THE FETAL ALCOHOL SYNDROME IN MICE
AN ANIMAL MODEL

by

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ABSTRACT

Although the adverse effects of prenatal exposure to alcohol have been suggested since antiquity, only recently has a 'fetal alcohol syndrome' been described in human beings. Since ethical considerations limit the types of studies possible with humans, an animal model was developed.

CBA and C3H female mice, maintained on a liquid Metrecal-ethanol diet, received from 0 to 35 percent ethanol derived calories (EDC) for at least 30 days prior to and throughout gestation. Prenatal death and fetal abnormalities on day 18 of gestation were related to maternal blood alcohol levels which increased with increasing EDC. Reduced fetal weights, skeletal, and neural anomalies were observed at both low and high maternal blood alcohol levels, while cardiac and ocular malformations, similar to those observed in the human syndrome, exhibited both a dose-response effect and strain difference in liability, indicating that maternal chronic alcoholism is embryolethal and teratogenic in mice.

In a second experiment, the strain difference in liability was investigated using CBA, C3H, and C57 females maintained on a 20 percent EDC diet and mated in a diallele cross. Prenatal death, malformations, and fetal weights were directly related to maternal blood alcohol levels,

indicating a maternal effect. Fetal abnormalities and maternal blood alcohol levels varied with maternal strain (CBA > C3H > C57), and were inversely related to maternal alcohol dehydrogenase activity. Microsomal ethanol oxidizing system induction was directly associated with increased fetal abnormalities, being greatest in CBA females and lowest in C57.

The results of this study indicate that malformations observed in the mouse and human syndrome are similar, and that liability for these malformations is dependent on maternal blood alcohol levels, which are determined by the rate of maternal alcohol metabolism as well as the amount of maternal alcohol consumption.

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INTRODUCTION

> "Betsy Martin, widow, one child and one eye. Goes out charing and washing by day; never had more than one eye, but knows her mother drank bottled stout, and shouldn't wonder if that caused it". Charles Dickens-1836

Alcohol, claimed by some to be the evil of mankind, and by others to be the salvation for a troubled mind, has been a controversial subject of medical research for at least 200 years. Recently, the description of a fetal alcohol syndrome in humans has contributed further to the controversy, and the question posed by Dickens' Betsy Martin still awaits an answer.

The fetal alcohol syndrome, which has been observed in offspring of chronic alcoholic women who continue heavy drinking during pregnancy, is characterized by developmental and psychomotor delay, pre- and postnatal growth deficiency, impaired intellectual performance, and craniofacial, cardiac, and joint anomalies (Jones et al., 1973). Because of the magnitude of alcohol abuse in North America, the existence of a fetal alcohol syndrome has major significance for the prevention of congenital malformations and mental retardation. However, it can, and has been, argued that alcoholism is only one variable common to the syndrome, and that other factors such as maternal malnutrition, disruptive life-style, and poor prenatal care may contribute to the malformations observed. While these arguments may not be of significance

to the clinician dealing with affected children, they are of relevance when delineating the etiology and possible prevention of the syndrome, and therefore are needed subjects of investigation.

Unfortunately, experiments to elucidate the possible etiologic variables of the syndrome are not feasible using human beings. Controlling confounding variables would necessarily impose restraints on individual freedom that would be of questionable ethics. Most important would be the question of allowing an alcoholic woman to continue a supervised pregnancy knowing there is an elevated risk for an affected offspring, regardless of the exact etiology. For these reasons, the only practical approach to studying the pathogenesis of this syndrome is by developing an appropriate animal model. Once such a tool is developed, it will be possible to determine the exact etiology of the syndrome as well as investigate several other clinically important implications of the syndrome.

Historical Perspective To 1973

The use of drgus to produce altered states of consciousness likely originated with prehistoric man, and alcohol, the product of fermentation, has probably been used by man as an intoxicating agent since the invention of glazed pottery (Green, 1974). With the use of alcohol by many cultures over long periods of time, various myths and suspicions have arisen surrounding its effects. One such suspicion, which existed over several cultures, was that alcohol intoxication at the time of conception or during pregnancy could result in abnormal offspring. Thus, Carthage and Sparta inacted laws prohibiting newly wed couples from drinking spirits on their wedding night; Vulcan, the deformed blacksmith

in Greek mythology was the result of Jupiter's drunkeness; and the Holy Bible declares in Judges 13:3-4, "Behold now, thou art barren, and barest not; but thou shall conceive, and bear a son. Now therefore beware, I pray thee, and drink not wine or strong drink, and eat not any unclean thing".

