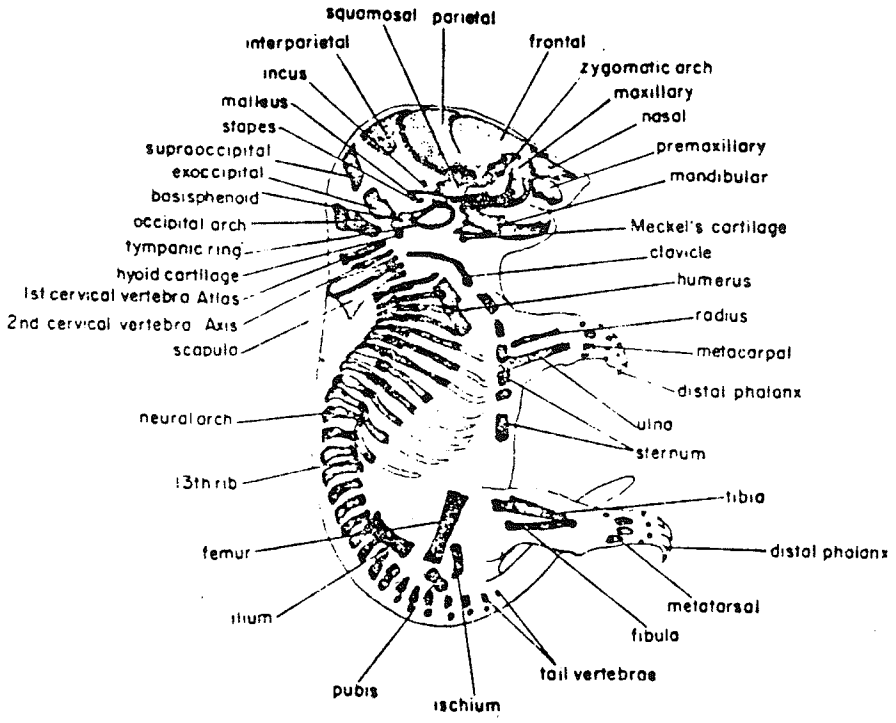

Figure 4. Skeletal system of mouse at 18 gestational days.

2. Hematocrit

Whole blood was drawn into a heparinized microcapillary tube, sealed, and centrifuged for four minutes. The percentage volume of packed red cells to that of whole blood was determined on a micro-Hematocrit Tube Reader*.

3. Blood ethanol

The gas chromtographic procedure of Freund (1967) was used. Blood was drawn directly from the heart during sacrifice into a heparinized micro-hematocrit capillary tube. Two microliters of blood were injected, using a 10 µl Hamilton syringe** into the gas chromatograph, Hy Fi Oven, Model 550-B***. The five foot column made of 0.125 inch O.D. stainless steel was packed with ethylvinylbenzene. A standard ethanol curve was constructed (2, 4, 6, and 8 ng/2 µl).

4. Serum iron and total iron binding capacity

Serum iron and total iron binding capacity (TIBC) were determined by the microtechnique of Caraway (1963). For serum iron determination, 100 µl serum were incubated with 1 percent ascorbic acid in 0.2 N HCl to liberate protein-bound iron. The protein was precipitated with trichloroacetic acid (20 percent) in the presence of chloroform and centrifuged for two minutes in a microcentrifuge at 10,000 rpm. One-hundred microliters of clear supernatant were transferred to a 6 x 50 mm tube. At this point, a blank and standard tube were included. To each tube, 10 µl of 0.1 percent of 2,4,6-tripyridyl-s-triazine (TPTZ) solution were added and mixed well. Then 10 µl of 40 percent ammonium acetate were added and mixed thoroughly.

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*The Drummond Scientific Company, Broomal, Pennsylvania.

**Hamilton Company, Reno, Nevada.

***Wilkin Instrument and Research, Inc., Walnut Creek, California.

The mixture was transferred to a Lowry-Bessey microcell. The absorbance of the blue colored complex was measured at 590 nm in a Beckman Model 25 Spectrophotometer*.

For TIBC, 50 µl of serum were saturated with ferric iron (500 µg/100 ml in 0.005 N HCl). The excess iron was removed by adsorption with magnesium carbonate. After this, measurement of the total bound iron was completed by employing the techniques used in the measurement of serum iron.

The percent saturation of transferrin was calculated as:

$$\frac{\text{Serum iron (µg/ml)}}{\text{TIBC (µg/ml)}} \times 100$$

5. Serum and red cell folate

Serum and red cell folates were determined according to the radioassay method of Longo and Herbert (1976). The principle of the assay is the competition of radioactive folate, tritiated folic acid ^{3}H-PGA and non-radioactive folate for a binding protein. ^{3}H-PGA, with 102 µCi/g** specific activity, was used after being diluted to 2.45 ng/ml with 0.05 M lysine-gelatin-sodium azide buffer, pH 9.3. Skim milk protein was used as a binding protein. A binding curve was constructed for each batch of skim milk. The milk was diluted to give 50 to 60 percent binding of ^{3}H-PGA. One-hundred microliters of diluted serum, 1:2, and 100 µl of diluted whole blood, 1:20, were used for serum and red cell folate determinations. Serum and whole blood were diluted on the day of sacrifice with 1 percent ascorbic acid and stored at -25°C for a period of two weeks. Previous studies indicated that serum samples diluted with ascorbic acid and stored at -25°C could be

*Beckman Instruments, Inc., Fullerton, California.

**Amersham Corporation, Arlington Heights, Illinois.

stored for two-six weeks prior to assay without loss of any folate activity (Tigner and Roe, 1979). On the day of sample assay, a standard folate curve was constructed (1, 2, 5, 10, and 20 ng/ml) using a log-logit transformation. The protocol of the assay is shown in Table 4. The sample radioactivity was counted in a Beckman Scintillation Counter, Model LS 9000*.

G. Liver analyses

1. Liver alcohol dehydrogenase activity

Activity of liver alcohol dehydrogenase (ADH) was determined in liver supernatant by the method of Vallee and Hoch (1955), where the rate of increase in absorbancy at 340 nm, as a result of NAD reduction, was measured in a Gilford Spectrophotometer Model 250**, as shown in the following reaction.

$$CH_3CH_2OH + NAD^+ \underset{}{\overset{ADH}{\rightleftharpoons}} CH_3CHO + NADH + H^+$$

In Experiment 1, ADH activity was calculated as the number of μmoles NADH formed/g liver/minute (unit activity), while in Experiment 2, ADH specific activity was determined as the number of nmoles NADH formed/mg soluble protein/minute, and μmoles NADH formed/g liver/minute (unit activity). The protein concentration of liver supernatant was determined by the method of Lowry et al. (1951).

Liver supernatant was prepared by homogenizing 0.5 g liver in 4.5 ml of 0.1 N potassium phosphate buffer, pH 7.5. The homogenate was then centrifuged at 30,000 rpm for an hour using an ultracentrifuge***. The

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*Beckman Instruments, Inc., Fullerton, California.

**Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

***Beckman Model L Ultracentrifuge, Beckman Instruments, Inc., Missouri.

Tabl . Protocol for radiometric assay for serum and d blood cell folacin

Tube no.	Lysine[1] buffer (µl)	Standard (µl)	Mice serum (µl)	Mice whole blood (µl)	Incubate	^{3}H-PGA[2] (µl)	Milk[3] binder (µl)	Incubate	Charcoal[4] (ml)	Incubate	Centrifuge
1,2	1400	-	-	-	Incubate all tubes at room temperature in dark for 15 minutes.	100	-	Cover tubes	-	Incubate all tubes at room temperature for 15 minutes.	Centrifuge all tubes (except 1 and 2) at top speed for 10 minutes. Clear supernatants were transferred to 10 ml/vial BCS liquid scintillation cocktail.
3,4	1000	-	-	-		100	-		0.4		
5,6	900	-	-	-		100	100		0.4		
7,8	800	100 A*	-	-		100	100		0.4		
9,10	800	100 B*	-	-		100	100		0.4		
11,12	800	100 C*	-	-		100	100		0.4		
13,14	800	100 D*	-	-		100	100		0.4		
15,16	800	100 E*	-	-		100	100		0.4		
Serum 1:1 or RBC in 1% ascorbic acid	800	-	100	100		100	100	Incubate all tubes at room temperature for 45 minutes.	0.4		
	-	Vortex	Vortex	-		Vortex	Vortex		Vortex		

[1]0.05 M lysine-gelatin-sodium azide buffer, pH 9.3.

[2](^{3}H-PGA) = 2.45 nm/ml.

[3]Skim milk diluted to bind 50-60% of tracer.

[4]Albumin coated charcoal.

*A, B, C, D and E = 1, 2, 5, 10, and 20 ng folacin/ml.

upper fat layer and the precipitate were discarded and the supernatant was used for determination of enzymatic activities.

2. Dihydrofolate reductase

The enzyme activity was measured in the liver supernatant by the method of Mathews et al. (1963). This assay is based on the measurement of the decrease in the absorbancy at 340 nm due to the disappearance of NADPH as shown in the following reaction:

$$H_2F + NADPH + H^+ \xrightarrow[\text{reductase}]{H_2F} H_4F + NADP^+$$

Dihydrofolate — Nicotinamide adenine dinucleotide phosphate (reduced form) — Tetrahydrofolate — (oxidized form)

The decrease in the absorbancy was measured with a Gilford Spectrophotometer Model 250*.

3. Liver lipids

Total lipids were determined by the method of Folch et al. (1957).

H. Statistical analyses

The statistical tests employed were General Analysis of Variance and chi square for Experiment 1 and Analysis of Variance (2 x 2 factorial), chi square, and correlation analyses (Steel and Torrie, 1960) for Experiment 2. In each experiment, the Least Significant Difference Test (LSD) was used to determine statistical differences among groups (Steel and Torrie, 1960). A probability value of $p < 0.05$ was taken at the level of significance.

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*Gilford Instrument Laboratories, Inc., Oberlin, Ohio.

CHAPTER IV

RESULTS AND DISCUSSION

The Effects of Chronic Ethanol Consumption on Both the Maternal and Fetal Organsim (Experiment 1)

A. Results

1. Body weight and daily caloric intake

Mean body weights, which were determined at the beginning of the experiment as well as before the initiation of mating and on day 18 of pregnancy, are presented in Table 5. Initial mean body weights were similar among all groups and ranged from 22.34 to 23.63 g. At the time of mating, when all groups receiving alcohol had been fed their respective levels of alcohol for at least 4 weeks, mean body weights were significantly lower in the groups fed either the stock diet or the diet providing 30 percent ethanol-derived calories (EDC) compared to the groups fed the diets providing 0, 10 or 20 percent EDC. These mean body weights were 31.19 (0% EDC), 30.82 (10% EDC), 29.89 (20% EDC), 25.92 (stock diet) and 27.59 g (30% EDC). There were no significant differences in the mean body weights among groups maintained on 0, 10 and 20 percent EDC diets prior to and during pregnancy. However, the mean body weight of the group maintained on 20 percent EDC prior to mating and 0 percent EDC during pregnancy (28.98 g) was significantly lower compared to the weights of 0 and 10 percent EDC groups, while it was comparable to groups fed 20 percent EDC and 30 percent EDC diets prior to and during pregnancy. When animals were sacrificed on day 18 of gestation, the mean body weight of the animals in the group fed 30 percent EDC was markedly lower (23.92 g) compared to the weights of all of the other groups. Mean body weights were 35.56 (stock diet), 38.55 (0% EDC), 39.65

Table 5. Effects of chronic ethanol consumption prior to and during pregnancy on mean body weight and daily caloric intake of maternal mice at day 18 of gestation[1]

	Dietary Treatments					
	Prior to Pregnancy					
	Stock diet (15)[3]	0% EDC[2] (14)	10% EDC (15)	20% EDC (15)	20% EDC (15)	30% EDC (13)
	During Pregnancy					
	Stock Diet (10)	0% EDC (10)	10% EDC (13)	20% EDC (13)	0% EDC (12)	30% EDC (8)
Body weights (g)						
At initiation of liquid diet	22.34 ± 2.35	22.93 ± 1.39	22.87 ± 2.25	23.63 ± 1.78	23.38 ± 1.78	23.08 ± 2.19
At mating	25.92 ± 1.90^{a}	31.19 ± 1.89^{b}	30.82 ± 3.77^{b}	29.89 ± 3.77^{b,c}	28.89 ± 2.53^{c,d}	27.59 ± 2.14^{a,d}
On day 18 of gestation	35.56 ± 7.54^{a}	38.55 ± 5.65^{a}	39.65 ± 5.69^{a}	36.52 ± 7.31^{a}	35.29 ± 5.35^{a}	23.92 ± 2.54^{b}
Daily caloric intakes (Kcal)						
4 weeks prior to mating	16.26 ± 2.33^{a}	18.15 ± 2.13^{b}	17.54 ± 2.33^{b,c}	17.15 ± 1.83^{c}	17.13 ± 1.60^{c}	14.53 ± 2.81^{d}
During pregnancy	16.89 ± 1.85^{a}	18.50 ± 1.16^{b}	18.11 ± 0.99^{a,b}	17.22 ± 0.98^{a,b}	18.16 ± 0.98^{a,b}	13.78 ± 1.44^{c}

[1]Results are expressed as mean ± SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2]EDC = ethanol-derived calories.

[3]Numbers of animals per group.

(10% EDC), 36.52 (20% EDC) and 35.29 g (20% EDC, discontinued during pregnancy).

Also shown in Table 5 are the daily caloric intakes during the 4 weeks prior to mating and during the 18 day gestational period. Mean daily caloric intakes prior to mating were significantly less in the groups maintained on the stock diet and 30 percent EDC diet than the mean intakes of all other groups. The difference in mean daily caloric intakes between the groups maintained on the stock diet and on 30 percent EDC was also statistically significant. No significant differences in daily caloric intakes were noted between groups fed 0 and 10 percent EDC diets or among those fed 10 percent and 20 percent EDC (continued or discontinued during pregnancy).

During pregnancy, animals receiving 30 percent EDC had a mean daily caloric intake that was significantly lower than those for groups fed either the stock diet or the other liquid diets. Mean caloric intakes of the groups during pregnancy were as follows (Kcal): 16.89 (stock diet), 18.50 (0% EDC), 18.11 (10% EDC), 17.22 (20% EDC), 18.16 (20% → 0% EDC) and 13.78 (30% EDC). No differences in daily caloric intakes were noted among groups receiving 0, 10 and 20 percent EDC during pregnancy. However, animals receiving 0 percent EDC had a mean daily caloric intake that was significantly greater than that for animals receiving the stock diet.

2. Maternal tissue analyses

a. Blood analyses

Mean hemoglobin concentration, hematocrit level and blood ethanol concentration on the day 18 of gestation are presented in Table 6. The hemoglobin concentration was significantly depressed only in animals fed

Table 6. Effects of chronic ethanol consumption prior to and during pregnancy on mean hemoglobin concentration, hematocrit level and blood ethanol concentration of maternal mice at day 18 of gestation[1]

	Dietary Treatments					
	Prior to Pregnancy					
	Stock Diet	0% EDC[2]	10% EDC	20% EDC	20% EDC	30% EDC
	During Pregnancy					
Blood parameter	Stock Diet (10)[3]	0% EDC (10)	10% EDC (13)	20% EDC (13)	0% EDC (12)	30% EDC (8)
Hemoglobin (g/100 ml)	18.40 ± 2.36[a]	16.88 ± 3.19[a]	17.62 ± 1.22[a]	17.48 ± 1.95[a]	16.86 ± 2.21[a]	14.32 ± 1.84[b]
Hematocrit (%)	34.75 ± 5.57	40.13 ± 4.11	39.38 ± 2.93	38.33 ± 0.97	38.18 ± 6.48	40.37 ± 6.75
Blood ethanol (mg/100 ml)	-	-	45.37 ± 16.88[a]	98.21 ± 82.99[a]	-	408.08 ± 190.00[b]

[1]Results are expressed as mean ± SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2]EDC = ethanol-derived calories.

[3]Number of animals per group.

the diet providing 30 percent EDC (14.32 g/100 ml blood) compared to the other groups (16.86 - 18.40 g/100 ml blood). Hematocrit levels did not differ among groups. Mean hematocrit values were 34.75, 40.13, 39.38, 38.33, 38.18 and 40.37 percent for stock diet, 0, 10, 20, 20 → 0 and 30 percent EDC groups, respectively. Mean whole blood ethanol concentrations in animals fed ethanol-containing diets were significantly higher in the 30 percent EDC group (408.08 mg/100 ml) than in the 10 and 20 percent EDC-fed groups (45.37 and 98.21 mg/100 ml, respectively). The difference in blood ethanol concentration between the 10 and 20 percent EDC groups was not statistically significant.

b. Liver analyses

Data on maternal liver weights, total lipids and alcohol dehydrogenase activity are summarized in Table 7. Maternal liver weights were significantly higher in the 10 and 20 percent EDC groups than in the stock diet group and the 30 percent EDC group. Mean liver weights were 2.63 (10% EDC) and 2.89 g (20% EDC) compared to 2.11 and 1.75 g in the stock diet and the 30 percent EDC groups, respectively. Mean liver weights of the groups receiving 0 percent EDC (2.42 g) and 20 percent EDC prior to mating (2.51 g) were comparable to all other groups.

Total lipid contents of the liver were significantly lower in the groups maintained on the stock diet (7.64 g/100 g) or the diet providing 30 percent EDC (7.09 g/100 g liver) compared to all of the other experimental groups (10.63 - 11.88 g/100 g liver).

No significant differences in alcohol dehydrogenase activity (ADH) were noted among groups maintained on the stock diet or the 0 or 10 percent EDC diets. Among the groups maintained on the liquid diets, the lowest

Table 7. Effects of chronic ethanol consumption prior to and during pregnancy on mean liver weight, total lipids, alcohol dehydrogenase activity and liver histopathology of maternal mice at day 18 of gestation[1]

	Dietary Treatments					
	Prior to Pregnancy					
	Stock Diet	0% EDC[2]	10% EDC	20% EDC	20% EDC	30% EDC
	During Pregnancy					
Liver parameter	Stock Diet (10)[3]	0% EDC (10)	10% EDC (13)	20% EDC (13)	0% EDC (12)	30% EDC (8)
Weight (g)	2.11 ± 0.70[a]	2.42 ± 0.55[a,b]	2.63 ± 0.64[b]	2.89 ± 0.72[b]	2.51 ± 0.48[a,b]	1.74 ± 0.13[a]
Total lipids (g/100 g liver)	7.64 ± 1.91[a]	10.74 ± 2.31[b]	11.49 ± 1.89[b]	11.88 ± 1.88[b]	10.63 ± 2.21[b]	7.09 ± 0.92[a]
Livers with fatty changes as determined histopathologically (No.)[4,5]	4	7	7	9	8	3
(%)	40.00	70.00	53.85	69.23	66.67	37.50
Alcohol dehydrogenase activity (μmoles/minute/g liver)	5.82 ± 1.62[a,b]	4.95 ± 2.08[a]	6.16 ± 1.66[a,b]	7.60 ± 2.44[b,c]	6.98 ± 0.83[b]	8.95 ± 1.83[c]

[1] Results are expressed as mean ± SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2] EDC = ethanl-derived calories.

[3] Number of animals per group.

[4] Number of livers examined for histological evidence of fatty infiltration.

[5] Chi square test indicated no significant difference in the number of livers with fatty changes among groups ($X^2 = 4.40$, $P > 0.05$).

mean alcohol dehydrogenase activity (4.95 μmoles/minute/g liver) was noted in the group receiving 0 percent EDC diet. The group fed the 30 percent EDC diet had a significantly higher alcohol dehydrogenase activity (8.95 μmoles/minute/g liver) than all other experimental groups, except the 20% EDC group.