The first documented reports to support those early suspicions appeared as a result of the English Gin Epidemic from 1720 to 1751. During this period, a law lifting traditional restrictions on distilling was put into effect providing new grain markets for the aristocratic farm interests. This resulted in an abundant supply of cheap gin to the English populace that eventually led to a social epidemic of major proportions. Birth rates dropped, death rates, especially for children under five, increased, eventually prompting the College of Physicians to petition Parliament for control on the distilling trades, citing gin as "A cause of weak, feeble, and distempered children" (quoted in George, 1965, p. 33). In such a social climate, it is not surprising that abstinence groups formed and often used the notion of prenatal insult as one of the evils of alcohol (see reviews by Ballantyne, 1902; Green, 1974; Warner and Rosett, 1975). Anecdotal reports, which seldom held scientific validity, were quoted by abstinence preachers to build arguments such as that stated in a temperance manual from 1849 which claimed, "Facts abundantly show that the children of mothers who drink alcohol are more likely than others to become drunkards, and in various other ways to suffer. Often they are not so large and healthy as other children. They have less strength of eye-sight, less firmness of quietness of nerves, less capacity of great bodily and mental achievement, and less power to withstand the attacks of disease or the vicissitudes of climates

and season" (quoted in Warner and Rosett, 1975, p. 1402).

By the late 1800s, animal experiments in teratology were being initiated in France, and the first experimental studies on the dangers of prenatal exposure to alcohol began. Treating chicken eggs by either exposure to alcohol vapor or by direct injection into the egg white, Fere (cited by Ballantyne, 1902, p. 274) produced a variety of malformations in chicks. However, no malformations were observed by Mairet and Cambemale in the offspring of cocker spaniel dogs exposed to alcohol during pregnancy (cited by Sandor and Elias, 1968, p. 53). Thus, the findings of these earliest experiments on the effects of prenatal exposure to alcohol were in direct disagreement, signalling the start of many years of contradictory reports.

This early scientific controversy on the effects of alcohol was best exemplified by Ballantyne in his 1902 classic monograph, Manual of Antenatal Pathology and Hygiene. In his review of alcoholism, Ballantyne (1902, p. 272-277) notes that soon after the first animal experiments were reported, Fournier, noting a similarity between alcoholism and syphilis in causing structural defects, reported observing ectrodactyly, defects of the occipital bone, hydrocephaly, cranial asymmetry, porencephaly, microcephaly, and infantilism in children of alcoholic parents. Support for this finding was cited in Carrara's 1899 report of neural malformations in the offspring of guinea pigs exposed to alcohol. However, in a subsequent study in rabbits prenatally exposed to alcohol, Ballantyne notes that no malformations were observed.

At the turn of the century, the demonstration that alcohol crossed the placenta in rabbits, guinea pigs, and human beings, supported the notion that alcohol could have deleterious effects on the developing

embryo and fetus (Ballantyne, 1902). Of particular interest were two papers by Nicloux (cited by Ballantyne, 1902, p. 273). The first study, using guinea pigs, found blood alcohol levels approximately equal in the mother and fetus after a one hour equilibration period. Similarly, in pregnant women given 60 cc of rum approximately one hour prior to delivery, examination of fetal cord blood as well as placenta revealed easily detectable amounts of alcohol, verifying that in humans and animals, alcohol could cross the placenta and enter the fetus.

During this same period, W.C. Sullivan (1899) used newly developed epidemiologic techniques to study the effects of maternal alcoholism in a Liverpool prison population. Of the 600 observed offspring born to 120 female alcoholics, 55 percent were stillborn or died under two years of age; a significant increase over a control population with a stillbirth and infant mortaility rate of 24 percent. This death rate of offspring from alcoholic mothers increased over succeeding pregnancies, with 34 percent for first born and 72 percent for sixth to tenth born. If the mothers began to drink at least two years prior to their first pregnancy, the perinatal mortality rate increased to 62 percent for first borns. Of particular interest was the observation that women who had adverse pregnancies while alcoholic later bore healthy children when forced to abstain during pregnancy because of imprisonment. This finding warranted a pun from Ballantyne (1902, p. 275): "For the female habitual drunkard, it is apparently the best thing to be committed for a term of imprisonment early in her pregnancy; the prison baby may be the best! A sad fact, but a fact pregnant with hope."

Following Sullivan's initial reproductive study, several investigators, in a variety of institutional settings, reported an association

between parental alcoholism and offspring with epilepsy, idiocy, and feeblemindedness (see review by Warner and Rosett, 1975). Then in 1910, Elderton and Pearson reported a study of school children from Edinburgh and Manchester that failed to demonstrate a relation between maternal alcoholism and abnormal physique, intelligence, or disease in the offspring. Instead, they concluded that the low birthweights and observed abnormalities were the result of sociological factors which might be associated with the tendency for abusive use of alcohol. This conclusion caused a furor among abstinence preachers, and also pointed out the necessity for further animal studies in a controlled laboratory setting.

For the next several years, investigators using different animals and administration techniques reported contradictory findings in offspring prenatally exposed to alcohol. Most exhaustive was the work of Stockard using fish embryos of Fundulus heteroclitus, guinea pigs, and chickens. His earliest work on fish blastomeres exposed to 2-5 percent dilutions of alcohol reported ocular malformations with the extremes of cyclopy and micropthalmia (Stockard, 1910). This was followed by a ten year study on guinea pigs in which matings were made between normal females and alcoholized (exposed to alcohol vapor) males, or between alcoholized females and normal males. The latter cross resulted in offspring with elevated perinatal mortality rates and increased rate of malformations. These effects persisted through several untreated generations. Because of the popularity of the then recently rediscovered genetic laws of Mendel, this result was interpreted as being due to the toxic action of alcohol on the germ cells (Stockard, 1913; 1923). Repeating Fere's experiments with chicken eggs exposed to alcohol vapor, Stockard (1914) reported 100 percent malformed embryos after 14 to 20

hours of treatment, and embryolethality with treatment beyond 23 hours. Malformations resulted from eye, neural and limb bud maldevelopment, as well as general retardation of embryonic growth.

In contradiction to Stockard's positive findings, Pearl (1917) reported that treating hens for one hour with alcohol vapors resulted in fewer viable eggs, however, those that did hatch were superior to a control series in terms of mortality rate. Again using the popular genetic notions of the day, the author suggested that alcohol was useful as an interuterine selection agent, since only the most resistant embryos would reach hatching. In 1923, McDowell replicated Stockard's guinea pig experiments using mice and found an increase in prental mortality but no increase in the frequency of malformations. Similar negative results were reported by Nice (1912; 1917) in a series of experiments using albino mice, and by Hanson and Cooper (1930) in a study examining ten generations of descendants from an original group of mice treated with alcohol vapor. But most damaging to Stockard's credibility was a study by Durhan and Woods (1932) that failed to find an increased malformation rate in the 6304 descendants of 83 female guinea pigs exposed to alcohol vapor.

The confusion resulting from these contradictory reports, as well as the social climate surrounding the end of the American prohibition, led to a general agreement that prenatal exposure to alcohol was not deleterious to embryonic and fetal development. Therefore, in a popular 1942 text on alcohol, Haggard and Jellinek attributed the fetal insult observed in offspring of alcoholic women to poor nutrition or home environment rather than to alcohol itself. This same attitude was reflected by Roe (1944) who argued that the high mortaility rate, epilepsy,

idiocy and psychosis observed in the offspring of alcoholic women were the result of social factors rather than physical or chemical damage due to alcohol.

Even in those animal studies that used the same route of administration, inconsistent results failed to provide insight into the action of alcohol on the embryo or fetus. Mirone (1952) was unable to produce malformed offspring in mice administered alcohol orally, however the offspring of female guinea pigs orally administered alcohol three to four times a week displayed specific malformations of the central nervous system including abnormal flattening of the gyri with resulting shallowness of fissures, cellular lesions in the cortex and basl ganglia, edema, dilation of blood vessels, hemorrhagic areas, and general retardation of myelination at birth (Papara-Nicholson and Telford, 1957). Lastly, rats orally administered alcohol prior to and during pregnancy delivered offspring that were normal at birth, but that failed to grow when nursed by alcohol treated mothers (Pilstrom and Kiessling, 1967).

Using a modification of Fere's early experiments, Sandor and Elias (1968) attempted to examine the pathogenesis of chick embryos exposed to alcohol. Two percent alcohol injected into the air space of eggs after 30 hours incubation resulted in a disturbance of circulation and growth retardation with generalized malformation of the brain vesicles and caudate nucleus, eventually resulting in chick mortality. By injecting eggs at 23 hours incubation and examining the embryos microscopically at 72 hours, three of twelve embryos were normal, while nine displayed deformed spinal chords, brain vesicles and somites, and developmental retardation (Sandor, 1968). These studies, which suggested the malformations occur in early organogenesis, were extended to rats. Females

were given 2 gm/kg alcohol intravenously on days six and seven of gestation, or 1.5 gm/kg alcohol intravenously on gestation days six through eight (Sandor and Amels, 1971). Half of each litter was removed and examined on day 9.5 of gestation, and the remaining half on day 19.5. In those embryos examined at the earlier period, there was an increase in resorption rate as well as an increase in the frequency of dysmorphology in central nervous system anlagen, while in those examined at 19.5 days, there was only an increase in fetal resorption and occurrence of bone retardation. This result suggests that prenatal exposure to alcohol in the rat most often results in lethal malformations, however in those offspring surviving, developmental retardation is more common than malformations.

This second finding in the rat was supported by a human study from the University of Washington that associated maternal alcoholism with 41 percent of a group of children underweight for their gestational age. Subsequently, 11 female alcoholics were identified retrospectively, and ten of their 12 children were small for gestational age. Five of the ten infants were retarded in performance on the Gesell or Denver developmental scales, and eight of the children failed to thrive given an adequate diet, with weight and head circumference remaining below the third percentile (Ulleland et al., 1970). Three years later, these eight children were to become the first cases to revive in a dramatic way, an awareness of the existence of a fetal alcohol syndrome.