The histological characteristics of the maternal livers are also presented in Table 7. Forty and 37.5 percent of the animals in groups maintained on the stock diet and the 30 percent EDC diet, respectively, had evidence of fatty changes compared to 70 (0% EDC), 53.85 (10% EDC), 69.23 (20% EDC) and 66.67 percent of animals (20% and 0% during pregnancy). However, these apparent differences in the percentages of fatty livers were not statistically significant ($X^2 = 4.41$). Nevertheless, histological examination revealed individual variations in each of the experimental groups. Some of the maternal livers were normal while the others showed various degrees of fatty changes as well as inflammatory and necrotic foci. The severity of fatty changes was greater in some individuals maintained on 20 percent EDC prior to and during pregnancy. Severity of fatty changes in maternal livers, as determined histologically was positively correlated with the values of total lipid contents of the maternal liver ($t = 2.38$, $P < 0.05$).

Figures 5 and 6 show photomicrographs of maternal livers of mice fed the stock diet and the 0 percent EDC diet, respectively. The livers were essentially normal. Figure 7 shows a photomicrograph of a liver from a mouse fed 10 percent EDC. The liver had a marked fatty change and inflammatory foci of polymorphonuclear leukocytes in opposition to necrotic hepatocytes. The liver of a maternal mouse fed 20 percent EDC is shown in Figure 8. The photomicrograph reveals extensive fatty changes. The

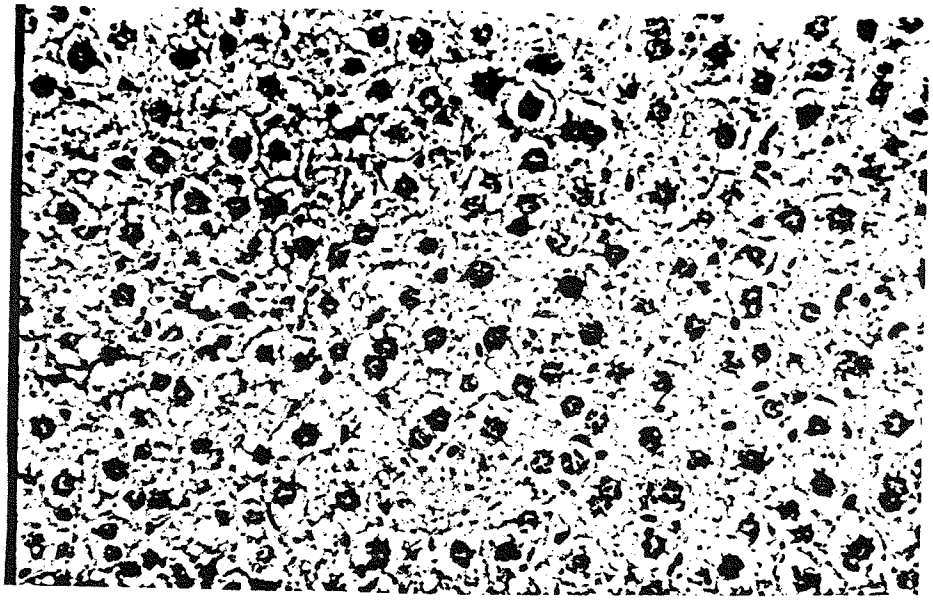


Figure 5. Photomicrograph of a maternal liver from an animal receiving the stock diet prior to and during pregnancy. Normal liver histology is evident (Hematolyline-eosin stain; x 400).

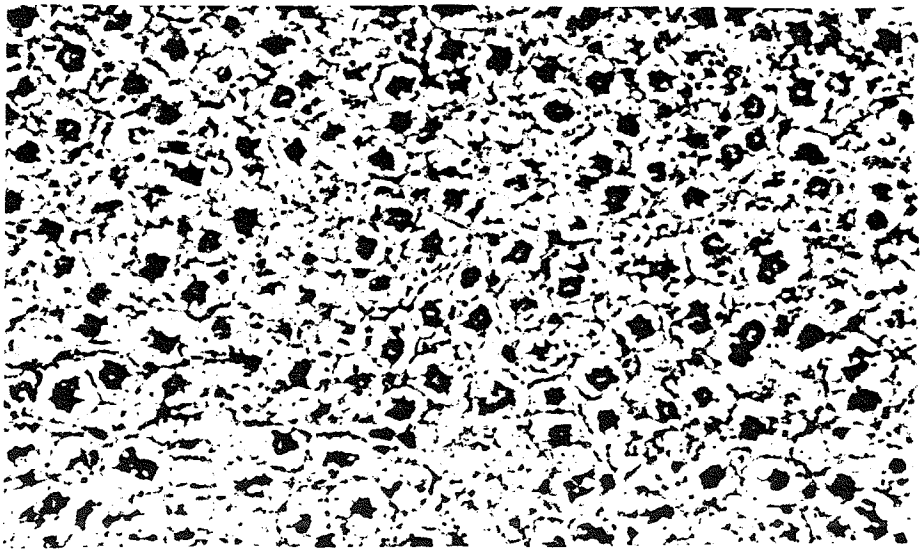


Figure 6. Photomicrograph of a maternal liver from an animal receiving a diet providing 0% EDC prior to and during pregnancy. Normal liver histology is evident (Hematoxyline-eosin stain; x 400).

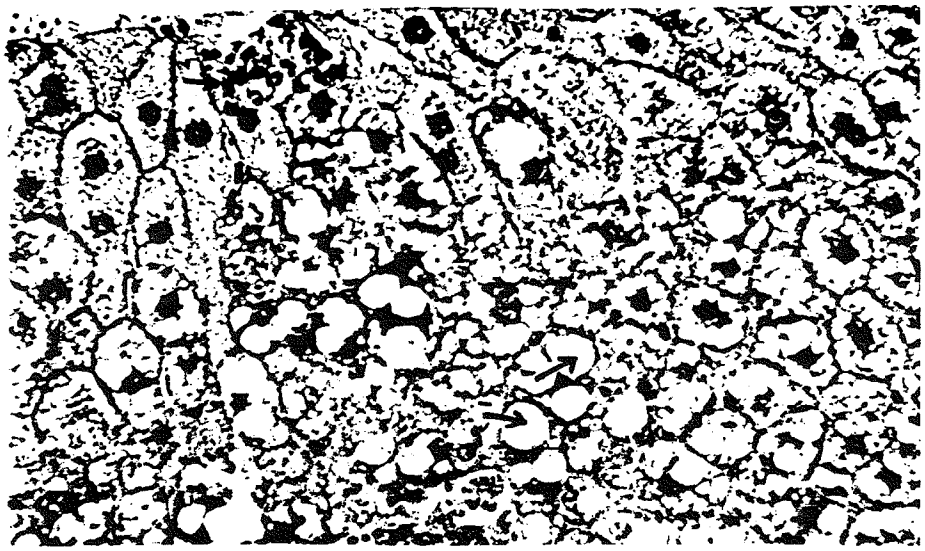


Figure 7. Photomicrograph of a maternal liver from an animal receiving a diet providing 10% EDC diet prior to and during pregnancy. Marked fatty change (arrow 1) and inflammatory foci of polymorphonuclear leukocytes in opposition to necrotic hepatocytes (arrow 2) are evident (Hematoxyline-eosin stain; x 400).

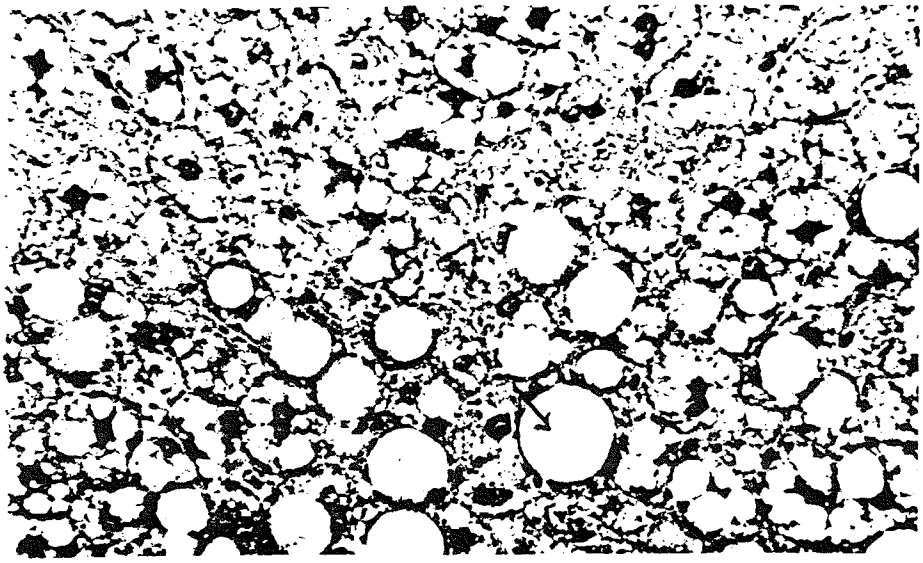


Figure 8. Photomicrograph of a maternal liver from an animal receiving a diet providing 20% EDC prior to and during pregnancy. Extensive fatty changes (arrow) is evident (Hematoxyline-eosin stain; x 400).

photomicrograph of a liver from a mouse fed 20 percent EDC diet prior to pregnancy and 0 percent EDC diet after mating reveals mild fatty changes, mild foci of necrosis and polymorphonuclear infiltration (Figure 9). The photograph of a liver from 30 percent EDC group (Figure 10) reveals mild degenerative changes, cloudy swelling primarily with one focus of necrotic hepatocytes and polymorphonuclear leukocytes.

3. Gestational performance of maternal mice

Data on the effect of alcohol consumption during pregnancy on the reproductive performance of maternal mice are presented in Table 8. Resorption of the entire litter occurred in all pregnant mice maintained on the 30 percent EDC diet. The number of total resorptions in this group was significantly greater than those of all other groups ($X^2 = 11.24$, $P < 0.05$). However, no significant differences were noted among the other groups ($X^2 = 2.31$).

No significant differences were noted in the mean numbers of implantations among groups. The mean numbers of implantations per dam ranged among groups from 8.29 - 10.92. The percentage of resorbed fetuses per litter was significantly lower in the group receiving the liquid diet providing 0 percent EDC when compared to all other groups. In contrast, the percentage for the group receiving the liquid diet providing 30 percent EDC was significantly higher than those for all other groups. The mean percentages of resorption of implanted fetuses per litter were 6.57 in the 0 percent EDC group compared to 31.94 (stock diet), 37.43 (10% EDC), 23.16 (20% EDC), 27.88 (20% → 0% EDC) and 100 percent (30% EDC).

As the alcohol content of the maternal diet increased, so did the percentage of dead fetuses per litter. The percentage of dead fetuses

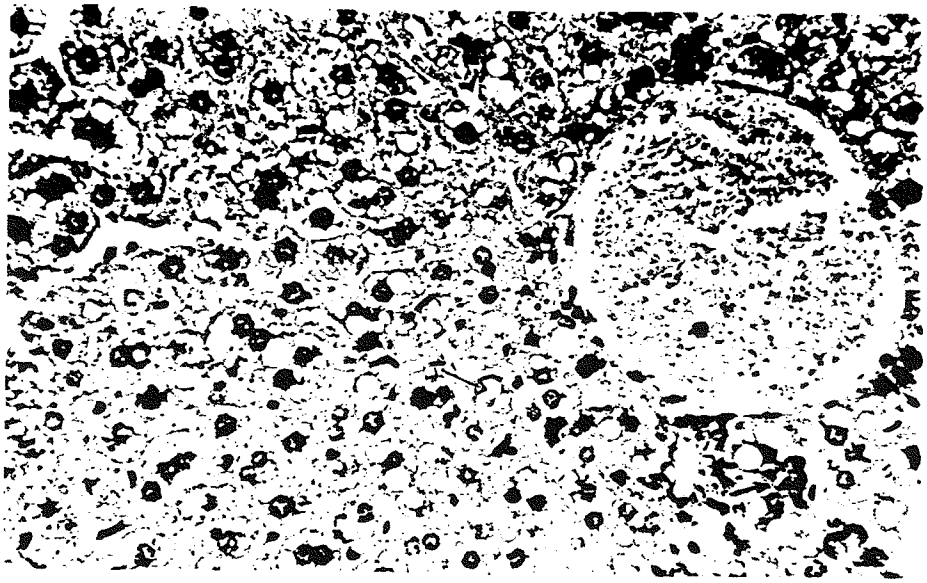


Figure 9. Photomicrograph of a maternal liver from an animal receiving a diet providing 20% prior to pregnancy and 0% EDC during pregnancy. Mild fatty changes (arrow 1) and mild focal necrosis and polymorphonuclear leukocytes infilerates (arrow 2) are evident (Hematoxyline-eosin stain; x 400).

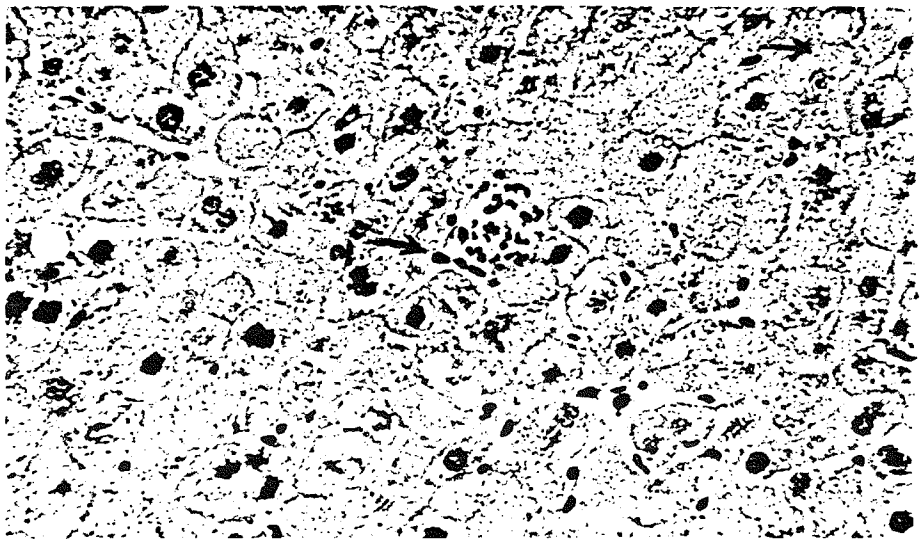


Figure 10. Photomicrograph of a maternal liver from an animal receiving a diet providing 30% EDC. Mild degenerative changes, cloudy swelling (arrow 1) and primarily one focus of polymorphonuclear leukocytes adjacent to a necrotic hepatocyte (arrow 2) are evident (Hematoxyline-eosin stain; x 400).

Table 8. The effect of chronic ethanol consumption prior to and during pregnancy on the reproductive performance of mice[1]

Dietary Treatment		Condition of pregnant females on day 18 of gestation[2]		Implantation Sites[1] (mean/dam)	Fetuses	Conditions Mean percentage/litter[1]		Mean Fetal Weight[1] (g)
Prior to pregnancy	During pregnancy	Females with live fetuses	Females with total resorption		Resorption %	Dead fetuses %	Live fetuses	
Stock diet (15)[3]	Stock diet (10)[3]	7	3	8.40 ± 1.90	31.94 ± 7.97[a]	1.69 ± 3.78[a]	66.63 ± 10.23[a]	0.81 ± 0.12[a]
0% EDC (14)	0% EDC (10)	6	4	9.80 ± 1.02	6.57 ± 5.02[b]	0.00 ± 0.00[a]	93.41 ± 5.17[b]	0.74 ± 0.14[b]
10% EDC (15)	10% EDC (13)	8	5	10.92 ± 2.22	37.43 ± 22.05[a]	4.55 ± 6.88[a,b]	58.00 ± 22.48[a]	0.61 ± 0.21[c]
20% EDC (15)	20% EDC (13)	8	5	9.69 ± 2.46	23.16 ± 12.12[a]	11.02 ± 12.63[c]	65.85 ± 10.58[a]	0.55 ± 0.16[c,d]
20% EDC (15)	0% EDC (12)	5	7	9.58 ± 1.69	27.88 ± 12.15[a]	9.62 ± 6.90[b,c]	61.54 ± 14.97[a]	0.51 ± 0.10[d]
30% EDC (13)	30% EDC (8)	0	8	8.29 ± 2.14	100.00 ± 0.0[c]	-	-	-

[1]Means within a column with unlike superscripts are significantly different at $P < 0.05$.

[2]Chi square tests indicated a significant difference between 30% EDC group and all of the other groups ($X^2 = 11.24$, $P < 0.05$).

[3]Number of animals per group.

[4]EDC = ethanol-derived calories.

per litter was significantly increased in the groups fed 20 percent EDC whether continued during pregnancy or not, compared to the groups fed either the stock diet or the diet providing 0 percent EDC. The percentages of dead fetuses per litter were 11.02 (20% EDC) and 9.62 (20% → 0% EDC), compared to 0.00 (0% EDC) and 1.69 percent (stock diet). The percentage of live fetuses per litter in the 0 percent EDC group was significantly higher than those of all other groups. Mean percentages of live fetuses per litter were 93.41 (0% EDC), 58.00 (10% EDC), 65.85 (20% EDC), 61.54 (20% EDC, discontinued) and 66.63 percent (stock diet).

The mean body weight of live fetuses was significantly higher in the group fed the stock diet (0.81 g) than those fed liquid diets. Among the groups fed liquid diets, fetal weights decreased significantly by increasing ethanol in the diet from 0.74 g (0% EDC) to 0.61 (10% EDC), 0.55 (20% EDC) and 0.51 g (20% → 0% EDC).

4. Gross morphological characteristics of fetuses

a. External and internal malformations

The number of fetuses examined for external and internal malformation and the percentage of fetuses with morphological abnormalities are presented in Table 9. No significant differences were noted between the fetuses examined from the stock diet group and the 0 percent EDC group ($X^2 = 0.67$). However, increased percentages of fetuses with external malformations were noted in the groups fed 10, 20 and 20 → 0% EDC, compared to the 0 percent EDC group ($X^2 = 62.14$, $P < 0.01$). Only 2.04 percent of fetuses from the 0 percent EDC group had morphological abnormalities compared to 15.69 (10% EDC), 65.21 (20% EDC) and 57.41 percent (20% → 0% EDC). The percent of external and internal malformation in the stock diet group was 5.36.

Table 9. Effects of chronic ethanol consumption prior to and during pregnancy on gross morphological characteristics of fetal mice

Dietary Treatments		Total number of fetuses examined	Number of malformed fetuses[2]	Percentage of malformed fetuses[1] (%)	Types of internal and external malformations[1]						
Prior to pregnancy	During pregnancy				Small[3] fetuses (%)	Open eye lids (%)	Wrinkled skin (%)	Internal hemorrhage (%)	Defective limbs (%)	Failure of abdominal wall to fuse (%)	Cleft Palate[4] (%)
Stock diet	Stock diet	38	2	5.36	5.36	0.00	0.00	0.00	0.00	0.00	0.00
0% EDC	0% EDC	49	1	2.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10% EDC	10% EDC	51	8	15.69	13.73	13.73	9.80	3.92	15.60	0.00	10.00
20% EDC	20% EDC	46	30	65.21	39.13	65.21	21.74	6.52	4.35	0.00	85.00
20% EDC	0% EDC	54	31	57.41	38.89	57.41	27.78	5.56	9.26	3.70	90.00
X^2 Values		-	62.14	-	28.51	76.26	18.32	3.22	9.91	5.46	55.31
p			< 0.01		< 0.01	< 0.01	< 0.01	> 0.05	< 0.05	> 0.05	< 0.01

[1]Results are expressed as a percentage of the total number of fetuses examined per group.

[2]Chi square test reveals no significant differences between the control groups fed the stock diet and 0% EDC, and significant differences among all other groups ($X^2 = 62.14$).

[3]Small fetuses were defined as those weighing less than 0.50 g.

[4]Only 20 fetuses/group were used to examine cleft palates.