The Fetal Alcohol Syndrome: 1973 to 1977

Clinical Studies

A follow-up study on the eight children noted by Ulleland to have

retarded growth was conducted by Jones and Smith at the Dysmorphology Unit, University of Washington. It was found that these children displayed a pattern of malformations characterized by growth deficiency, which was more severe with regard to birth length than birth weight; severe postnatal growth deficiency with linear growth rate averaging 65 percent of normal, and average rate of weight gain only 30 percent of normal; mental retardation with an average intelligence quotient of 63 in those children in whom it was measured; microcephaly; short palpebral fissures; joint anomalies that included congenital hip dislocations, inability to extend the elbows completely, camptodactyly of toes and inability to flex at the metacarpal-phalangeal joints; alterations of palmar crease patterns including rudimentary palmar creases, aberrant alignment of the palmar creases, and/or single upper palmar crease; cardiac anomalies, the majority of which were ventricular septal defects; and fine motor dysfunction manifested by a weak grasp, poor eye-hand coordination, and tremulousness in the newobrn period (Jones et al., 1973). The common prenatal factor shared by these children was a severe chronically alcoholic mother who drank throughout pregnancy; thus the term 'fetal alcohol syndrome' was coined to describe this specific pattern of malformations.

Once this initial description was published, case studies from around the world began appearing in the literature describing children with the syndrome (Barry and O'Nuallain, 1975; Bierich et al., 1976; Christoffell and Salafsky, 1975; de Chateau, 1975; Ferrier et al., 1973; Goetzman et al., 1975; Hall and Orenstein, 1974; Ijaiya et al., 1976; Jones and Smith, 1973; Loiodice et al., 1975; Loser et al., 1976; Manzke and Grosse, 1975; Mulvihill and Yeager, 1976; Mulvihill et al.,

1976; Noonan, 1976; Palmer et al., 1974; Reinhold et al., 1975; Root et al., 1975; Saule, 1974; Tenbrink and Buchin, 1975). As experience with the syndrome increased, anomalies other than those originally noted by Jones et al. were reported. Urogenital anomalies including crossed fused renal ectopia, renal hypoplasia with uretero-pelvic junction obstruction, and pyelocalyectasis were reported by Tenbrinck and Buchin (1975), and Goetzman et al. (1975). Cardiovascular anomalies initially reported in 70 percent of the cases were shown to occur in approximately 50 percent of the affected patients (Barry and O'Nuallain, 1975; Loser et al., 1976; Noonan, 1976). Autopsies on 13 children who died near or shortly after birth revealed extensive developmental anomalies of the brain including aberation of neuronal migration resulting in multiple heterotopias; fusion of the anterior superior gyri through infiltration by leptomeningeal hematomata of glial and neuronal cells; incompletely developed cerebral cortex as shown by relative agyria and large lateral ventricles; and agenesis of the corpus callosum (Jones and Smith, 1973; Jones, 1975; Clarren, 1977).

Of major importance in demonstrating the prevalance of the syndrome were three large studies from France, Russia, and the United States. Originally overlooked at the time of its publication, the first study, by Lemoine and colleagues in 1968, described 127 infants born to 69 French families in which there was chronic alcoholism. Twenty percent of these children had anomalies including cleft palate, micropthalmia, limb malformations, congenital heart disease, viseral anomalies, protruding forehead, sunken nasal bridge, short upturned nose, retracted upper lip, receding chin, and deformed ears. Many appeared hyperactive, had delayed psychomotor and language development, showed mental

retardation with an average I.Q. of 70, and, with increasing age had difficulty attending to tasks with resultant behavioral problems at school. In the Russian study describing 98 pregnancies to 18 chronically alcoholic women, 50 of the pregnancies ended in abortions, five in miscarriages, and one in a stillborn, giving a total perinatal mortality rate of 57 percent. Of the 42 resulting live births, 19 who were born before their mother developed alcohlism demonstrated vegative, emotional and behavioral disorders which improved with favorable microsocial change. However, in the 23 children born after prenatal exposure to alcohol, 14 were mentally retarded and demonstrated organic impairment of the central nervous system early in infancy (Shruygin, 1974). The University of Washington study examined dysmorphogenic features in 41 children born to chronic alcoholic women who continued to drink during pregnancy (Hanson et al., 1976). In addition to previously reported malformations of the fetal alcohol syndrome, two to five of the children had eye anomalies including micropthalmos, intraocular defects, strabismus, and ptosis of the eyelids; cleft palate; muscular skeletal anomalies including pectus excavatum; diaphragmatic anomalies; nail hypoplasia; and cutaneous anomalies including pigmented nevi and hirsuitism. Thus, these major studies from separate parts of the world, along with the many case reports, verified the long suggested association between maternal alcoholism and adverse outcome of pregnancy.