Description and percentages of each of the external and internal malformations detected in live fetuses are likewise presented in Table 9. The morphological abnormalities noted were open eye lids ($X^2 = 76.26$, $P < 0.01$), small fetuses ($X^2 = 28.51$, $P < 0.01$), wrinkled skin ($X^2 = 18.32$, $P < 0.01$), internal hemorrhage ($P > 0.05$), limb defects ($X^2 = 9.91$, $P < 0.05$), failure of fussion of abdominal wall ($P > 0.05$) and cleft palate (55.31, $P < 0.01$).

b. Skeletal defects

Numbers of fetuses stained for bone examination and the percentages of those fetuses with skeletal abnormalities are presented in Table 10 along with a characterization of skeletal abnormalities. Some of the normal and abnormal skeletons found in experimental groups are photographically presented in Figures 11 to 15. Fetuses from litters of mothers who were maintained on the stock diet and the diet providing 0 percent EDC had few defective skeletons. The defects noted in these two groups were the absence of phalanges in 16.67 percent and a skull abnormality (8.33%). Significantly increased numbers of fetuses with bone defects were noted in the groups fed a diet containing ethanol at any phase of the experiment ($X^2 = 27.00$, $P < 0.01$). Percentages of the fetuses with bone defects were 50 percent in the 10 percent EDC group and 100 percent in the 20 percent EDC-fed groups whether alcohol consumption was continued or discontinued. Bones defects noted most frequently were incomplete ossification and missing bone from skulls ($P > 0.05$), pelvices ($X^2 = 8.0$, $P > 0.05$), sternum ($X^2 = 10.95$, $P < 0.01$), tail ($X^2 = 8.6$, $P < 0.05$), ribs ($P > 0.05$) and limbs ($X^2 = 27.00$, $P < 0.05$).

Figures 11 and 12 show photographs of fetal skeletons from groups fed the stock diet and the diet providing 0 percent EDC, respectively.

Table 10. Effects of chronic ethanol consumption prior to and during pregnancy on morphological characteristics of fetal skeleton[1]

Dietary Treatments											
Prior to pregnancy	During pregnancy	Total no. of stained skeletons	No. of[2] fetuses with defects	Percentage of fetuses	Small skeletons %	Skull %	Sternum %	Pelvic bones %	Limb %	Ribs %	Tail bones %
Stock diet	Stock diet	12	2	16.67	0	0	0	0	16.67	0	0
0% EDC[3]	0% EDC	12	2	16.67	0	8.33	0	0	16.62	0	0
10% EDC	10% EDC	12	6	50.00	41.67	25.00	16.67	16.67	50.00	0.0	8.33
20% EDC	20% EDC	12	12	100.00	58.33	58.33	41.67	41.67	100.00	25.00	41.67
20% EDC	0% EDC	12	12	100.00	41.67	41.67	0.00	41.67	100.00	16.67	33.33
X^2 Values		-	27.00	-	9.80	7.5	10.95	8.0	27.00	6.26	8.6
p			< 0.01		< 0.05	P > 0.05	< 0.05	< 0.05	< 0.01	> 0.05	< 0.05

[1] Results are expressed as percentages of malformation among the examined fetal skeletons.

[2] Chi square test revealed a significant difference among the groups (X^2 = 27.00).

[3] EDC = ethanol-derived calories.

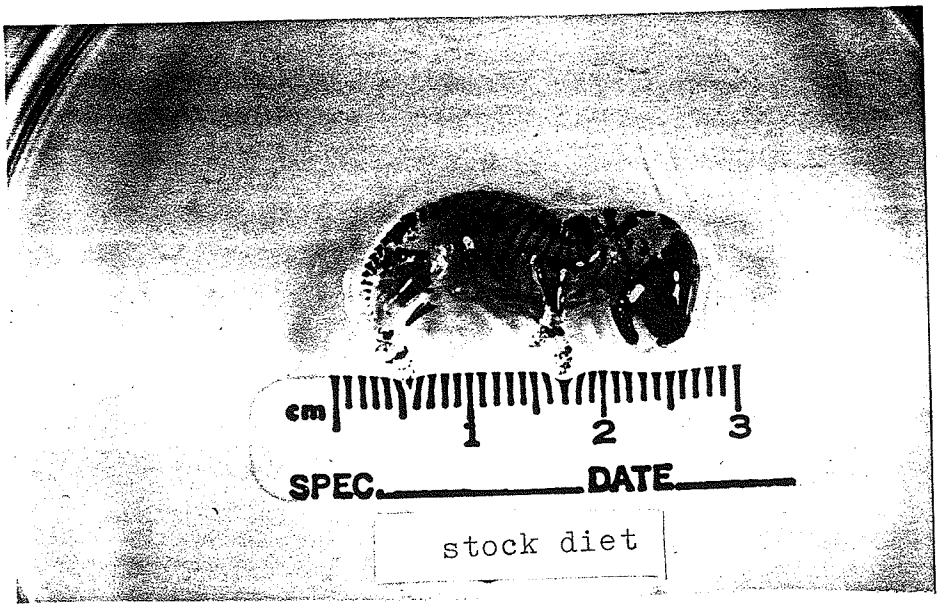


Figure 11. Photograph of a fetal skeleton from an animal receiving stock diet prior to and during pregnancy. Normal skeleton is evident (Alizarin Red S stain).

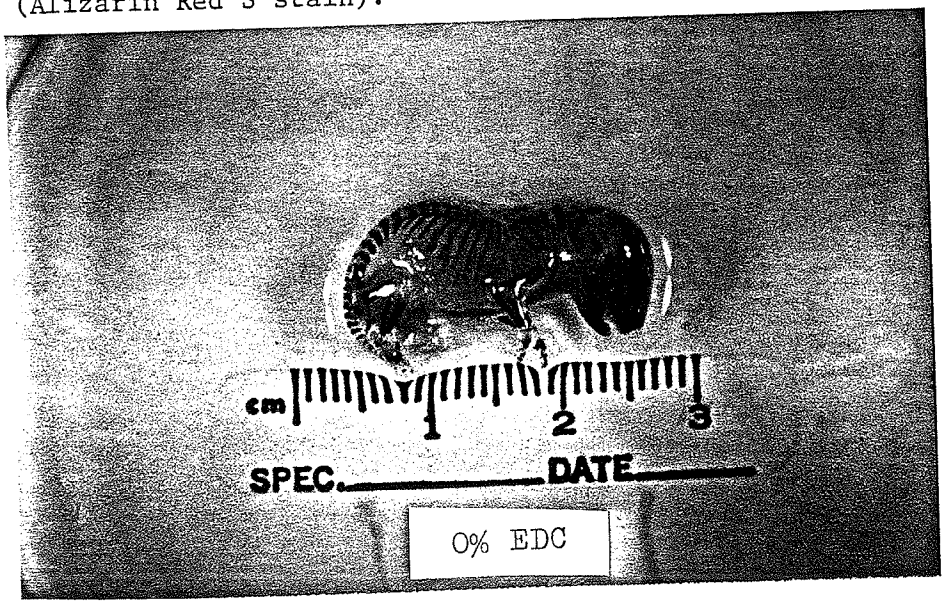


Figure 12. Photograph of a fetal skeleton from an animal receiving 0% EDC diet prior to and during pregnancy. Normal skeleton is evident (Alizarin Red S stain).

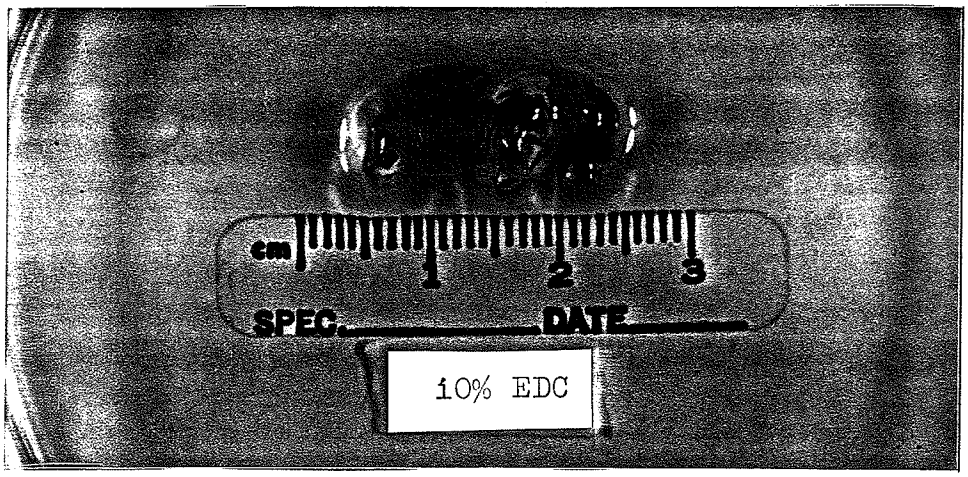


Figure 13. Photograph of a fetal skeleton from an animal receiving 10% EDC diet prior to and during pregnancy. Missing phalanges, metacarpal and metatarsal bone are evident (Alizarin Red S stain).

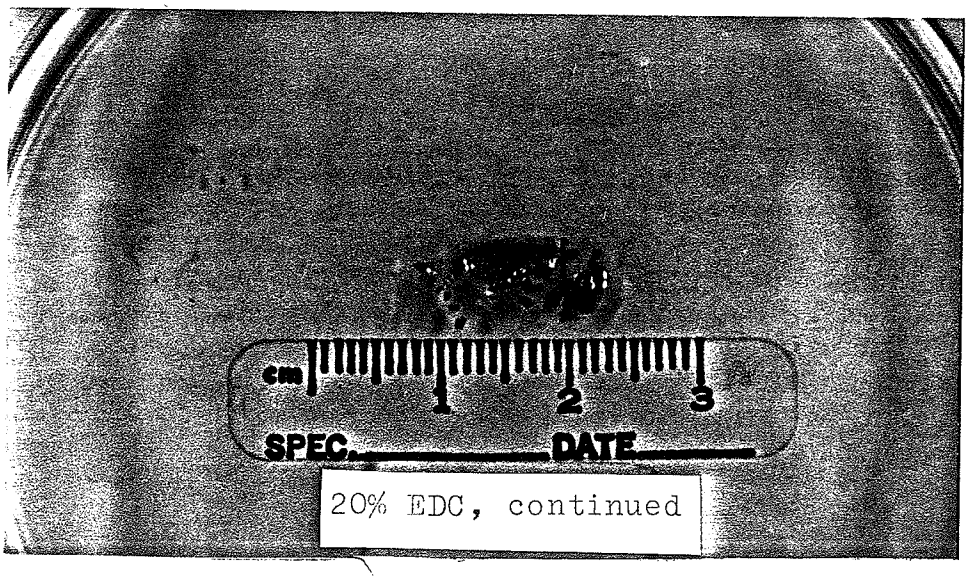


Figure 14. Photograph of a fetal skeleton from an animal receiving 20% EDC diet prior to and during pregnancy. Incomplete ossification of skull bones and missing phalanges are evident (Alizarin Red S stain).

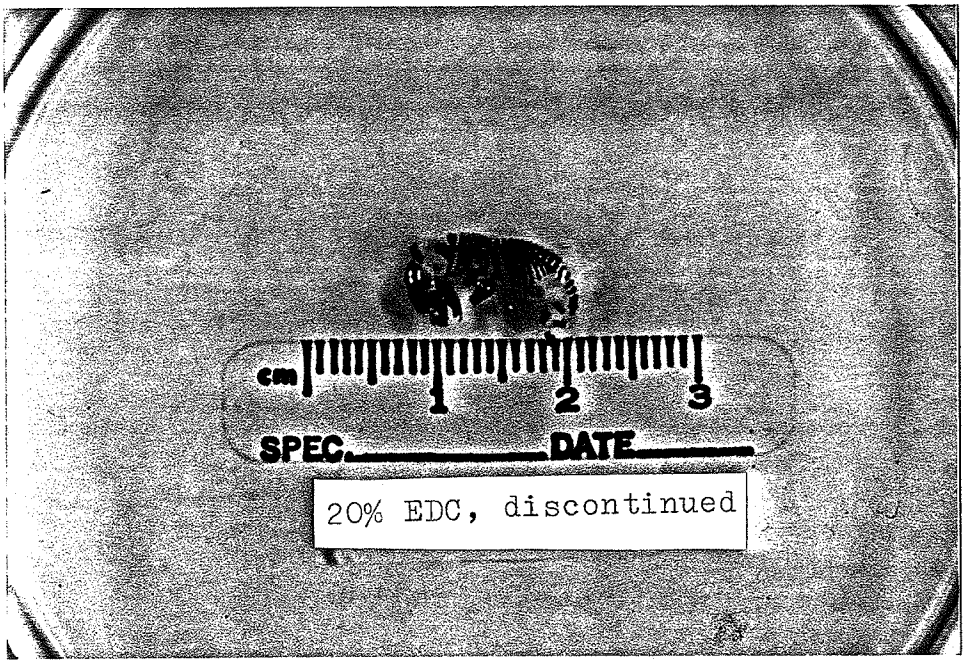


Figure 15. Photograph of a fetal skeleton from an animal receiving 20% prior to pregnancy and 0% EDC during pregnancy. Small size, incomplete ossification of skull bones, missing phalanges and metacarpal and metatarsal bones are evident (Alizarin Red S stain).

The skeletons were essentially normal. Figure 13 shows a photograph of a fetal skeleton from the group fed the diet providing 10 percent EDC. Missing phalanges, metacarpal and metatarsal bones are evident. Fetal skeletons from the group fed 20 percent EDC prior to and during pregnancy and the group fed 20 percent EDC prior to and 0 percent EDC during pregnancy (Figures 14 and 15, respectively, show incomplete ossification of the skull bones and missing metacarpal, metatarsal and phalanges bones.

5. Histological characteristics of fetal livers

Some of the fetal livers were examined histologically, but the number of fetuses examined were too few to do statistical analysis. Livers of fetuses from groups fed the stock diet and the diets providing 0 and 10 percent EDC were essentially normal (Figures 16, 17 and 18, respectively). These photomicrographs reveal normal hematopoietic activity. Some of the fetal livers from the groups fed 20 percent EDC, whether continued or discontinued during pregnancy, show a marked dimunition of hematopoietic activity (Figures 19 and 20, respectively).

B. Discussion

Recent reports indicate that alcohol use by pregnant women is associated with adverse effects on the offspring (Jones et al., 1973; Jones and Smith, 1975; and Hanson et al., 1978). However, the mechanisms through which the effects are produced remain unclear. The question of whether it is the direct toxic effect of ethanol on fetuses as suggested by Jones and Smith (1975), Chernoff (1977) and Mulvihill and Yeager (1977), or indirectly caused through nutritional imbalances as a result of ethanol consumption as suggested by Davidson (1975) and Lin et al. (1978) is unanswered. It is generally accepted that alcoholism and pregnancy,

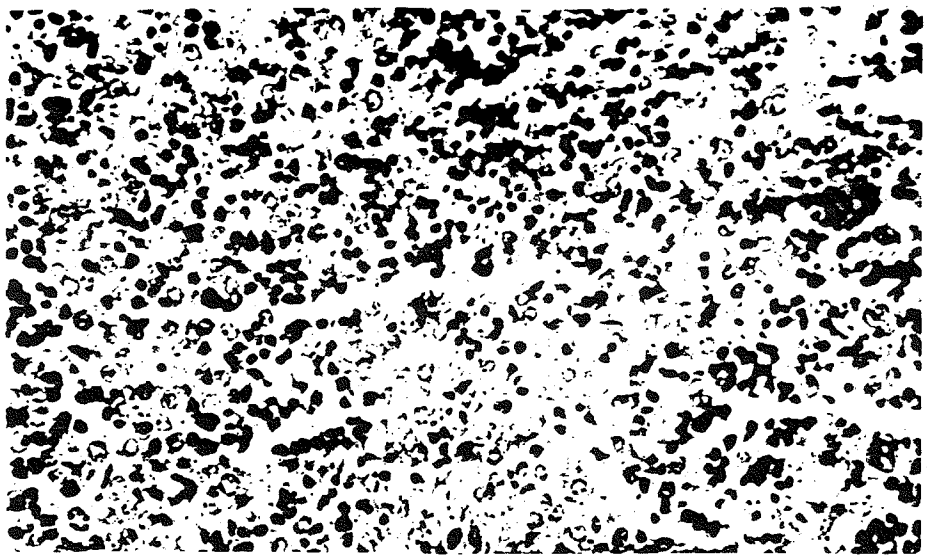


Figure 16. Photomicrograph of a fetal liver from an animal receiving stock diet prior to and during pregnancy. Normal hematopoietic activity is evident (Hematoxyline-eosin stain; x 400).

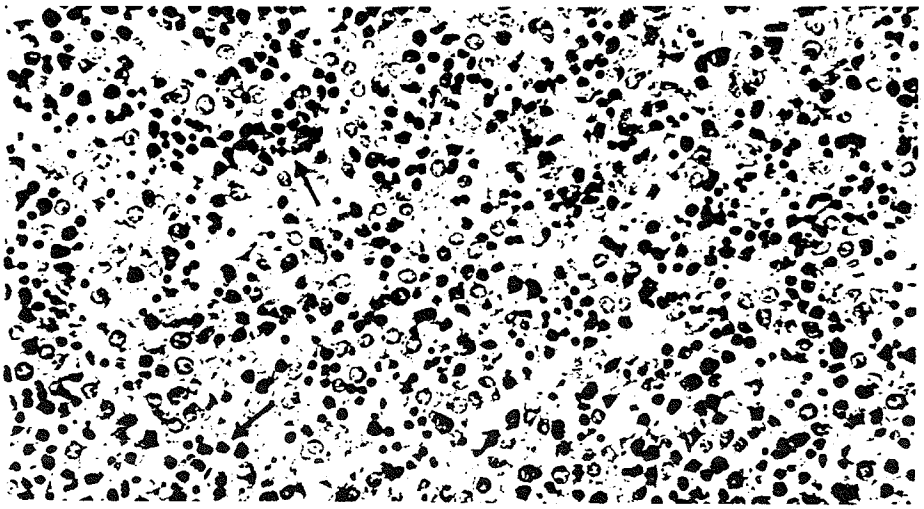


Figure 17. Photomicrograph of a fetal liver from an animal receiving a diet providing 0% EDC prior to and during pregnancy. Normal hematopoietic activity is evident (Hematoxyline-eosin stain; x 400).

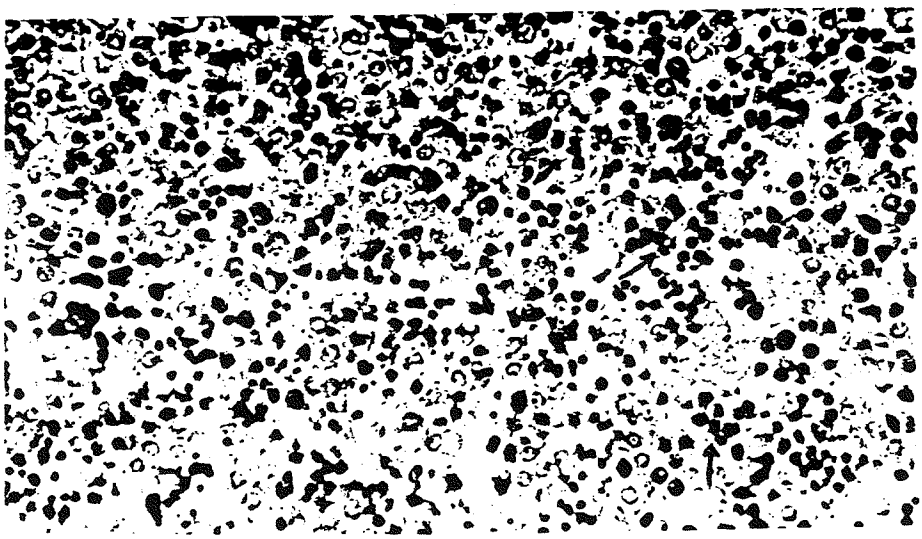


Figure 18. Photomicrograph of a fetal liver from an animal receiving a diet providing 10% EDC prior to and during pregnancy. Normal hematopoietic activity is evident (Hematoxyline-eosin stain; x 400).