Animal Studies

Stimulated by the human data, several investigators attempted to replicate the observed malformations in animals. In vitro tests on day 9 and 10 rat embryos demonstrated a decrease in somites and retarded brain differentiation at a low alcohol concentration (0.1 mg ethanol

per ml), while embryolethality was observed at higher concentrations (10 mg ethanol per ml). Extending the study to in utero testing, 5 ml of 40 percent ethanol per kg was administered to 13 female rats on days 8-14 of gestation. Examination of fetuses at term revealed an increase in postimplantation mortality and a significant decrease in fetal weight; however, no teratological effects were observed (Skosyreva, 1973). When alcohol was orally administered in drinking water five weeks prior to and during gestation, litter size and fetal weight was reduced, but the only anomalies reported in the live offspring were microcephaly and generalized dermatologic problems (Tze and Lee, 1975). Thus, attempts to use the rat as a model were unsuccessful. Possible explanations for this failure are that the maternal blood alcohol levels (average of 61 mg per 100 ml blood in the Tze study) were not sufficient to cause fetal insult; offspring were not examined for internal malformations; and in the Tze study, offspring may have been cannibalized by the mothers at birth.

In contrast, mouse studies have been successful in producing alcohol induced malformations. Using the hybrid strain $B6D2F_1/J$ injected intraperitoneally with 0.030 ml per gram body weight of a 25 percent (v/v) solution of 95 percent ethanol, Kronick (1976) demonstrated that fetal malformations were most prevalent when maternal treatment occurred on days 8, 9, or 10 of gestation. Malformations included coloboma of the iris, which was prevalent in fetuses exposed on days 8 or 9; ectrodactyly of the forepaws, prevalent on day 10; hypoplastic atria, which was infrequently observed from all three treatment days; hydronephrosis, infrequently observed in fetuses treated on days 9 or 10; and exencephaly occasionally observed in fetuses treated on day 8 or 9. Fetal mortality increased from 7 percent with treatment on gestation

day 7 to approximately 45 percent when females were treated on day 10, 11, or 12. This study, which demonstrated critical time periods for both the teratogenicity and embryolethality of ethanol administered intraperitoneally, was supported by a second study using C57Bl/6J mice that were orally administered a nutritionally adequate diet of Metrecal-ethanol (Randall, 1977). Diets containing 17, 25, or 35 percent ethanol derived calories (EDC) were administered to pregnant females on days 5 through 10 of gestation resulting in blood alcohol levels ranging from less than 40 mg per 100 ml blood to 300 mg per 100 ml blood. Fetal mortality, measured as resorptions or fetuses dead at birth, increased from 15 percent at the lowest diet to 25 percent on the highest diet while malformations, including syndactyly, adactyly and ectrodactyly of the forepaws; micropthalmia and anopthalmia; cardio-vascular anomalies involving the major branches of the aorta as well as intercardiac abnormalities; hydronephrosis and hydroureter; and hydrocephalus and gastroschisis, increased from 8 percent at the low EDC diets to 40 percent at the highest diets. Although both these mouse studies reported malformations associated with the human syndrome, unlike the human syndrome, no clear pattern was observed. This failure to reproduce systematic malformations casts doubt on the applicability of equating prenatal exposure to alcohol during organogenesis with pre-natal exposure throughout gestation.

Behavioral Studies

The observation of extensive neural malformations in autopsied off-spring of chronic alcoholic women (Jones and Smith, 1973; Jones, 1975; Clarren, 1977) is consistent with reports of intellectual and behavioral abnormalities in children prenatally exposed to alcohol. In addition

to mental deficiency, I.Q. range of 45 to 105, 12 children with the syndrome exhibited behavioral aberations including fine motor problems, weak and primitive grasp, poor figer articulation and delay in establishing hand dominance. As preschoolers, they often had severe feeding problems, and tended to be hyperactive with poor attentional skills and problem solving abilities (Streissguth, 1976). In the 12 children of chronic alcoholic women who were measured for I.Q. at 7 years of age, six who lived with their mothers had a mean I.Q. of 73, six who had spent some time with relatives had a mean I.Q. of 84, and the control population had a mean I.Q. of 95. While this indicates than an enriched home environment might facilitate development in mildly affected children with only borderline retardation, children with the most severe stigmata of the syndrome often were the most severely retarded, and did not improve in spite of excellent postnatal care (Jones et al., 1974).

In studies on neonates prenatally exposed to alcohol, decreased rapid eye movement and quiet sleep, as well as increased fussiness and crying have been reported (Landesman-Dwyer and Keller, 1977). In addition, decreased activity with poor muscle tone and habituation as evaluated by the Brazelton Neonatal Behavioral Scale has been noted (Streissguth and Barr, 1977) as has reduced learning in an operant testing situation (Martin, 1977). Unfortunately the significance of these findings is unclear. Because of hospital discharge procedures, the infants were tested in the first three days of life, and the results may have been confounded by anesthesia or other medications given at the time of delivery which were not fully eliminated from the infant at the time of testing. Furthermore, since there is no evidence that performance during the neonatal period is predictive of later learning

abilities, meaningful results must await further studies when the children are older.