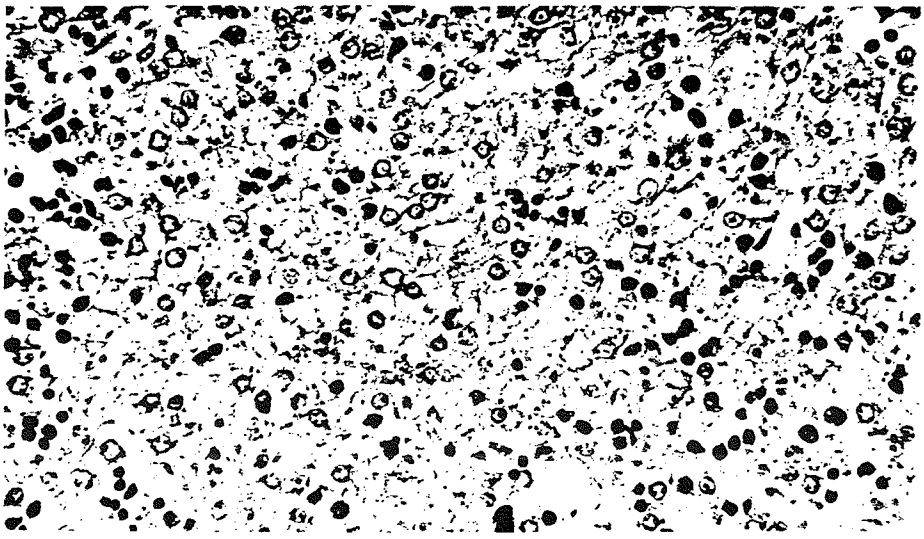


Figure 19. Photomicrograph of a fetal liver from an animal receiving a diet providing 20% EDC prior to and during pregnancy. A marked dimunition of hematopoietic activity is evident (Hematoxyline-eosin stain; x 400).

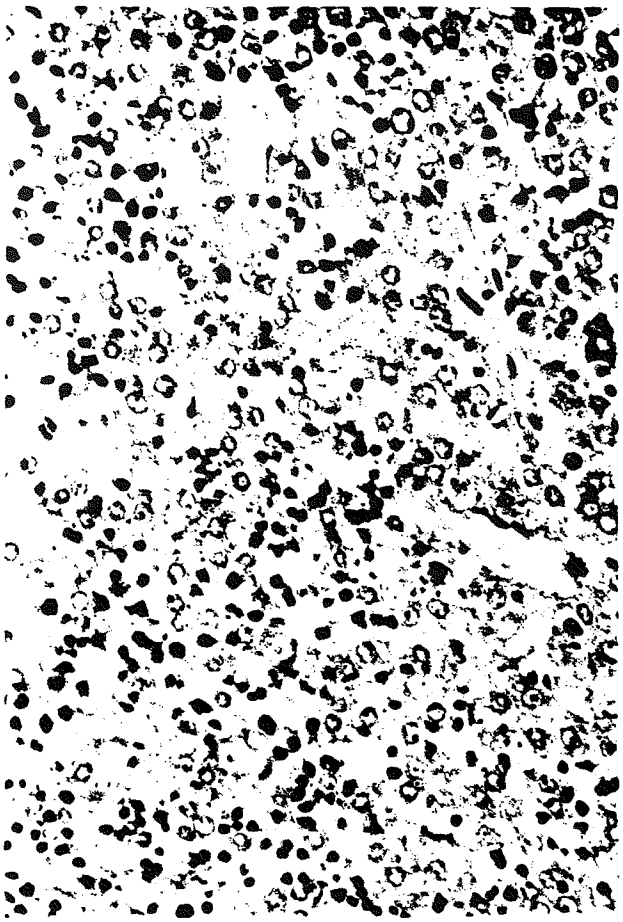


Figure 20. Photomicrograph of a fetal liver from an animal receiving a diet providing 20% prior to pregnancy and 0% EDC during pregnancy. A marked dimunition of hematopoietic activity is evident (Hematoxyline-eosin; x 400).

particularly among poor women, are frequently associated with nutritional imbalance and deficiencies (Herbert et al., 1963; Ouellette et al., 1977; and Roe, 1979).

In Experiment 1, the effect of maternal alcoholism, when nutritionally-adequate diets were fed, was determined. Results showed that the mean body weight of mice fed a liquid diet providing 30 percent of the calories from ethanol, was lower than those on a liquid diet without ethanol prior to pregnancy and on day 18 of gestation. Although significant, slight decreases in the body weights of animals maintained on the stock diet and the diet providing 20 percent EDC prior to mating, but discontinued during pregnancy, were observed in the prepregnancy period compared to the 0 percent EDC diet-fed group, no other changes in body weights were noted (Table 5). Other investigators (Kronick, 1976; and Tze and Lee, 1975) noted decreases in maternal body weights. However, these investigators used different methodology. Kronick (1976) injected pregnant mice intraperitoneally with ethanol during the period of organogenesis, while Tze and Lee (1975) introduced ethanol to rats in drinking water.

In the present study, significant decreases in daily caloric intakes prior to and during pregnancy were observed in the animals fed 30 percent EDC (Table 5). Significantly lower daily caloric intakes on the stock diet compared to the 0 percent EDC diet also occurred. A significant decrease in the daily caloric intake was noted in the groups fed 20 percent EDC prior to pregnancy, but no other differences in daily caloric intakes were observed among the groups fed 0, 10 and 20 percent EDC (continued or discontinued during pregnancy). The decreases in daily caloric intakes of the group fed 20 percent EDC prior to pregnancy only were probably due

to animal acclimation to ethanol. In contrast to these findings, Chernoff (1977) noted no differences in daily caloric intake prior to pregnancy in mice fed 30 percent EDC or a stock diet compared to those fed 0, 15, 20 and 25 percent EDC diets. Tze and Lee (1975) also noted no changes in daily caloric intakes of pregnant rats given ethanol in the drinking water. The results in Experiment 1 showed that mice ingested more calories on the liquid diet (0% EDC) than on the stock diet and that ethanol in the highest concentration (30% EDC) depressed the daily caloric intake of mice. Therefore, it can be concluded that any changes in maternal and fetal mice fed 10 and 20 percent EDC, continued or discontinued during pregnancy, are due to ethanol ingestion. While, in the case of the 30 percent EDC diet, the changes might be due to direct ethanol effects and/or decreases in daily caloric intakes and resulting nutritional imbalances.

The mean hemoglobin concentration of maternal blood was significantly decreased only in the group fed 30 percent EDC (Table 6). Low levels of blood hemoglobin were found in patients admitted to the hospital for treatment of alcoholism (Herbert et al., 1963). In the present study, no significant differences in hematocrit percentages were noted among any of the groups. A marked increase in blood ethanol level was observed in the pregnant mice maintained on 30 percent EDC. This increase in blood ethanol level was approximately ten times that obtained for the group fed 10 percent EDC and five times that observed in the group fed 20 percent EDC. However, the difference in blood ethanol concentration between the 10 and 20 percent EDC groups was not statistically significant.

As shown in Table 7, maternal liver weights were significantly higher in animals fed ethanol at levels of 10 and 20 percent of the kilocalories

prior to and during pregnancy than in those fed a stock or 30 percent EDC diet. The lower liver weights for the latter group might be in part attributed to the differences in the amount of fat accumulated in the livers and the degenerative changes as evidenced by liver necrosis in the 30 percent EDC group. Earlier, Mirone (1965) noted an increase in the liver weights of male rats drinking 15 percent ethanol in water.

Lieber (1975) found that fatty livers were common among alcoholics. In advanced cases, more cases of liver cirrhosis were detected. However, Leevy et al. (1975) indicated that the pattern of alcohol intake was not a critical determinant of alcoholic liver cirrhosis. Leiber (1975) proposed that ethanol consumption caused fatty livers because its metabolism replaced fatty acids as a normal fuel for the hepatic mitochondria. Thus, a decrease in lipid oxidation and a concomitant increase in fat accumulation was responsible. Also, the excess hydrogen generated by ethanol oxidation could be used to synthesize more lipid. Results from Experiment 1 support a direct link between ethanol ingestion and fatty accumulation and eventual necrosis of livers. Higher fat concentrations were found in the pregnant animals fed 10 and 20 percent EDC. On the other hand, less fat accumulated in the maternal livers of animals fed stock and 30 percent EDC diets. Although no significant differences were noted in the number of animals within the groups with fatty livers as determined by histological examination (Table 7), a positive correlation was obtained between the values of total lipids as measured chemically and the intensity of fatty changes. The severity of fatty changes was obvious in the 20 percent EDC group (Figure 8). Evidence of inflammation and necrotic foci was observed in maternal livers of the 30 percent EDC group (Figure 10). The

degree of fatty change in the 30 percent EDC group was less marked. These results suggested that ethanol induced pathological changes in maternal livers that in turn might affect liver metabolism. In contrast to these findings, Chernoff (1977) noticed no pathological changes in liver sections among mice fed liquid diets with 10, 15, 25 and 30 percent EDC. However, histological examination was performed on livers of animals prior to mating and not subsequent to gestation.

In Experiment 1, liver alcohol dehydrogenase (ADH) activities in the ethanol-fed groups increased with increasing ethanol level in the diet. The highest value was observed in the 30 percent EDC group (Table 7). The relationship between ADH activity, the main enzyme of ethanol oxidation, and alcohol ingestion is a controversial issue in the literature. Some investigators notedan increase in ADH activity during chronic ethanol ingestion (Raskin and Sokoloff, 1970; Mirone, 1965; Dajani et al., 1963; and Sze et al., 1976) which was confirmed by the present data. On the other hand, Figueroa and Klotz (1962) and Greenberger et al. (1965) failed to note any significant change in ADH activity with alcohol ingestion. These differences in reported ADH activity are probably due to the differences in species, sex and physiological status of the laboratory animals as well as ethanol dose and duration of ethanol intake.

Data from the present experiment indicate that liquid diets can be used successfully to study the effects of maternal alcoholism in mice. Essentially normal gestational performance was observed in the group maintained on a liquid diet providing no ethanol (0% EDC) compared to mice on a stock diet (Table 8). The number of implantation sites, dead fetuses and external, internal and skeletal abnormalities were not significantly

different between the group fed a stock diet and the group receiving 0 percent EDC. However, a lower percentage of implantations was resorbed in the group receiving 0 percent EDC than in the group receiving the stock diet while the weights of living fetuses were lower in the 0 percent EDC group than in the stock diet group (mean fetal weight was 0.81 g in the stock diet versus 0.74 g in the 0% EDC group). Chernoff (1977) found no significant differences in fetal weight between animals fed a stock diet and those fed 0 percent EDC. Liquid diets are now widely used for the study of the ethanol metabolism in experimental animals (Lieber, 1975; Chernoff, 1977; and Boggan et al., 1979).

Among the groups fed liquid diets in the present study total reproductive failure occurred in the dams fed a diet providing 30 percent EDC, even though they had a similar number of implantation sites per dam as the other groups. Chernoff (1977) noticed similar reproductive failure in mice among those fed 30 percent EDC. In Experiment 1, the implantation sites per dam were not significantly different among the groups fed 0, 10 and 20 percent EDC. By day 18 of gestation, significant numbers of implantation sites were resorbed in mice fed ethanol-containing diets. Also, the weights of viable fetuses were lower in ethanol-fed groups (10 and 20% EDC) than in the 0 percent EDC group. In addition, high percentages of fetuses had morphological abnormalities in these two ethanol-fed groups. With the 20 percent EDC group, a higher percentage of dead fetuses and increased numbers of morphological abnormalities (external, internal and skeletal) were observed (Tables 8 and 9). In the study with mice (Chernoff, 1977) in which 0, 15, 20, 25 and 30 percent EDC were fed prior to and during pregnancy, similar results were obtained. In the present experiment, the

principal external and internal malformations observed in the 10 and 20 percent EDC groups were mainly small body size, open eye lids, cleft palates and internal hemorrhages, while skeletal abnormalities noted were in the skull, pelvic bones, limb, tail and sternum bones. These results supported previous findings by Kronick (1976) and Chernoff (1977). Studies with human beings indicated that light and moderate ethanol consumption by pregnant women resulted in low birth weights and a small degree of congenital anomalies (Little, 1977; and Hanson et al., 1978). Also, heavy ethanol consumption in humans was reported to result in a high degree of congenital anomalies in the offspring (Jones et al., 1973; and Ouellette et al., 1977).

Ethanol consumption prior to mating (20% EDC) and abstinence during pregnancy was studied in Experiment 1 as well. Ethanol withdrawal during gestation did not completely reverse all indications of abnormal gestational performance produced by feeding 20 percent EDC prior to and during pregnancy (Tables 8, 9 and 10). A high percentage of resorptions, a high number of dead fetuses per dam, a low percentage of viable fetuses and low fetal weights were observed in the group receiving 20 percent EDC prior to pregnancy only. Similar numbers and types of morphological defects were noted in the 20 percent EDC group that continued ethanol consumption during pregnancy. In support of this finding, histological examination of some fetal livers from the groups fed 20 percent EDC, whether continued or discontinued during pregnancy, revealed decreased hematopoietic activity (Figures 19 and 20). However, the number of livers examined was not sufficient to do statistical analysis. In view of the significant abnormalities in the gestational performance of the pregnant mice maintained on ethanol-containing diets

prior to and during pregnancy, it is concluded that ethanol is teratogenic to mice. Moreover, ethanol withdrawal immediately before conception does not minimize the risk of excessive ethanol ingestion.

In the retrospective study by Little (1980), infants born to alcoholic women who reportedly abstained during pregnancy had lower birth weights than those born to women who did not drink alcohol. Thus, excessive alcohol consumption prior to pregnancy in reproductively active women may represent a hazard to the fetus. Although studies using laboratory animals (Tze and Lee, 1975 and Kronick, 1976) all indicated teratogenicity of ethanol, the morphological abnormalities were not consistent. This can be attributed to the differences in the periods of gestation during which ethanol was given, species or strain of laboratory animals, route and dose of ethanol given. Another important consideration is the fact that kilocaloric intakes from sources other than ethanol were not completely controlled in these aforementioned studies. Thus, the possibility of concomitant nutritional imbalances as the teratogenic agent cannot be ignored.

In general, the results of Experiment 1 showed that the degree of the effect varied as a result of the level of ethanol in the diet. Total reproductive failure occurred in these animals fed a diet containing 30 percent EDC. Teratogenic effects were even present when ethanol was withdrawn prior to mating. Chronic maternal alcoholism also affected the maternal organism. The effects were severe in the case of animals fed a high ethanol diet (30% EDC), where body weight gains and daily caloric intakes were low. Also, found in this latter group were high blood ethanol levels, low hemoglobin concentrations and necrosis of the liver. These findings illustrate the extreme toxicity of ethanol fed at this level.

Particularly noteworthy findings are the low maternal hemoglobin level in the group fed 30 percent EDC, the histological evidence suggesting altered hematopoiotic activity of fetal livers from the 20 percent EDC groups. These findings suggested altered hematopoiesis in both the maternal animal and her conceptus that might either be due to direct ethanol toxicity or be mediated through altered metabolism of iron and folate. Thus, Experiment 2 was designed to examine the possible roles of iron and folate nutrition in mediating the adverse effects of alcohol consumption during pregnancy on both maternal and fetal mice. These nutrients are of special interest because they were reported (Herbert et al., 1975; and Prasad, 1978) to be limiting in the diets of pregnant women both nationally and internationally.

CHAPTER V

Effects of Iron Deficiency and/or Ethanol Consumption on Maternal and Fetal Mice (Experiment 2)

A. Results

1. Prepregnancy period

a. Body weight and daily caloric intake

Mean body weights at the beginning of the experiment and just prior to mating as well as mean daily caloric intakes during the 4 week period prior to mating are shown in Table 11. Analysis of variance statistics (2 x 2 factorial design) are presented in Appendix 1. Mean body weights at the beginning of the experiment were similar among all groups and ranged from 18.6 to 21.0 g. At the time of mating, mean body weights were significantly lower for the groups fed diets deficient in iron and/or containing ethanol than the mean body weight of the control group which was fed an iron-adequate diet without ethanol. Analysis of variance statistics showed a significant iron-ethanol interaction on body weight. Mean body weights at the time of mating were 29.4 g for animals maintained on an iron-adequate diet which provided no ethanol (CA), 26.4 g for animals fed an iron-deficient diet without ethanol (CD), 26.0 g in the group maintained on a diet similar to that of the CA group but providing 20 percent ethanol-derived calories, EDC (EA) and 25.6 g in the group maintained on an iron-deficient diet providing 20 percent EDC (ED). Mean daily caloric intakes prior to mating are similar among all groups. Mean daily caloric intakes ranged from 17.2 to 17.9 Kcal.

b. Tissue analyses

i. Liver

Data on liver weights, total lipids, alcohol dehydrogenase (ADH) and dihydrofolate reductase activities are presented in Table 12 and

Table 11. Effects of iron deficiency and/or ethanol consumption on mean body weight and daily caloric intake of mice prior to pregnancy[1]

	Dietary Treatments			
	CA 30 ppm Fe 0% EDC[2] (22)[3]	CD 2 ppm Fe 0% EDC (22)	EA 30 ppm Fe 20% EDC (22)	ED 2 ppm Fe 20% EDC (22)
Body weight				
At initiation of liquid diet (g)	20.94 ± 1.76	18.60 ± 2.29	19.10 ± 1.21	20.98 ± 1.42
At mating (g)[4]	29.41 ± 2.58[a]	26.42 ± 2.88[b]	26.03 ± 1.87[b]	25.65 ± 1.97[b]
Daily caloric intake (Kcal) 4 weeks prior to mating	17.90 ± 0.25	17.81 ± 0.87	17.29 ± 1.18	17.23 ± 1.24

[1]Results are expressed as means ± SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2]EDC, ethanol-derived calories.

[3]Number of animals/group.

[4]Analysis of variance statistics showed a significant interaction between iron deficiency and ethanol consumption on body weight at $P < 0.05$.

Table 12. Effects of iron deficiency and/or ethanol consumption on liver weight, total lipids, alcohol dehydrogenase (ADH) and dihydrofolate reductase activities of mice prior to pregnancy

Liver parameter	Dietary Treatments			
	CA 30 ppm Fe 0% EDC[2] (5)[3]	CD 2 ppm Fe 0% EDC (5)	EA 30 ppm Fe 20% EDC (5)	ED 2 ppm Fe 20% EDC (5)
Weight (g)	1.22 ± 0.17^{a}	1.56 ± 0.26^{b}	$1.34 \pm 0.23^{a,b}$	1.56 ± 0.10^{b}
Total lipids (g/100 g liver)[4]	5.70 ± 1.80^{a}	8.37 ± 2.41^{b}	10.28 ± 1.74^{b}	8.60 ± 1.63^{b}
ADH (nmoles/minute/g protein)	19.31 ± 2.28^{a}	19.88 ± 1.62^{a}	25.85 ± 2.85^{b}	25.13 ± 1.70^{b}
Dihydrofolate reductase[4] (nmoles/minute/mg protein)	1.07 ± 1.15^{a}	0.67 ± 0.15^{b}	0.67 ± 0.08^{b}	0.65 ± 0.17^{b}

[1]Results are expressed as mean ± SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2]EDC, ethanol-derived calories.

[3]Number of animals per group.

[4]Analysis of variance statistics showed significant interactions between iron deficiency and ethanol consumption on liver total lipids ($P < 0.05$) and on dihydrofolate reductase activity ($P < 0.01$).

analyses of variance statistics are tabulated in Appendix 1. Liver weights were significantly increased in animals maintained on iron-deficient diets (CD, ED) compared to the control group (CA). Mean liver weights were 1.6 g in both CD and ED groups compared to 1.2 g in the CA group. The mean liver weight of the EA group was 1.3 g which was comparable to all other groups.

Total liver lipid content of the CA group was 5.7 g/100 g liver and was significantly elevated to 8.4, 10.3 and 8.6 g/100 g in CD, EA and ED groups, respectively. A significant iron-ethanol interaction on liver lipid was noted. Liver alcohol dehydrogenase (ADH) activity increased significantly in ethanol-fed groups compared to those on diets providing no ethanol. Mean ADH activity was 19.3 and 19.9 nmoles/minute/mg soluble protein in the CA and CD groups, respectively, and increased to 25.8 (EA) and 25.1 (ED) nmoles/minute/mg soluble protein. No effect of iron content of the diet was evident with regard to ADH activity.

Activity of liver dihydrofolate reductase was significantly decreased in the three experimental groups compared to the control group. Mean dihydrofolate reductase was 1.1 nmoles/minute/mg soluble protein in the CA group compared to 0.7 (CD and EA) and 0.6 (ED) nmoles/minute/mg soluble protein. Statistical analysis indicated that iron, ethanol and iron-ethanol interaction had significant effects on dihydrofolate reductase activity.

ii. Blood analysis

Mean hemoglobin, hematocrit, serum iron, percentages saturation of transferrin, serum folate, red cell folate and blood ethanol concentrations prior to pregnancy are tabulated in Table 13, and analyses of variance statistics presented in Appendix 2. Hemoglobin and hematocrit levels were

Table 13. Effects of iron deficiency and/or ethanol consumption on mean blood hemoglobin, hematocrit, serum iron, percentage saturation of transferrin, serum folate, red blood cell folates and blood ethanol levels prior to pregnancy[1]

Blood parameter	Dietary Treatments			
	CA 30 ppm Fe 0% EDC[2] (5)[3]	CD 2 ppm Fe 0% EDC (5)	EA 30 ppm Fe 20% EDC (5)	ED 2 ppm Fe 20% EDC (5)
Hemoglobin (g/100 ml)	16.39 ± 1.99[a]	10.87 ± 0.69[b]	11.31 ± 0.99[b]	7.93 ± 2.26[c]
Hematocrit (%)	45.40 ± 3.43[a]	35.00 ± 4.89[b]	38.60 ± 4.15[c]	31.60 ± 6.10[b]
Serum iron (µg/100 ml)	119.26 ± 21.06[a]	57.62 ± 19.13[b]	134.86 ± 20.50[a]	45.38 ± 11.79[b]
Percentage saturation of transferrin (%)	36.43 ± 5.66[a]	18.64 ± 5.50[b]	36.49 ± 4.39[a]	16.31 ± 5.35[b]
Serum folate (ng/ml)	61.50 ± 5.81[a]	28.84 ± 23.16[b]	48.06 ± 6.49[a]	26.18 ± 13.43[b]
Red cell folates[4] (ng/ml packed RBC)	400.65 ± 81.56[a]	109.57 ± 40.29[b]	147.01 ± 102.89[b]	138.05 ± 75.23[b]
Blood ethanol (mg/100 ml)	-	-	127.78 ± 56.39	190.19 ± 44.21

[1]Results are expressed as means ± SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2]EDC, ethanol-derived calories.

[3]Number of animals per group.

[4]Analysis of variance statistics revealed a significant interaction between iron deficiency and ethanol consumption on red blood cel folate at $P < 0.01$.

significantly lower in CD, EA and ED groups compared to the CA group. The mean hemoglobin concentration was 16.4 g/100 ml blood (CA) compared to 10.9 (CD), 11.3 (EA) and 7.9 (ED) g/100 ml blood. The mean value for hemoglobin concentration of the ED group was the lowest compared to all other groups. The mean hematocrit (percent) was 45.4 in the CA group compared to 35.0 in CD, 38.6 in EA and 31.6 in ED. The lowest mean values for hematocrits were noted in the iron-deficient groups.

Serum iron and percentage saturation of transferrin were significantly decreased in animals maintained on iron-deficient diets. No significant ethanol effect was noted. Mean serum iron levels were 119.3 and 134.9 µg/100 ml in CA and EA groups, respectively, compared to 57.6 (CD) and 45.4 µg/100 ml (ED). Mean percentages saturation of transferrin were 36.4 (CA) and 36.5 (EA) compared to 18.6 (CD) and 16.3 (ED).

Serum folate dropped significantly in the groups maintained on iron-deficient diets compared to those maintained on iron-adequate diets. Serum folate levels were 61.5 (CA) and 48.1 ng/ml (EA) compared to 28.8 (CD) and 26.2 ng/ml (ED). Statistically, ethanol had no effect on serum folate prior to pregnancy. However, red cell folates were significantly lower in the groups maintained on iron-deficient diets and/or those providing ethanol as compared to the control group (CA). Mean red cell folates were 400.6 ng/ml packed red cells in the CA group and decreased markedly to 109.6, 147.0 and 138.0 ng/ml packed red cells in CD, EA and ED groups, respectively. A statistically significant ($P < 0.05$) iron-ethanol interaction on red cell folate was noted. No significant difference was noted in blood ethanol levels in animals fed 20 percent EDC diets regardless of the iron content of the diets. Mean blood ethanol concentrations were 127.8 and 190.2 mg/100 ml blood in the EA and ED groups, respectively.

2. Pregnancy period

a. Body weight and daily caloric intake

Body weights on day 18 of gestation and daily caloric intakes during pregnancy are shown in Table 14. Statistical analyses are tabulated in Appendix 3. Mean body weights were significantly lower in the groups maintained on iron-deficient and/or ethanol-containing diets compared to the control group. The mean body weights were 43.1 g in the CA group compared to 34.7 g in the CD group, 33.7 g in the EA group and 32.9 in the ED group. A significant iron-ethanol interaction was noted for body weights during pregnancy. Daily caloric intakes during pregnancy were lower in animals fed iron-deficient diets (CD and ED groups) compared to the CA group. Mean daily caloric intakes were 15.0 (CD) and 14.8 Kcal (ED) versus 16.3 Kcal (CA). The mean daily caloric intake of the EA group (15.7 Kcal) was comparable to the CA and CD groups. Ethanol consumption alone had no significant effect on daily caloric intake.

b. Maternal tissue analyses

i. Liver

Data for mean maternal liver weights, total lipid contents, alcohol dehydrogenase and dihydrofolate reductase activities are summarized in Table 15 and analysis of variance statistics are presented in Appendix 3. Mean maternal liver weights varied slightly among the groups. The least significant difference test indicated that the mean maternal liver weight was lower in the CD group (1.8 g) compared to the EA group (2.2 g). Mean liver weights of CA (2.1 g) and ED (2.0 g) were comparable to those of CD and EA groups. Mean maternal liver lipid contents were significantly higher in the ethanol-fed groups (EA and ED) compared to the CA group. Mean total lipids were 14.8 and 13.6 g/100 g liver in EA and ED groups, respectively, compared

Table 14. Effects of iron deficiency and/or ethanol consumption on mean body weight and daily caloric intake of maternal mice[1]

	Dietary Treatments			
	CA 30 ppm Fe 0% EDC[2] (17)[3]	CD 2 ppm Fe 0% EDC (17)	EA 30 ppm FE 20% EDC (17)	ED 2 ppm Fe 20% EDC (17)
Body weight (g)				
At day 18 of gestation[4]	43.12 ± 3.26^{a}	34.73 ± 3.91^{b}	33.69 ± 5.23^{b}	32.90 ± 6.72^{b}
As % of CA	100	79.66	78.10	76.30
Daily caloric intake (Kcal) during pregnancy	16.33 ± 1.08^{a}	$14.99 \pm 1.26^{b,c}$	$15.70 \pm 1.11^{a,b}$	14.75 ± 0.92^{c}
As % of CA	100	91.70	96.10	90.30

[1] Results are expressed as means + SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2] EDC, ethanol-derived calories.

[3] Number of animals per group.

[4] Analysis of variance statistics revealed a significant interaction between iron deficiency and ethanol consumption on body weight on day 18 of gestation at $P < 0.01$.

Table 15. Effects of iron deficiency and/or ethanol consumption on liver weight, total lipids, alcohol dehydrogenase (ADH) and dihydrofolate reductase activities and liver histology of maternal mice[1]

Liver parameter	Dietary Treatments			
	CA 30 ppm Fe 0% EDC[2] (17)[3]	CD 2 ppm Fe 0% EDC (17)	EA 30 ppm Fe 20% EDC (17)	ED 2 ppm Fe 20% EDC (17)
Weight (g)	$2.12 \pm 0.36^{a,b}$	1.85 ± 0.42^{b}	2.24 ± 0.51^{a}	$2.04 \pm 0.48^{a,b}$
Total lipids (g/100 g liver)	10.70 ± 2.77^{a}	$11.13 \pm 4.93^{a,c}$	14.83 ± 3.02^{b}	$13.64 \pm 4.10^{b,c}$
No. of liver with histological evidence of fatty changes[5]	11 (16)[6]	7 (15)	8 (17)	9 (17)
As % of total livers examined	68.75	46.67	47.06	52.94
ADH (nmoles/minute/mg protein)	20.44 ± 3.38^{a}	19.41 ± 2.38^{a}	24.91 ± 3.25^{b}	23.06 ± 2.87^{b}
Dihydrofolate reductase[4] (nmoles/minute/mg protein)	1.02 ± 0.15^{a}	0.75 ± 0.10^{b}	0.80 ± 0.17^{b}	0.63 ± 0.15^{c}

[1] Results are expressed as means $\pm$ SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2] EDC, ethanol-derived calories.

[3] Number of animals/group.

[4] Analysis of variance statistics showed a significant interaction between iron deficiency and ethanol consumption on liver dihydrofolate reductase activity at $P < 0.05$.

[5] Chi square value is 2.06; $P > 0.05$.

[6] Number of maternal livers examined.

to 10.7 g/100 g in the CA group. The mean total lipid content of the CD group (11.1 g/100 g liver) was comparable to those of the CA and ED groups. Iron in the diet had no significant effect on total liver lipids.

Results of histological examination of maternal livers for fatty changes are also presented in Table 15. No statistical differences in the frequencies of maternal fatty livers from all groups were noted ($X^2 = 2.06$). The percentages of fatty livers were 68.8 (CA), 46.7 (CD), 47.1 (EA) and 52.9 (ED). As in Experiment 1, histological examination revealed individual variations within each group. Figures 21 and 22 show examples of the histological examination of maternal livers from CD and ED groups. Values from chemical analysis for total lipid content in maternal livers were positively correlated with the intensity of fatty changes determined histologically ($t = 3.73$, $P < 0.05$).

Mean maternal liver ADH activity was significantly higher in the groups maintained on ethanol-containing diets than in the groups consuming no ethanol. Mean liver ADH activities were 20.4 and 19.4 nmoles/minute/mg soluble protein in the CA and CD groups, respectively, compared to 24.9 (EA) and 23.1 nmoles/minute/mg soluble protein (ED). Iron had no significant effect on ADH activity. Liver dihydrofolate reductase activity of CD, EA and ED groups were lower than that of the CA group. Mean dihydrofolate reductase activity was 1.0 nmoles/minute/mg soluble protein in the CA group compared to 0.8 in CD and EA and 0.6 nmoles/minute/mg soluble protein in ED group. Statistical analyses indicated a significant ($P < 0.05$) iron-ethanol interaction on dihydrofolate reductase activity.

ii. Blood analyses

Data on blood analyses of pregnant mice on day 18 of gestation are tabulated in Table 16 and analyses of variance statistics are presented in

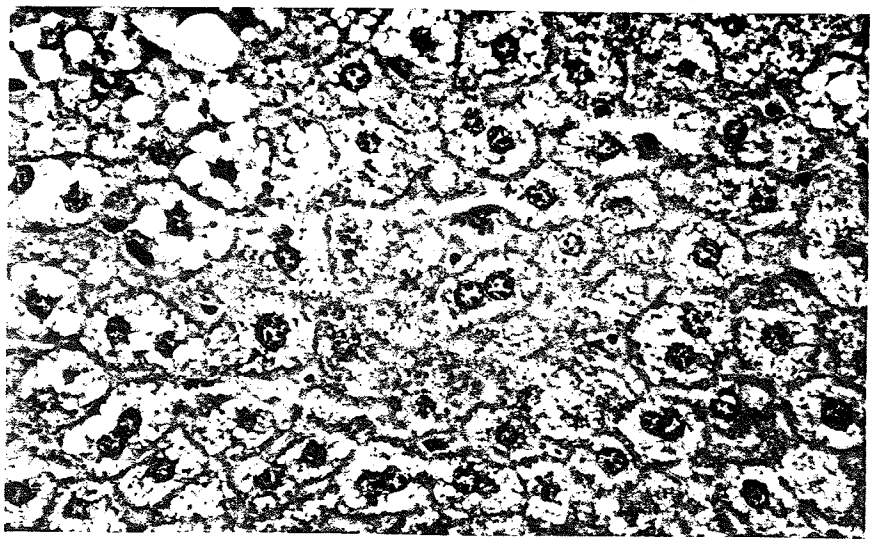


Figure 21. Photomicrograph of a maternal liver from an animal receiving an iron-deficient diet with 0% EDC (CD). Hepatocytes containing fat vacuoles and occassional hepatocytes degeneration are evident (Hematoxyline-eosin stain; x 400).

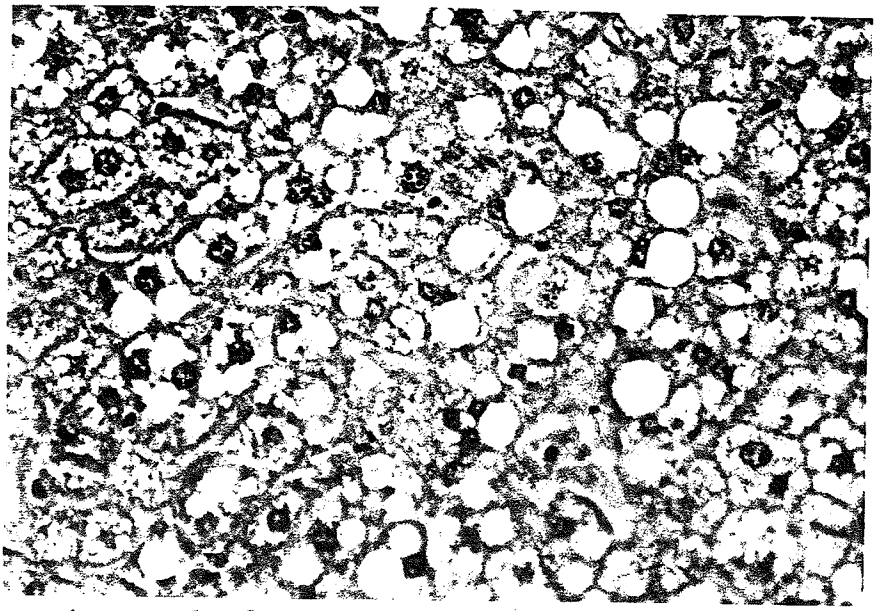


Figure 22. Photomicrograph of a maternal liver from an animal receiving an iron-deficient diet with 20% EDC (ED). Numerous hepatocytes containing fat vacuoles, degenerative hepatocytes and focal necrosis are evident (Hematoxyline-eosin stain; x 400).

Table 16. Effect of iron deficiency and/or ethanol consumption on mean hemaglobin, hematocrit, serum iron, percentage saturation of transferrin, serum folate, red blood cell folate and blood ethanol levels of maternal mice[1]

Blood parameter	Dietary Treatments			
	CA 30 ppm Fe 0% EDC[2] (17)[3]	CD 2 ppm Fe 0% EDC (17)	EA 30 ppm Fe 20% EDC (17)	ED 2 ppm Fe 20% EDC (17)
Hemoglobin (g/100 ml)[4]	14.86 ± 1.64^{a}	9.06 ± 0.75^{c}	12.34 ± 2.08^{b}	9.64 ± 1.45^{c}
Hematocrit (%)	44.17 ± 3.41^{a}	31.88 ± 2.14^{b}	41.06 ± 4.21^{a}	32.47 ± 6.82^{b}
Serum iron (µg/100 ml)	130.86 ± 40.93^{a}	48.90 ± 14.90^{b}	113.89 ± 38.14^{a}	44.89 ± 22.18^{b}
Percentage saturation of transferrin (%)	35.14 ± 5.28^{a}	16.42 ± 5.43^{c}	30.59 ± 7.02^{b}	14.63 ± 5.82^{c}
Serum folate (ng/ml)	36.49 ± 17.13^{a}	20.02 ± 9.75^{b}	32.08 ± 9.55^{a}	16.01 ± 8.92^{b}
Red blood cell folate (ng/ml packed RBC)[4]	383.24 ± 139.60^{a}	126.16 ± 77.34^{b}	127.68 ± 81.02^{b}	111.24 ± 58.67^{b}
Blood ethanol (mg/100 ml)	-	-	100.82 ± 58.00	111.49 ± 39.82

[1] Results are expressed as means ± SD. Means within a horizontal row with unlike superscripts indicate significant differences at $P < 0.05$.

[2] EDC, ethanol-derived calories.

[3] Number of animals per group.

[4] Analysis of variance statistics revealed a significant interaction between iron deficiency and ethanol consumption on these blood parameters ($P < 0.01$).

Appendix 4. Significantly lower hemoglobin concentrations were noted in groups that received diets which were deficient in iron and/or contained ethanol. Mean blood hemoglobin was 14.9 g/100 ml in the control group (CA) compared to 9.1, 12.3 and 9.6 g/100 ml blood in CD, EA and ED groups, respectively. The lowest values were noted in the iron-deficient groups. A significant iron-ethanol interaction for hemoglobin concentration of pregnant animals was noted. Hematocrit values decreased significantly in the groups maintained on iron-deficient diets compared to those on iron-adequate diets. Mean hematocrit percents were 44.2 and 41.1 in the CA and EA groups, respectively, compared to 31.9 (CD) and 32.5 (ED). Iron level of the diet, but not ethanol consumption, significantly affected hematocrit values.

Serum iron concentrations decreased sharply in animals maintained on diets deficient in iron (CD and ED) compared to those on iron-adequate diets (CA and EA). Mean serum iron values were 48.9 and 44.9 µg/100 ml in CD and ED, respectively, compared to 130.9 (CA) and 113.9 µg/100 ml (EA). Ethanol consumption had no effect on serum iron concentration. Percentage saturation of transferrin was markedly decreased in animals fed iron-deficient diets and to a lesser extent in animals fed an iron-adequate diet providing 20 percent EDC compared to the control group. Mean percentage saturation of transferrin was 35.1 in the CA group and decreased to 16.4 in CD, 30.6 in EA and 14.6 in ED group. Therefore, either iron deficiency or ethanol consumption had a significant depressing effect on percentage saturation of transferrin.

Serum folate levels decreased significantly in the CD and ED groups compared with the CA and EA groups. Mean serum folate levels were 20.0 and 16.0 ng/ml in the CD and ED groups, respectively, compared to 36.5 (CA)

and 32.1 ng/ml (EA). Statistical analyses indicated that iron content of the diet, but not ethanol consumption had a significant effect on serum folates. However, using red cell folates as an index of folate status, both iron deficiency and/or ethanol consumption had a significant influence. Mean red cell folates decreased from 383.2 ng/ml packed red cells in the CA group to 126.2, 127.7 and 111.2 ng/ml packed red cells in CD, EA and ED groups, respectively.

No significant difference was noted in blood ethanol levels of animals fed 20 percent EDC, regardless of iron content of the diet. Mean blood ethanol levels were 100.8 and 111.5 mg/100 ml blood in the EA and ED groups, respectively.

c. Reproductive performance of maternal mice

Patterns of reproductive performances of pregnant mice differed greatly among the control and experimental groups (Table 17). A significant increase in the number of dams with abnormal indicators of reproductive performance was noted in CD, EA and ED groups compared to the control group (CA) ($X^2 = 18.52$, $P < 0.01$). Only one mother in the CA group had an indication of abnormal reproductive performance (total fetal resorption) compared to 11 (CD), 11 (EA) and 12 dams (ED) in the other groups. In the CA group, other than the dam with total fetal resorption, the rest of the pregnant mice had normal litters. The litter size and the arrangement of the live fetuses between right and left horns of the uteri in group CA were nearly similar and symmetrical (Figure 23).

In the group maintained on the iron-deficient diet without ethanol (CD), 5 dams had total fetal resorption. Four dams had live fetuses only in one of the two uterus horns while the other horn was free of them (Figure 24);

Table 17. Indices of reproductive performance at day 18 of gestation

Groups[1]	No. of animals with live fetuses	No. of animals[2] with abnormal indices	Implantation[3] Sites (mean/litter)	Fetal Condition (mean/litter)[3]		
				Resorption %	Live fetuses %	Fetal weight (g)
CA	16	1	8.2 ± 1.1^{a}	10.1 ± 11.4^{a}	89.9 ± 11.4^{a}	0.97 ± 0.12^{a}
CD	12	11	7.5 ± 3.4^{a}	26.8 ± 17.9^{b}	55.0 ± 16.8^{b}	0.65 ± 0.12^{b}
EA	15	11	9.8 ± 1.6^{b}	34.1 ± 17.5^{b}	49.0 ± 14.0^{b}	0.64 ± 0.11^{b}
ED	13	12	10.1 ± 1.9^{b}	34.2 ± 15.5^{b}	42.5 ± 13.3^{b}	0.59 ± 0.14^{b}

[1] n = 17 pregnant mice/group.