Animal studies on behavior and learning in offspring prenatally exposed to alcohol, while generally supportive of the human data, fail to give unequivocal results. Learning to discriminate contingencies in a shock reinforcement punishment situation was deficient when rats, prenatally exposed to alcohol from day one of gestation to weaning were tested at eight months (Martin et al., 1977). Similarly, exposure to alcohol from day two of gestation to weaning impaired T-maze and shuttle-box learning when tested at 21 to 33 days post parturition (Shaywitz et al., 1976). This same deficiency in shuttle-box learning was observed when rats, exposed to alcohol throughout gestation, were tested at 1.5 months. However, when these animals were tested at a later age, 4.5 and six months, the deficiency was not observed, indicating that the nature of the early deficit in shuttle-box learning was transient (Auroux, 1973). In two out of three studies, ambulation in an open-field situation was increased in rats prenatally exposed to alcohol; however with age, the activity decreased (Martin, 1977; Bond and Digiusto, 1976; Shaywitz et al., 1976). While these studies are of interest, the results should be interpreted carefully, because as mentioned earlier, prenatal exposure to alcohol has not been shown to produce the characteristic morphologic abnormalities of the fetal alcohol syndrome in neonatal rats (Sandor and Amels, 1971; Skosyreva, 1973; Tze and Lee, 1975). This fact, along with the confounding effects of continued alcohol administration through weaning, make conclusions from these studies tentative at best.

In a series of studies using mice, behavioral abnormalities in

animals prenatally exposed to alcohol were reported. Offspring of C57Bl/10Bg and DBA/1/Bg females administered alcohol in drinking water prior to pregnancy and through day 14 post-parturition displayed a reduction in latency to attack and fighting when measured as adults (Yanai et al., 1976). When the offspring were tested for ambulation, unlike the rat studies, scores were reduced with a noted increased latency to leave the initial square (Ginsberg et al., 1976). Of particular interest was the finding that C57Bl mice perinatally exposed to alcohol had an increased susceptibility to audiogenic seizures. However, when cross fostering techniques were employed to separate the pre- and postnatal effects of alcohol on this increased susceptibility, it was found that the major determinant was the postnatal exposure (Yanai et al., 1975). Therefore, while these studies demonstrate that early exposure to alcohol can produce behavioral changes, the data indicate that at low levels of alcohol exposure (maternal blood alcohol levels ranging from 20 to 45 mg per 100 ml blood), it is the early postnatal exposure that is critical. This postnatal effect could be the result of faulty neural maturation in early life, either as a direct insult from alcohol, or from a decrease in the quantity or quality of maternal milk production. It is clear that behavioral studies of the future, if they are to produce meaningful results, must employ cross fostering techniques.

Biochemical Studies

Biochemical studies have been performed on rats pre- and postnatally exposed to alcohol in an attempt to explain the functional deficits observed in the syndrome. By feeding pregnant animals alcohol for two weeks prior to term and through lactation, protein synthesis measured by leucine incorporation into ribosomes was decreased in both

fetal and neonatal brain, heart and liver (Rawat, 1975a; 1976; 1977). Using the same treatment protocol, acetylcholine, the brain excitatory neurotransmitter, was decreased in prenatally exposed fetuses and neonates, while the levels of gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter and its precursor, glutamate, were increased (Rawat, 1975b). Recently, the demonstration that GABA-transferase and glutamate decarboxylase activities are decreased in fetal and neonate brains of animals exposed to alcohol led Rawat (1977) to suggest that the metabolism of GABA and glutamate is reduced, resulting in the higher observed levels. While it is tempting to interpret these alterations in neurotransmitter levels and protein synthesis as being factors in the deficits observed in the human syndrome, it should first be remembered that the rat has not been shown to be an appropriate model for the syndrome, and secondly, the effects of maternal alcohol treatment during lactation, which had significant effects in the mouse behavioral study, have not been carefully controlled. Therefore, while this work has promising possibilities, the significance of the results should be viewed with caution.

Epidemiological Studies

Of major importance from a clinical point of view is a determination of the frequency and variability of the fetal alcohol syndrome in the human population. Several epidemiological studies, both retrospective and prospective have been initiated to answer these questions, but in general the results have been inconclusive. Studies from France (Kaminski et al., 1976) and Germany (Mau and Netter, 1974) have reported that mothers who consume alcohol during gestation have an increased incidence of short pregnancies (less than 240 days) and stillbirths; but live births of normal weight and physical appearance. On

the other hand, an American study by Russell (1977) reported low birth weights in offspring born to women classified as having an alcohol related psychiatric disorder. In the German study, women who frequently drank coffee during pregnancy had children of low birth weight, suggesting that in the human population, nutritional and life style differences may confound the effect of alcohol, making an absolute determination of risk very difficult.