[2] Females with total fetal resorption, small litter size, no sites of implantations or all resorbed and/or all dead fetuses in one horn of the uterus and assymmetrical arrangement of fetuses ($X^2 = 18.52$, $P < 0.01$).

[3] Results are expressed as means $\pm$ SD. Means within a column with unlike superscripts are significant at $P < 0.05$.

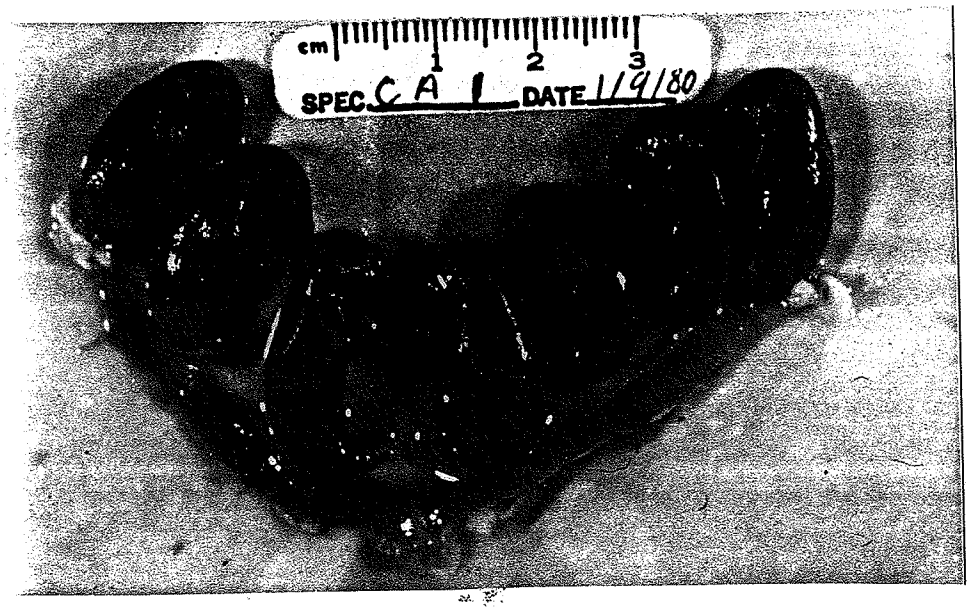


Figure 23. Photograph of a litter from an animal receiving an iron-adequate diet with 0% EDC (CA). Normal live fetuses with one early resorption in left horn are evident.

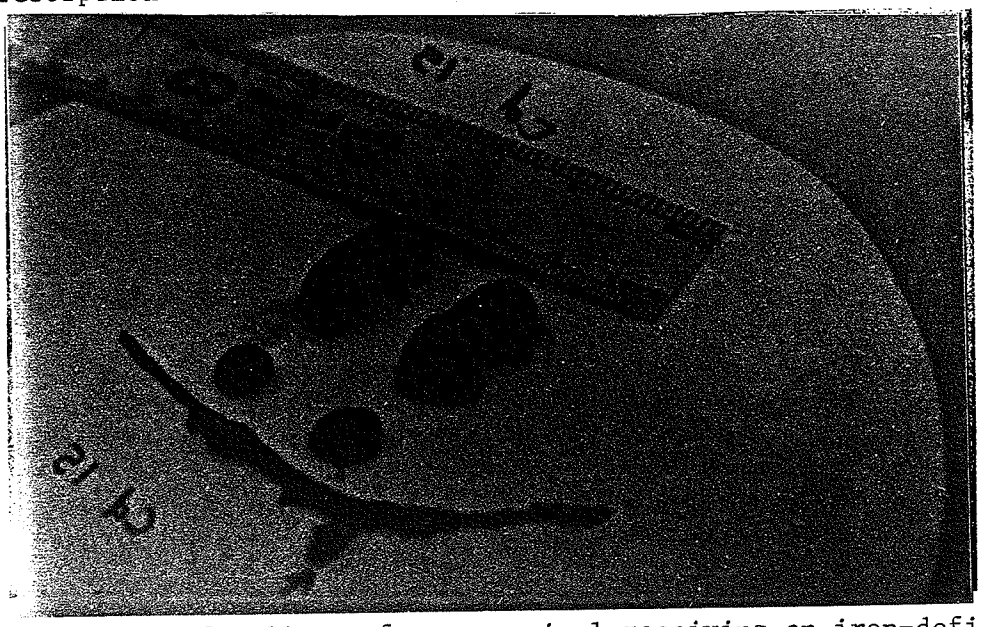


Figure 24. Photograph of a litter from an animal receiving an iron-deficient diet with 0% EDC (CD). Two live fetuses in the right horn and no sites of implantation in left horn are evident.

all of these 4 dams had small-sized litters. Another 2 pregnant mice in this group had asymmetrical arrangements of live fetuses between the two horns of the uterus.

In the EA group, 2 dams had total fetal resorptions, 1 dam had live fetuses only in one horn of the uterus and 4 dams had assymmetrical arrangements of live fetuses between the uterus horns. The litters of the last 4 dams were small-sized litters. Also, another 4 dams in the EA group had small-sized litters (Figure 25).

In the group maintained on an iron-deficient diet providing ethanol (ED), 4 females had total fetal resorptions. Three dams had their live fetuses in one horn of the uterus and another 3 dams had asymmetrical arrangements of live fetuses between the two horns of the uterus (Figure 26a). In this group, 4 dams had small-sized litters of 3 or less live fetuses (Figure 26b); 2 of these 4 dams were among those with live fetuses in only one horn.

Data on numbers of implantations, percentages of resorption, and live fetuses and live fetal weights when dams were sacrificed on day 18 of gestation are also tabulated in Table 17. Analyses of variance statistics are presented in Appendix 5. The mean numbers of implantations were higher in the EA and ED groups compared to the CA and CD groups. The mean numbers of implantations were 9.8 (EA) and 10.1 (ED) versus 8.2 (CA) and 7.5 (CD). Statistical analyses indicated that ethanol in the diet had a positive effect on the number of implantations, while iron content of the diet was without an effect. However, the percentage of resorbed fetuses was lower in the CA group compared to the other groups. The percentages of resorption per litter were 10.1 (CA), 26.8 (CD), 34.1 (EA) and 34.2 (ED). Analysis of variance statistics for a 2 x 2 factorial

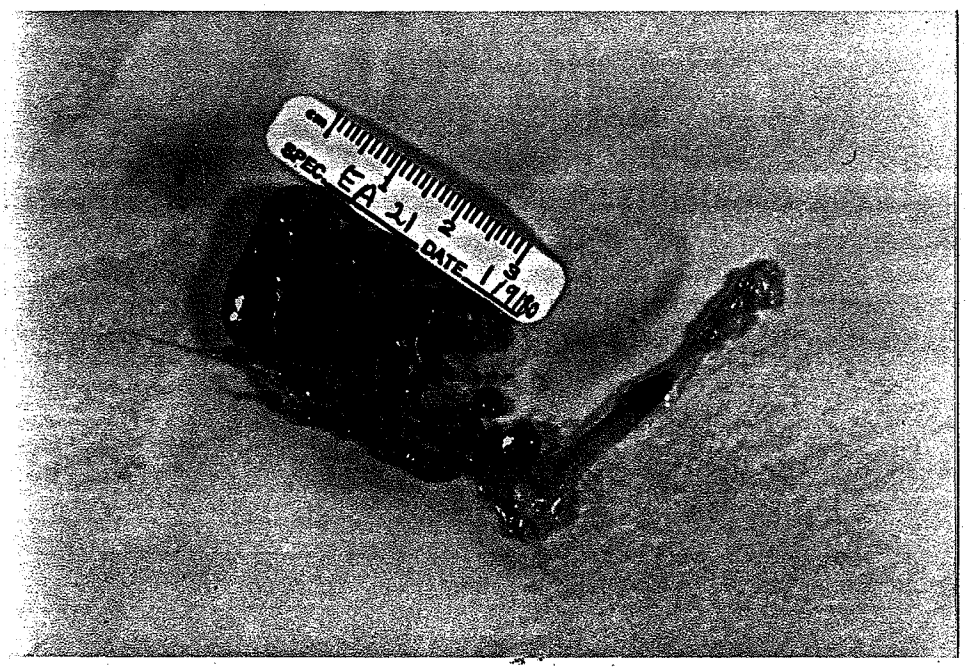
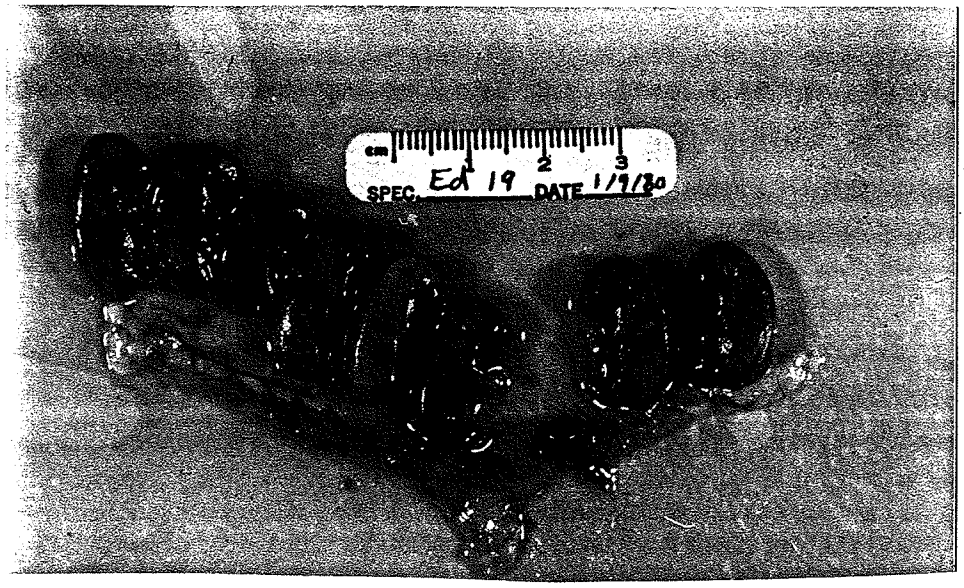


Figure 25. Photograph of a litter from an animal receiving an iron-adequate diet with 20% EDC (EA). Live fetuses in the left horn and one late resorption (arrow) in the right horn.

a)



b)

Figure 26. Photographs of 2 litters from animals receiving an iron-deficient diet with 20% EDC (ED).

a) Assymmetrical distribution of live fetuses between the 2 uterine horns is evident.

b) Litter of 3 live fetuses, two dead fetuses (arrows) and 2 resorptions (crosses) are evident.

design indicated significant iron and ethanol effects. Least significant difference tests substantiated these findings. No dead fetuses were noted in the litters of the control group (CA). In the experimental groups, 18.1, 16.1 and 23.3 percent of implanted fetuses per litter died in CD, EA and ED groups, respectively. Statistical analyses show that iron or ethanol in the diet caused an increase in percentages of dead fetuses.

The mean percentages of live fetuses per litter decreased from 89.9 in CA to 55.0 in CD, 49.0 in EA and 42.5 in ED. Analysis of variance statistics indicated an iron-ethanol interaction as well. Mean live fetal weights were significantly decreased in CD, EA and ED groups compared to that of CA group. The mean live fetal weight was 0.97 g (CA) versus 0.65 (CD), 0.64 (EA) and 0.59 g (ED). Therefore, either iron deficiency and/or ethanol consumption significantly decreased fetal weights.

d. Relationship between maternal iron and folate status to gestational performance

Maternal serum iron concentration was positively correlated with maternal red cell folates ($t = 3.72$, $P < 0.01$). However, no significant correlation was found between serum iron and serum folate. Maternal serum iron, measured on day 18 of gestation was positively correlated with live fetal body weight ($t = 3.52$, $P < 0.01$) (Table 18). No significant relationships were noted between serum iron and the percentages of resorbed and live fetuses per litter.

Correlations between red cell folates and the percentages of resorbed fetuses, live fetuses per litter and fetal weights are also presented in Table 18. Red cell folates were negatively correlated with the percentage of resorption per litter ($t = 2.15$, $P < 0.05$) and positively correlated with both the percentages of live fetuses per litter ($t = 3.69$, $P < 0.01$) and live fetal weights ($t = 6.72$, $P < 0.01$).

Table 18. Relationships between maternal iron and folate status and gestational performance in mice

Related Variables	Regression Equation (y = ax + b)	r[1]	t Value
Serum iron concentration and live fetal weight	y = 0.002 + 0.59	0.443	3.52[2]
Red blood cell folates and percentage resorption	y = 0.07X + 50.19	-0.29	2.15[3]
Red blood cell folates and percentage live fetuses/litter	y = 0.12X + 29.51	0.46	3.69[2]
Red blood cell folates and live fetal weight	y = 0.001X + 0.56	0.69	6.72[2]

[1] r = correlation coefficient.

[2] Significant at $P < 0.01$.

[3] Significant at $P < 0.05$.

e. Gross morphological characteristics of fetuses

i. External and internal malformation

The total number of fetuses examined and percentages with external and internal malformations as well as the types of abnormalities noted are presented in Table 19. The number of fetuses with external and internal morphological abnormalities in the CD, EA and ED groups were significantly higher than the CA group ($X^2 = 93.01$, $P < 0.01$). Fetuses examined from the control group were essentially normal, only one fetus had a morphological defect, while in the group fed an iron-deficient diet providing no ethanol (CD) 37.7 percent of fetuses had morphological defects. The percentages of external anomalies were 55.4 in the EA and 59.6 in the ED group As in Experiment 1, the abnormalities noted were small fetal size ($X^2 = 66.8$, $P < 0.01$), open eye lids ($X^2 = 96.4$, $P < 0.01$), wrinkled skin ($X^2 = 35.5$, $P < 0.01$), internal hemorrhage ($X^2 = 17.7$, $P < 0.01$), defective limbs ($P > 0.05$) and failure of abdominal wall to fuse ($X^2 = 7.86$, $P < 0.05$). Only 30 (CA and EA) and 26 fetuses (CD and ED groups) were sectioned for internal defects. The number of fetuses with cleft palate was higher in the experimental groups (CD, EA and ED) than in the control group (CA) ($X^2 = 66.39$, $P < 0.01$). The percentage of fetuses with cleft palate was 0 percent (CA) versus 46.1 (CD), 80.0 (EA) and 100 percent (ED). Figures 27 to 29 illustrate some of the external and internal malformations noted among the experimental groups. Figure 27 is a photograph of fetuses from CA, ED and EA groups. Abnormalities including reduced fetal size, open eye lids (EA and ED), small head (ED) and wrinkled skin (EA) are evident. Figure 28 is a photograph of fetuses from a litter of group CD. Abnormal heads and failure of abdominal walls to fuse with part of the viscera outside the abdominal cavity are evident. Figure 29 shows a photograph of a

Table 19. Effects of maternal iron deficiency and/or ethanol consumption on the frequency of external and internal fetal malformations[1]

Dietary Treatments	n*	External and internal defects		Types of Internal and External Malformations						
		Number of fetuses[2]	Percentage of fetuses	Small fetuses[4]	Open eye lids	Wrinkled skin	Internal hemorrhage	Defective limbs	Failure of abdominal wall to fuse	Cleft palate[5]
CA	119	1	0.84	0.84	0	0	0	0	0	0
CD	53	20	37.74	26.42	37.74	20.75	5.66	5.66	7.54	46.15
EA	74	41	55.40	32.43	55.40	27.02	4.05	4.05	4.05	80.00
ED	57	34	59.65	52.64	59.65	10.54	14.04	5.26	5.26	100.00
X^2 Values		93.01[2]	-	66.83[2]	96.4[2]	35.5[2]	17.7[2]	6.2	7.66[3]	66.39
p		< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	> 0.05	<0.05	< 0.01

[1]Results are expressed as a percentage of the total number of fetuses examined/group.

[2]Chi square reveals significant differences between the control (CA) and experimental groups (CD, EA and ED) at $P < 0.01$.

[3]Chi square reveals significant differences between the control (CA) and experimental groups (CD, EA and ED) at $P < 0.05$.

[4]Small fetuses = fetal weight < 0.60 g.

[5]Thirty fetuses (CA and EA) and 26 fetuses (CD and ED) were used to examine cleft palates.

*n = n-mber of fetuses examined/group.

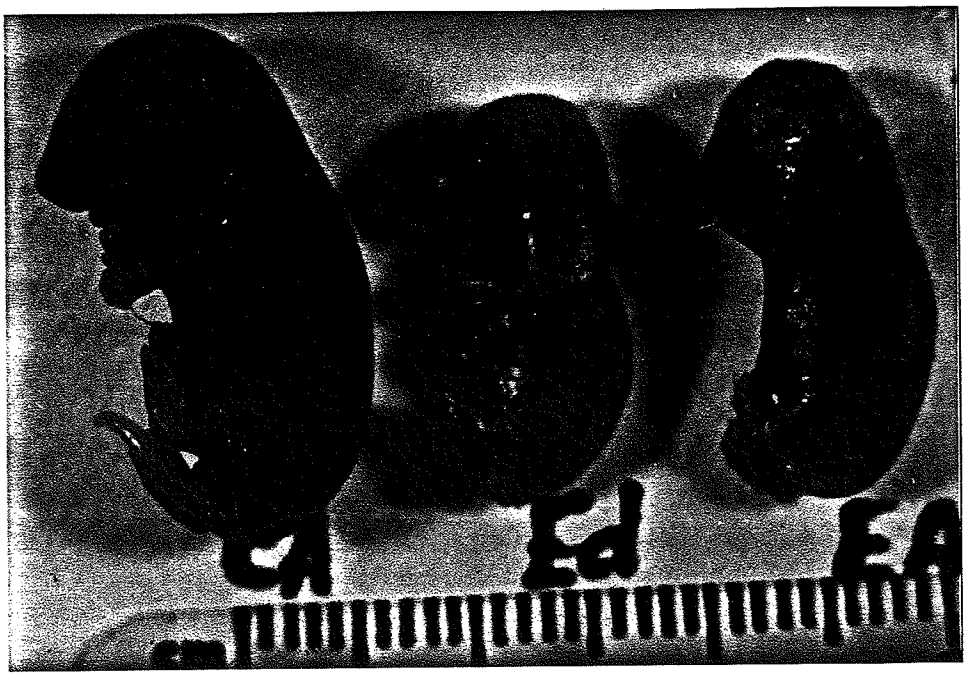


Figure 27. Photograph of fetuses from CA, ED and EA groups. The difference in size, open eye lids (EA and ED), small head (ED) and wrinkled skin (EA) are evident.

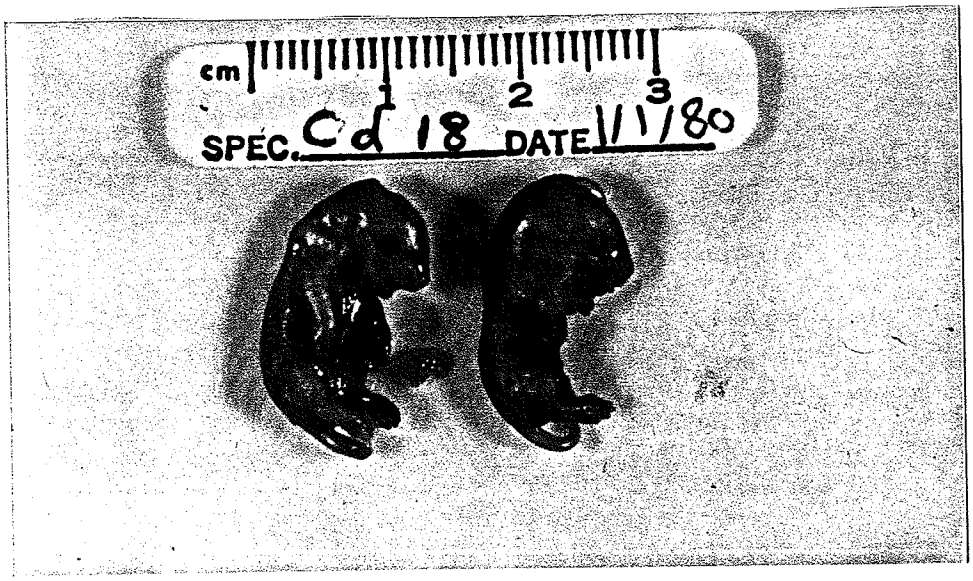


Figure 28. Photograph of 2 fetuses (CD). Abnormal heads and failure of abdominal wall to fuse with part of the viscera outside the abdomen are evident.

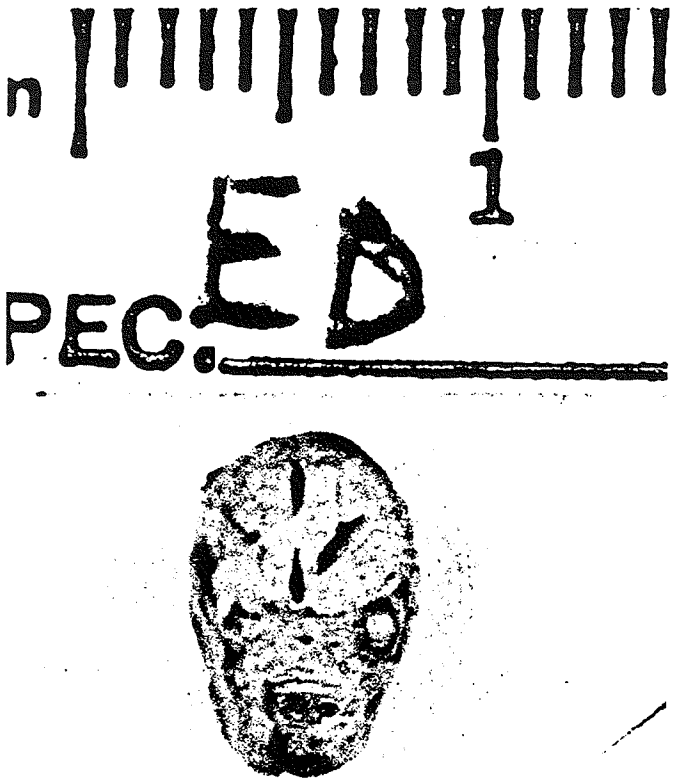


Figure 29. Photograph of a longitudinal section of a fetal mouse head (ED). Missing left eye and cleft palate are evident.

longitudinal section of a fetal mouse head from group ED. A missing left eye and cleft palate are evident.

ii. Skeletal defects

Results of examinations of skeletons for bone defects are presented in Table 20. There were increased numbers of fetal skeleton defects in CD, EA and ED groups compared to the control group (CA) ($X^2 = 66.69$, $P < 0.01$). The examined skeletons from the CA group had 5 percent of fetuses with missing phalanges. Other than this, skeletons were essentially normal (Figure 30). Ninety percent of skeletons stained from the CD group had bone defects, while 100 percent of skeletons from EA and ED groups had bone defects. The skeletal defects noted most often in the experimental groups (CD, EA and ED) were as follows: missing and incomplete ossification of skull ($X^2 = 16.3$, $P < 0.01$), sternum ($X^2 = 24.9$, $P < 0.01$), pelvic bones ($X^2 = 23.6$, $P < 0.01$), limb ($X^2 = 66.5$, $P < 0.01$) and tail bones ($X^2 = 11.9$, $P < 0.05$) (Figures 31 to 33). Figure 34 shows photographs of fetal forelimb bones from group CA and CD. Presence of metacarpal bones and phalanges in CA fetus and absence of these bones in CD are evident. Figure 35 is a comparison photograph of stained fetal skeletons representative of all groups where the differences in the skeleton size within and among the groups are evident.

f. Histological characteristics of fetal livers

Some of the fetal livers were examined histologically. As in Experiment 1, livers of fetuses from the control group (CA) were essentially normal. Some of the fetal livers from CD, EA and ED groups show a marked dimunition of hematopoietic acitivity ($X^2 = 0.9$) (Figures 36a,b).

Table 20. Effects of maternal iron deficiency and/or ethanol consumption on morphological characteristics of mice fetal skeleton in mice[1]

Dietary Treatment		Fetal skeletal defects No. 3	%	Small skeleton "unmature" %	Types of Skeletal Defects[2] Skull %	Sternum %	Pelvic Bones %	Limb %	Ribs %	Tail Bones %
CA	20	1	5	0	0	0	0	0	0	0
CD	19	17	90	36.80	36.8	0	5	90	5	10
EA	22	22	100	54.50	54.50	45.00	54.50	100	0	27.30
ED	20	20	100	65	50	50	45	100	15	40
X^2 Values	-	66.69	-	20.62[3]	16.28[3]	24.96[3]	23.60[3]	66.52[3]	6.5	11.95[4]
P	-	< 0.01	-	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	> 0.05	< 0.05

[1]Results are expressed as percentage of the total number of fetuses examined per group.

[2]Immature or missing bones.

[3]Chi square test reveals significant differences among the groups at $P < 0.01$.

[4]Chi square test reveals significant differences among the groups at $P < 0.05$.

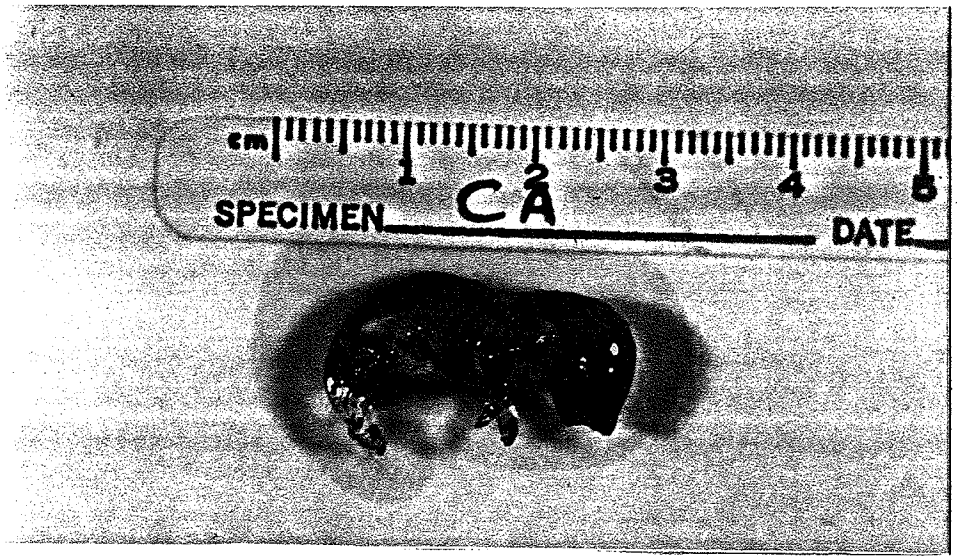


Figure 30. Photograph of a fetal skeleton (CA); the skeleton is essentially normal.

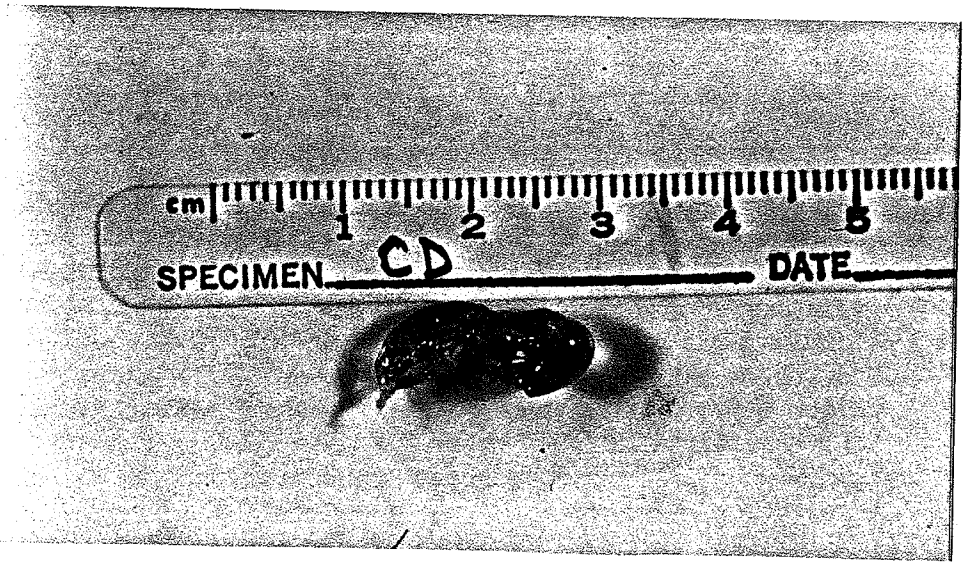


Figure 31. Photograph of a fetal skeleton (CD), incomplete ossification of skull, limbs, pelvic and missing phalanges and tail bone are evident.

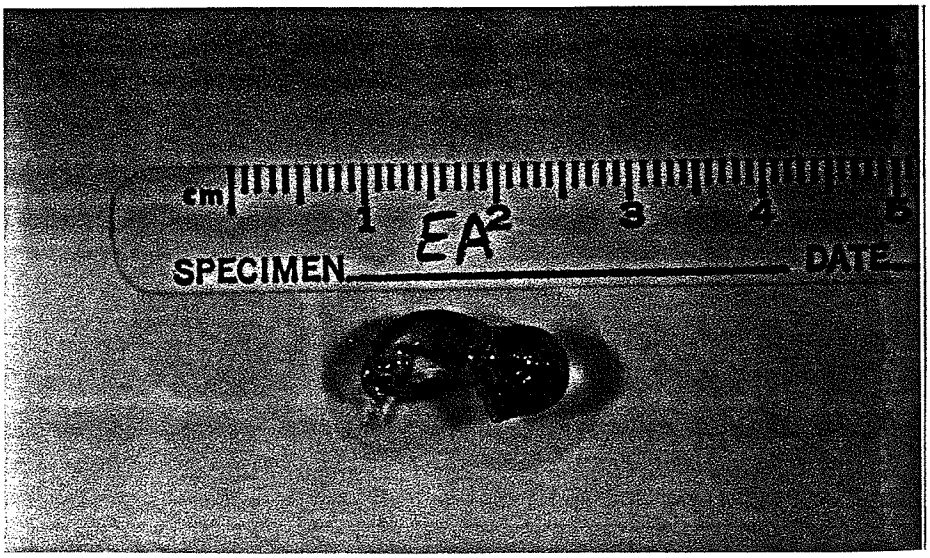


Figure 32. Photograph of a fetal skeleton (EA), incomplete ossification of skull, limbs, pelvic and missing of phalanges and tail bones are evident.

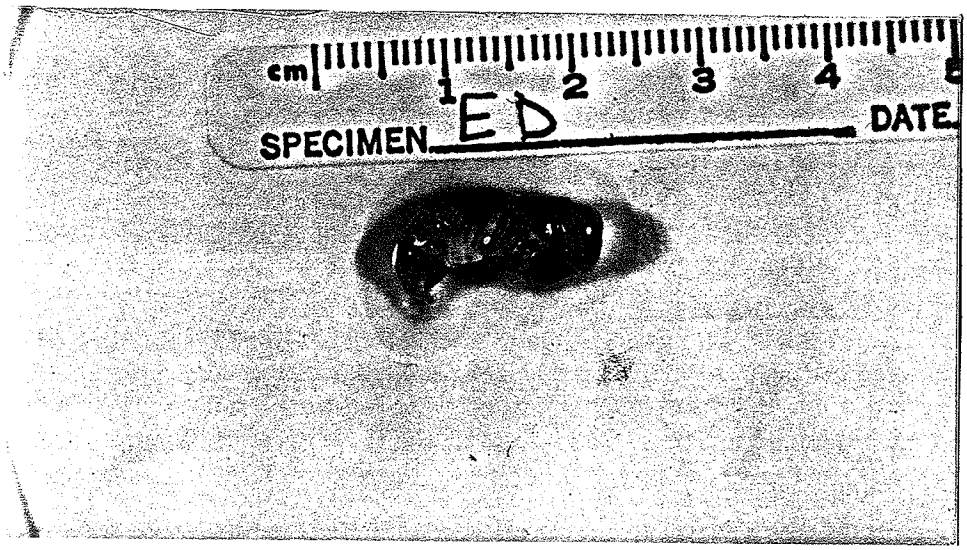


Figure 33. Photograph of a fetal skeleton (ED), incomplete ossification of skull, ribs, limbs, pelvic and missing phalanges and tail bones are evident.

a)

b)

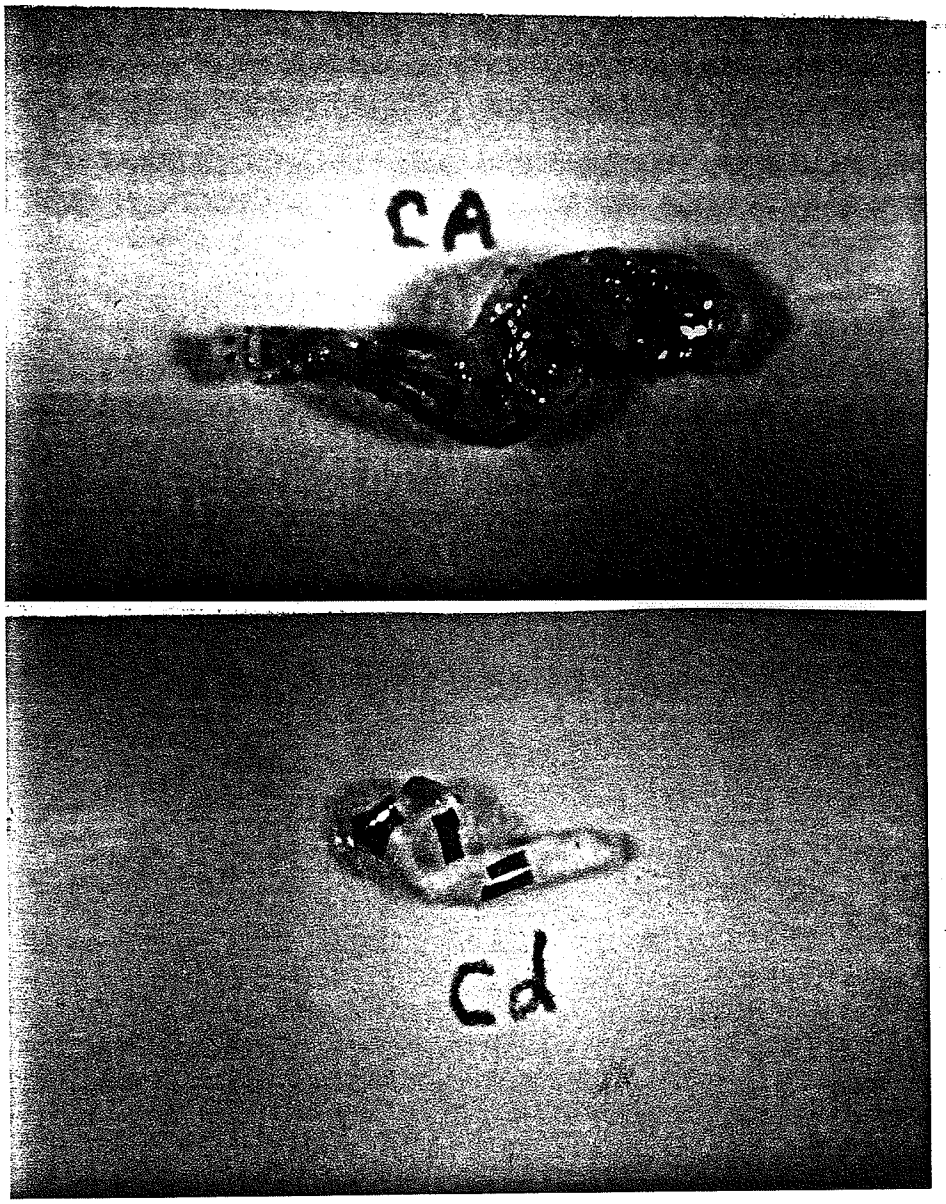


Figure 34. Photograph (a). Bones of a forelimb from a fetus (CA), presence of metacarpal and phalanges bones are evident.

Photograph (b). Bones of a forelimb from a fetus (CD), missing of metacarpal and phalanges bones are evident.

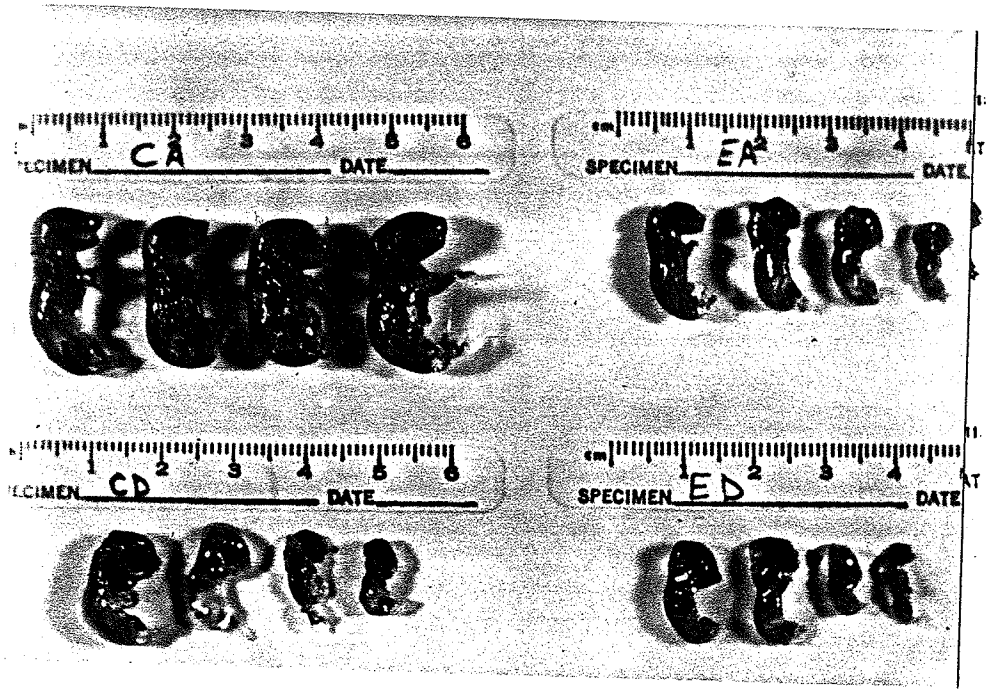
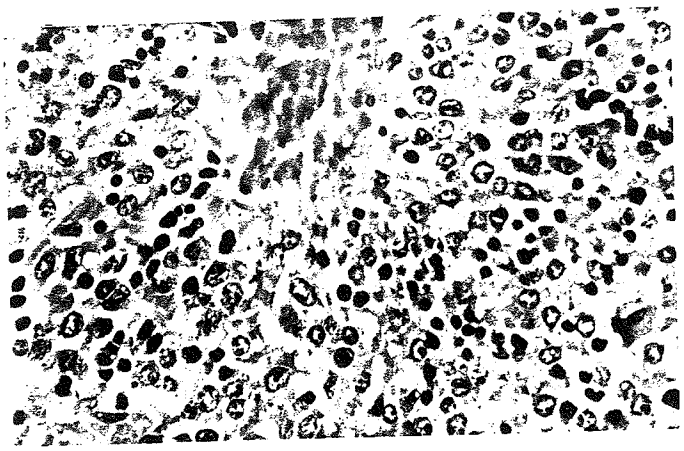


Figure 35. Photograph of fetal skeletons from all groups; iron-adequate diet without ethanol (CA), iron-deficient diet without ethanol (CD), iron-adequate diet providing 20% of the calories from ethnaol (EA) and iron-deficient diet providing 20% of calories from ethanol (ED). The differences in skeletons size within and among the groups are evident.

a



b

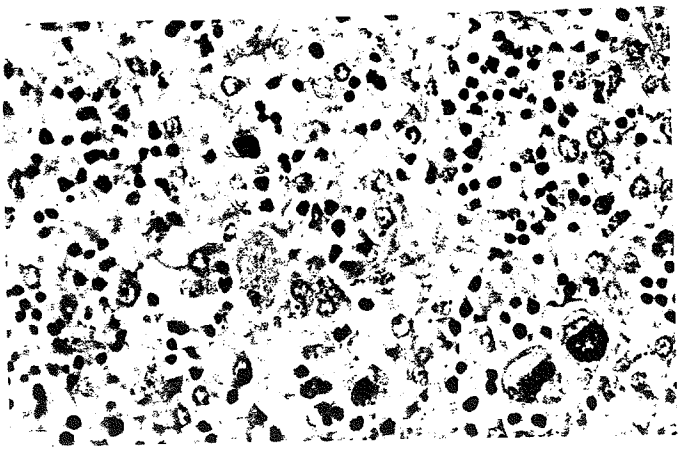


Figure 36 (a,b). Photomicrographs of fetal livers (CD and ED, respectively), marked dimunition of hematopoietic activity is evident (Hematoxyline-eosin stain; x 400).