Similar confounding results have been noted in two prospective studies now being carried out in the United States. The largest study, being conducted at Loma Linda University, has now reported that in the 1500 births studied to date, the only notable effect of alcohol drinking during pregnancy is low birth weight. However, when cigarette smoking is taken into consideration, the low birth weights can be attributed to a combination of smoking and drinking during pregnancy, with smoking having the major effect (Kuzma, 1977). In the Boston City Hospital study (Rosett et al., 1976; Ouellette et al., 1976; 1977), preliminary reports on 322 offspring failed to show a correlation between the amount of alcohol consumed by the mother and the degree of malformation in the offspring. Hence, women classified as heavy drinkers (minimum of one and one-half drinks per day with the occasional intake of at least five or six drinks) bore children with the same degree of fetal insult as women classified as moderate drinkers (more than one drink per month). Even more confusing was the finding that in women who had been heavy drinkers in the first trimester, but who reduced or abstained in the third trimester, the rate of congenital malformations in their offspring was seven percent as opposed to 41 percent in the offspring of women who continued heavy drinking throughout pregnancy. Since the majority of

congenital malformations have their origin during organogenesis in the first trimester, such results are to be questioned. The most reasonable explanation is that these women who did reduce their drinking would have had children with a low congenital malformation rate even if they had continued their heavy drinking practice throughout gestation.

Two studies conducted at the University of Washington have been most useful in giving an indication of the risk involved in the syndrome. The first, using data from the Collaborative Perinatal Project of the National Institute of Neurologic Disease and Stroke, estimated the frequency of adverse outcome of pregnancy for chronically alcoholic women to be 43 percent (Jones et al., 1974). This figure, which was arrived at by noting that of the offspring of 23 women who chronically drank alcohol before and during pregnancy, four died prior to one week of age, and six had abnormal features suggestive of the fetal alcohol syndrome, has been criticized on the grounds of sampling methodology (Rosett, 1974; Sturdevant, 1974). Since national surveys have estimated that five percent of the female population are heavy drinkers, among the 55,000 women taking part in the Collaborative Study, 2,700 would be expected to fall into the 'heavy' classification. Since only 23 cases of maternal chronic alcoholism were reported, the sample could represent an extreme confounded by common nutritional, socioeconomic and drinking variables, or an inaccurate reporting of maternal drinking histories. The preliminary results from the second study, prospective in nature, suggest that the early estimate may apply only to chronically alcoholic women. In this study, 164 infants, 74 born to mothers consuming in excess of one ounce of alcohol per day, and 90 mothers consuming lesser amounts, were examined. Eleven of these children exhibited clinical

features of altered growth and morphogenesis with nine coming from the heavy drinkers and two from the low drinking group, giving a total incidence rate of 12 percent (Hanson and Smith, 1977). From these two studies it appears that women who drink in excess of one ounce of alcohol daily throughout pregnancy have a risk of 12 percent for having a child with altered growth and dysmorphogenesis, while women who are chronic alcoholics and continue drinking through pregnancy have an even greater (43 percent) risk for an adverse outcome of pregnancy. Whether these women have common nutritional and socio-economic factors contributing to the abnormalities observed can not be determined at this time, nor can the effects of smaller amounts of alcohol.

CHAPTER II

PURPOSE AND RATIONALE OF THE PRESENT STUDY

Although many studies have reported investigating the epidemiologic, dysmorphic, biochemical and behavioral etiology of the fetal alcohol syndrome, little insight has been gained. The human studies are confounded with unknown or poorly understood environmental variables, and the animal studies are based on the assumption that fetal insult from prenatal exposure to alcohol is equivalent in all mammalian species; an assumption not supported by data. Clearly, an animal model of the syndrome is required if further studies are going to elucidate the etiology and pathogenesis of the abnormalities observed in the human population. It is the purpose of this study to establish such a model and to investigate some of the factors that may influence the degree of insult found in the prenatally exposed offspring.

Criteria For An Animal Model

When seeking an appropriate model for the fetal alcohol syndrome, one must question the applicability of the traditional teratological method of administering the suspected agent during organogenesis only. It must be remembered that the features of the human syndrom have been observed only in offspring of chronic alcoholic women, and therefore the suspected teratogenic agent is not simply alcohol, but the action

of chronic maternal alcoholism. With this in mind, it becomes readily apparent that testing for alcohol only during organogenesis is not equivalent to testing for the teratogenicity of maternal chronic alcoholism, and therefore a new set of criteria for testing must be established. Since chronic alcoholism is a disease unique to the human population, the animal model, if it is to be effective in delineating the etiology, prevention, and cure of the human syndrome, must replicate where possible, the condition as observed in the human population (Falk et al., 1972; Mello, 1973; Lester and Freed, 1973). Furthermore, since it is the teratogenicity of the maternal action that is under question, criteria applicable to teratological testing must also be fulfilled. By paralleling criteria for a diagnosis of human alcoholism (Criteria Committee, 1972) and the pattern of consumption in mothers of children with the fetal alcohol syndrome (Ulleland, 1972), the following set of criteria was established.