B. Discussion

Significant physiological changes take place during the course of human pregnancy. The physiological adaptive mechanisms of pregnancy involve marked changes in systemic response factors of the mother as well as in metabolism. These adaptive mechanisms may modify the response to ethanol consumption in pregnant women. It is unfortunate that little information is available about ethanol metabolism in females; most of the studies involving ethanol have been done in males. However, Jones and Jones (1976) and Morgan and Sherlock (1977) found that females were more susceptible to the physiological effects of ethanol than males. On the other hand, chronic ethanol consumption during pregnancy is always complicated with nutritional problems (Ouellette et al., 1977). Iron and folate deficiencies are the most commonly recognized forms of nutritional deficiency in pregnancy (Prasad, 1978; and Herbert et al., 1975). Although many studies indicate a direct ethanol-folate interaction, little attention, if any, has been given to an ethanol-iron deficiency interaction. Iron-ethanol studies have always been related to iron overload in cases of alcoholism (Williams, 1975; and Prasad, 1978). MacDonald (1963) postulated that this iron overload was due to increased iron absorption secondary to the heavy ingestion of iron contained in wine. In another study, Charlton et al. (1964) suggested that alcohol increased iron absorption at the luminal level. However, Celada et al. (1978) found a decrease in total iron absorption in alcoholic patients, while in an earlier study they found that organic iron absorption was diminished by ethanol consumption (Celada et al., 1977). Investigations with rats drinking ethanol solution over long periods of time did not show an increase in iron absorption

(Murray and Stein, 1965; and Loh and Juggi, 1975). Prevalence of iron deficiency during pregnancy and the recent information about iron absorption in alcoholics suggested the need to study the effect of iron deficiency combined with chronic ethanol consumption on the maternal organism and her products of conception.

In Experiment 2, significant decreases in body weight of animals prior to and during pregnancy were observed with iron deficiency and/or ethanol consumption (Tables 11 and 14). In contrast, Toskes et al. (1974) reported no significant difference in body weights between the control and iron-deficient male rats. However, other investigators reported decreases in maternal body weights because of ethanol consumption (Kronick, 1976; and Tze and Lee, 1975). Previously (Experiment 1), no significant difference in body weights was observed between pregnant mice fed 0 or 20 percent EDC diet or in the daily caloric intakes of these groups. In Experiment 2, decreases in daily caloric intakes were observed during pregnancy in animals fed iron-deficient diets (CD and ED), while no significant difference in daily caloric intake was observed with feeding 20 percent EDC prior to and during pregnancy. Also, iron-deficient diets fed prior to pregnancy had no significant effects on daily caloric intakes (Tables 11 and 14). Since we observed significant decreases in maternal body weights and daily caloric intakes in iron-deficient groups, it can be concluded that maternal iron deficiency had adverse effects on the animals which may be of the same magnitude as the maternal ethanol effects.

As shown in Table 12, liver weights prior to pregnancy were significantly increased only in animals fed iron-deficient diets. Significant increases in liver total lipids were observed when dietary iron was deficient or ethanol was ingested. However, on day 18 of gestation

(Table 15), liver weights of iron-deficient mice or mice consuming 20 percent ethanol-derived calories were not significantly different from those of control animals. Total lipid contents of the liver were high in the ethanol-fed groups, but not in the group with only iron deficiency. The iron content of the diets had no significant effects on alcohol dehydrogenase or blood ethanol concentration. Therefore, it can be concluded that iron had no direct effect on ethanol metabolism.

Most investigators agree about the relationship between alcohol abuse and development of fatty liver. Lieber (1975) explained the alcoholic fatty liver by a direct toxic effect of ethanol, since the task of ethanol oxidation falls primarily on hepatocytes, which switches from fat to ethanol as a preferred fuel. This change promotes the oxidation of ethanol and raises the NADH/NAD ratio and decreases fatty acid oxidation which leads to fat deposition in the hepatocytes. On the other hand, Davidson (1975) reported that alcohol consumption causes malnutrition and interferes with lipotropic factors which in turn are responsible for fat deposition in the liver. In the present experiment, as in Experiment 1, liver total lipids were significantly increased in ethanol-fed groups (Tables 12 and 15). Values from chemical analysis for total lipid content in maternal livers were positively correlated with the intensity of fatty changes determined by histological examination, and support the well-known development of fatty liver in alcoholism.

A significant increase in alcohol dehydrogenase (ADH) activity was also observed in the ethanol-fed groups in the present study. This finding is in agreement with reports of Raskin and Sokoloff (1970); Mirone (1965); and Sze *et al*. (1976) that there is an increase in ADH activity due to chronic ethanol ingestion.

Folate deficiency is very common among alcoholics as well as during pregnancy. Low folate levels in alcoholics have been attributed to malnourishment (Herbert et al., 1963), alcoholic interference with folate absorption (Halsted et al., 1967) or folate metabolism (Lane et al., 1973). Although several explanations have been given concerning how ethanol affects folate metabolism, no information is available on the effect of ethanol consumption on the enzymes involved in folate metabolism. Also, recent research indicates the importance of folate binding proteins in folate metabolism (Colman et al., 1981), but the effect of ethanol upon folate binding protein has not yet been explored. On the other hand, maternal folate deficiency is probably due to malnourishment and an increased requirement because of synthesis of new cells in very large numbers (Rodriguez, 1978).

Folate deficiency, secondary to iron deficiency, has been reported by some investigators (Velez et al., 1966; Roberts et al., 1971; and Toskes et al., 1974). Das et al. (1978) observed covert folate deficiency in fifteen patients with apparently uncomplicated iron deficiency anemia. The effect of iron deficiency on folate metabolism is poorly understood. Toskes et al. (1974) suggested that folate deficiency, secondary to iron deficiency, is probably due to an additional demand for folate caused by increased ineffective erythropoiesis, while Vitale et al. (1966) observed a decrease in the glutamate formaminotransferase activity, an iron-dependent enzyme, involved in folate metabolism, in the livers of iron-deficient rats. However, Roberts et al. (1971) indicated that iron deficiency had no effect on this enzyme. On the other hand, Das et al. (1978) suggested that masked folate deficiency in patients with uncomplicated iron deficiency anemia

may be unmasked by treatment with iron alone due to an increase of hematopoietic activity. Experiment 2 indicates significant decreases in body folates, measured as serum folate and red cell folates, in the groups fed iron-deficient diets (Tables 13 and 16). Therefore, it can be concluded that iron deficiency can induce secondary folate deficiency in mice.

Our data show significant decreases in body folates, measured as red cell folates, in groups fed ethanol-containing diets. However, no significant difference in serum folate was observed in ethanol-fed groups. Jacob et al. (1976) indicated that red cell folate values are a better indicator of the folate status than serum folate value. Plasma folates are merely a reflection of recent intake. Folate deficiency in alcoholics was reported by Herbert et al. (1963) who observed that 93 percent of alcoholic patients examined had low serum folate activity (< 5.7 ng/ml). However, these investigators did not determine if folate deficiency was due to malnutrition or ethanol interference since dietary assessments were not performed. In addition, they did not measure red cell folates.

Dihydrofolate reductase is known as an essential enzyme which catalyzes the reduction of the oxidized folate into the active form as well as dihydrofolate, formed in thymidylate synthesis, into tetrahydrofolate. Evans (1978) indicated that the teratogenic effects of some anticonvulsants in mice were due to interference in folate metabolism through inhibition of dihydrofolate reductase. In the present experiment, a significant decrease in dihydrofolate reductase activity was observed prior to and during pregnancy in the groups maintained on iron-deficient and/or ethanol-providing diets (Tables 12 and 15). Although there is no data on the effect of ethanol upon dihydrofolate reductase activity, Lieber (1975) indicated

that NADPH (the source of electrons for the dihydrofolate reductase-dependent reaction, Mathews et al., 1963), is required as a cofactor for the Microsomal Ethanol Oxidizing System (MEOS). Lieber (1975) also indicated that the NADP/NADPH and NAD/NADH systems are linked and that both are affected by ethanol consumption. Accordingly, the depression of dihydrofolate reductase activity in the ethanol-fed groups might be attributed to the depression of NADPH/NADP system, also it might be due to ethanol effect on hepatocytes. Since we observed a decrease in blood folates and dihydrofolate reductase activity in groups fed iron-deficient and/or ethanol-providing diet, it can be concluded that adverse effects of maternal iron deficiency and/or ethanol consumption on the fetus may be mediated via alteration of folate metabolism.

Significant decreases in iron status as evidenced by low hemoglobin concentration, hematocrit, serum iron and percentage saturation of transferrin levels (Tables 14 and 17) were found when the diets were deficient in iron. These findings are in agreement with those reported by other investigators (Toskes et al., 1974; and Shepard et al., 1980). Toskes et al. (1974) reported a significant decrease in hemoglobin concentration and serum iron of male rats fed an iron-deficient diet. Shepard et al. (1980) demonstrated a significant decrease in hemoglobin concentration of pregnant rats fed an iron-deficient diet.

In Experiment 2, significant decreases in blood hemoglobin and hematocrit values prior to pregnancy were observed in animals fed diets containing ethanol. However, no significant changes were observed in serum iron and the percentage saturation of transferrin with ethanol-containing diets. These findings are comparable to those of Herbert et al. (1963),

where low hemoglobin concentration was reported in alcoholic patients. In contrast, Loh and Juggi (1975) found no significant differences in hemoglobin concentration and hematocrit percentage between the control and alcohol-fed rats. On day 18 of pregnancy, we observed decreases in hemoglobin concentration and percentage saturation of transferrin in animals fed ethanol-containing diets. No significant differences in hematocrit percentages or serum iron measured on day 18 of gestation were observed in groups fed ethanol-containing diets. The results of histological study of some of the fetal livers in Experiments 1 and 2 showed a decrease in the hematopoietic activity. These findings suggest that ethanol consumption has an adverse effect on the iron status of mice. Blood ethanol concentration was not influenced by the iron content of the diet (Tables 13 and 16).

As previously reported (Experiment 1), maternal ethanol consumption in Experiment 2 had significant adverse effects on gestational performance in mice as indicated by a significant increase in the number of resorptions per litter and significant decreases in the number of live fetuses per litter and the live fetal weights. Data on gestational performance also indicated similar adverse effects of maternal iron deficiency as evidenced by significant decreases in the percentages of live fetuses per litter and live fetal weights as well as a significant increase in the percentages of resorption of implantations per litter. In this experiment, abnormal gestational patterns were observed in iron-deficient and/or ethanol-fed groups as evidenced by small litter size, no implanatations in one horn of the uterus, total fetal resorption or dead fetuses in one horn and assymmetrical arrangement of live fetuses between the two uterine horns (Figures 24 to 27).

As previously observed (Experiment 1), significantly increased numbers of fetal morphological defects (external, internal and skeletal) were observed in groups maintained on ethanol-containing diets (Tables 19 and 20). Also, significantly increased numbers of fetal morphological defects were observed in groups fed iron-deficient diets. These findings of the effects of iron deficiency are comparable to those of the recent study (Shepard et al., 1980) where the effects of severe iron deficiency on reproductive performance and fetal growth in rats was investigated. They had female rats fed iron-deficient diets (5-10 ppm iron) prior to and during pregnancy and found a significant decrease in the numbers of viable fetuses, lower fetal weight, significant increases in fetal resorption per dam and growth defects. However, they failed to observe any significant bone defects. Also, O'Dell et al. (1961) reported a decrease in hemoglobin concentration and increased eye defects in fetuses born to rats maintained on iron-deficient diets. In contrast, Sisson and Lund (1958) failed to demonstrate a reduction in hemoglobin concentration in fetuses born to rats fed iron-deficient diets.

Although some of the maternal characteristics, such as body weight and hemoglobin concentration, were different between our two experiments, the adverse effects of chronic maternal ethanol consumption on maternal and fetal growth in mice were successfully reproduced. These confirm the teratogenic effects of ethanol in mice.

Our experimental data indicate that severe maternal iron deficiency or chronic maternal ethanol consumption as well as the combined effect of these two factors have adverse effects on the reproductive performance of mice. Also, our results indicate that iron deficiency and/or ethanol

consumption prior to and during pregnancy interfere with folate metabolism. In addition, red cell folates were positively correlated with the percentages of live fetuses per litter and fetal weight and negatively correlated with resorption of implantation per litter. These findings combined with the fact that folates are required in higher amounts during pregnancy and that folate deficiency is teratogenic (Nelson, 1952) suggest that the adverse effects of severe iron deficiency and/or maternal ethanol consumption on the maternal and fetal organism are mediated via alteration of folate metabolism.

CHAPTER VI

SUMMARY

The present study was undertaken to determine the influence of chronic alcohol ingestion on the maternal organism and the fetus under conditions of nutritional adequacy and the effects of chronic ethanol ingestion and/or iron deficiency on reproductive performance as well as iron and folate metabolism. A mouse model, CBA/J strain, was employed, and morphological and biochemical assessments were performed.

In Experiment 1, the influence of chronic ethanol consumption, using isocaloric nutritionally-adequate liquid diets, was investigated. The results of Experiment 1 indicated that liquid diets can be successfully used to study the effects of maternal alcoholism in mice. Chronic maternal alcoholism adversely affected maternal and fetal growth of CBA/J mice, the degree of this effect varied as a result of the level of ethanol in the diet. Total reproductive failure, low body weight, decreased daily caloric intake, high blood ethanol, low hemoglobin concentration and liver necrosis were noted in the group which had 30 percent of their calories derived from ethanol (EDC) suggesting that ethanol at this level was toxic. Ethanol fed to provide 10 or 20 percent of total kilocalories adversely affected maternal and fetal development as evidenced by increased liver total lipids as well as an increased percentage of resorption of implantation sites, decreased fetal weights and increased gross morphological fetal defects. In addition, similar fetal anomalies were present when ethanol was withdrawn prior to mating. Ethanol at the level of 20 percent EDC produced in fetal mice effects comparable to the fetal alcohol syndrome in human studies.

Experiment 2 was performed to determine the adverse effects of maternal iron deficiency and/or ethanol consumption on maternal and fetal mice. A 2 x 2 factorial design was employed, with ethanol-derived calories and iron as independent variable. Isocaloric liquid diets adequate or deficient in iron without or with ethanol (20% EDC) were used. Experiment 2 was also designed to determine the effect of maternal iron deficiency and/or ethanol consumption on folate metabolism. The results of this experiment indicate that hemoglobin concentration, percentage saturation of transferrin, red cell folate and dihydrofolate reductase activity were decreased by iron deficiency and/or ethanol consumption. Liver total lipids and alcohol dehydrogenase activity were increased by ethanol consumption. Iron deficiency had no effect on blood ethanol level or alcohol dehydrogenase activity. Iron deficiency and/or ethanol consumption adversely affected gestational performance in mice as evidenced by an abnormal pattern of gestation, increased fetal resorption and decreased fetal weights as well as an increase in the number of external and internal morphological and skeletal defects in fetuses. Positive correlations were noted between serum iron and red cell folates and between red cell folates and the percentage of live fetuses/litter and fetal weight while a negative correlation was noted between red cell folate and the percentage of fetal resorptions/litter.

Folate deficiency or altered folate metabolism secondary to iron deficiency and/or ethanol consumption prior to and during pregnancy were seen. These findings suggest that the adverse effects of iron deficiency and/or ethanol consumption on maternal CBA/J mice, their reproductive performance and fetuses were mediated via alteration of folate metabolism.

Appendix 1. Analysis of variance for a 2 x 2 factorial design of mice characteristics prior to pregnancy

	F Value			
	Treatment	Ethanol	Iron	Ethanol x iron Interaction
Body weight				
At initiation of liquid diet	2.21[1]	-	-	-
At mating	4.64**	1.45	5.20**	7.28**
Daily caloric intake 4 weeks before mating	0.27	0.32	0.15	0.35
Liver weight	3.49**	0.41	9.70*	0.41
Total lipids	4.76**	7.66*	0.32	6.31*
Alcohol dehydrogenase (ADH)	11.61*	17.05*	0.01	0.50
Dihydrofolate reductase	9.61*	10.50*	9.92*	8.41*

*F values presented with level of significance $P < 0.01$.

**F values presented with level of significance $P < 0.05$.

[1]Body weight at the beginning of the experiment was not significant using general analysis of variance.

Appendix 2. Analysis of variance with a 2 x 2 factorial design of blood parameters of mice prior to pregnancy

Blood parameter	F Value			
	Treatment	Ethanol	Iron	Ethanol x iron interaction
Hemoglobin	23.28*	30.32*	37.34*	2.18
Hematocrit	7.71*	5.75*	16.74*	0.63
Serum iron	28.75*	0.04	83.39*	2.82
Percentage saturation of transferrin	21.92*	0.23	65.28*	0.27
Serum folate	7.04*	1.63	18.76*	0.73
Red blood cell folate	14.97*	10.33*	18.35*	16.23*

*F values presented with level of significance $P < 0.01$.

Appendix 3. Analysis of variance for a 2 x 2 factorial design of maternal characteristic of mice during pregnancy

	F Value			
	Treatment	Ethanol	Iron	Ethanol x iron interaction
Body weight (day 18 of pregnancy)	15.31*	21.63*	14.47*	9.84*
Daily caloric intake during pregnancy	7.22*	2.69	18.43*	0.54
Liver weight	2.35	1.29	5.58*	0.32
Total lipids	4.91**	13.74*	0.18	0.81
Alcohol dehydrogenase	12.13*	30.38*	3.97	2.03
Dihydrofolate reductase	21.99*	38.77*	22.42*	4.77**

*F values presented with level of significance $P < 0.01$.

**F values presented with level of significance $P < 0.05$.

Appendix 4. Analysis of variance for a 2 x 2 factorial design of maternal blood parameter of mice during pregnancy

	F Value			
	Treatment	Ethanol	Iron	Ethanol x iron interaction
Hemoglobin	50.13*	6.34**	127.31*	16.46*
Hematocrit	31.62*	1.52	90.79*	2.53
Serum iron	36.32*	2.05	106.11*	0.78
Transferrin percent saturation	52.73*	5.09**	152.15*	0.95
Serum folate	11.42*	2.17	32.10*	0.01
Red cell folate	32.87*	35.04*	35.83*	27.73*
Blood ethanol	0.80	-	-	-

*F values presented with level of significance $P < 0.01$.

**F values presented with level of significance $P < 0.05$.

Appendix 5. Analysis of variance for a 2 x 2 factorial design of gestational performance in mice[1]

	F Value			
	Treatment	Ethanol	Iron	Ethanol x iron interaction
Implantation number/litter	4.82**	13.39*	0.08	0.97
Percent of resorption/litter	8.19*	16.63*	4.59**	3.37
Percent of dead fetuses/litter	8.92*	15.01*	18.59*	2.09
Percent of live fetuses/litter	33.94*	58.70*	32.30*	10.82*
Fetal weight	27.31*	21.48*	51.37*	9.07*

* F values presented with level of significance $P < 0.01$.

** F values presented with level of significance $P < 0.05$.

VITA

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