1. Oral Route of Administration: Most, if not all chronic alcoholics ingest rather than inject their alcohol. This in turn impairs intestinal absorption of vitamins, which may result in altered metabolic processes (Vitale and Coffey, 1971). Therefore, to simulate the condition of chronic alcoholism in animals, the alcohol must be taken orally.

2. Circadian Distribution of Alcohol Intake: In human chronic alcoholics, drinking takes place throughout the waking hours. Blood alcohol levels, while fluctuating over a 24 hour period, seldom fall low enough to initiate alcohol withdrawal symptoms. Therefore in an animal model it must be required that blood alcohol levels remain relatively constant over a 24 hour period, thereby necessitating an even intake of alcohol over the night and day period.

3. Blood Alcohol Levels In the Human Chronic Alcoholic Range:

Human chronic alcoholics maintain blood alcohol levels ranging from 100 to 400 mg per 100 ml blood. Consequently, the embryo/fetus of a chronic alcoholic woman is potentially exposed to these same amounts. Therefore, in an animal model it must be possible to achieve blood alcohol levels greater than 100 mg per 100 ml blood.

4. Behavioral Manifestation of Intoxification: Characteristically, the first signs of alcohol intoxication are ataxia with initial hyperkinesis eventually subsiding to lethargy. With time a tolerance develops and the behavioral manifestations decrease. The actual basis for this tolerance is unknown, however it is a common feature of alcoholism and should be observed in the animal model.

5. Dependence: Pharmacological dependence resulting from chronic alcoholism is manifested by characteristic withdrawal symptoms when alcohol is removed from the alcoholic's diet. Therefore, a sure sign that an animal is alcoholic is the ability to demonstrate withdrawal symptoms after removal of alcohol.

6. Diet of Adequate Nutritional Source: Since one of the confounding variables in human studies has been the maternal dietary habits, it is essential that the animal model employ a diet that meets the daily nutritional requirements of the animal used. This is not to imply that the animal is not malnourished as a result of alcohol disturbing absorption and metabolic processes, but rather that the diet available to the animal is not deficient.

7. Maternal Alcoholism Prior to and During Pregnancy: Chronic alcoholism has an adverse effect on almost every organ of the body. Disease of the liver, gastrointestinal tract, and the cardiovascular and hematopoietic system are not uncommon (Seixas, 1975). Liver

metabolism is altered and faulty absorption can lead to folate, thiamine, and magnesium deficiencies (Lieber, 1975). Since all of these maternal factors may contribute to the fetal alcohol syndrome, it is necessary to administer alcohol before pregnancy in order to allow time for the various alterations caused by alcoholism to become manifest. To further simulate the human condition, alcohol treatment must then continue throughout the pregnancy.

8. Malformations in Offspring Similar to Those in the Human Syndrome: If the model is to be useful in further studies of this syndrome, it must provide a specific pattern of malformations that affect the systems observed in the human syndrome.

9. Dose-Response Effect: A characteristic of teratogenic agents is a dose related increase of affected offspring, going from 0 to 100 percent (Wilson, 1965). Therefore, if maternal chronic alcoholism is indeed teratogenic, then such a dose-response effect should be observed in the model.

It is possible to meet the first six criteria by inducing alcoholism with scheudle induced polydipsia (Falk et al., 1972), or with a Metrecal-ethanol liquid diet (Freund, 1969). The polydipsia technique is attractive because it allows the level of alcohol consumption in a 24 hour period to be varied by the investigator. Unfortunately, for the method to work, animals must be kept at a reduced weight in special cages that deliver the specified amount of food at specific intervals. The liquid diet utilizes the dietary source of chocolate Metrecal to mask large quantities of alcohol. With this diet as the sole source of calories, animals tend to lose weight in the early stages of treatment, but soon return to their pretreatment weights. By using the liquid diet to fulfill the first six

requirements, it is possible to test the teratogenicity of maternal chronic alcoholism in the mouse.

Etiologic Considerations

Teratogenic agents of environmental origin require a biological system to act upon. This interaction can best be described in terms of a 'multifactorial model' (Fraser, 1976), where both environmental and biological factors determine how liable an embryo is for teratogenic insult. In the fetal alcohol syndrome, the most likely candidate for the environmental factor is maternal chronic alcoholism. Biological factors depend on the mechanism of teratogenesis, which can occur either by direct insult on the developing organism, or indirect insult via alcohol induced alteration in maternal and/or fetal metabolism.

Direct insult, possible in this syndrome since alcohol freely crosses the placenta (Akesson, 1974), implies that liability for malformation increases as the amount of teratogen available for insult increases. The amount of alcohol the embryo/fetus is exposed to will then be dependent on the amount of maternal drinking and the rate at which alcohol is metabolized, and thus eliminated from the system.

Acute doses of alcohol are metabolized almost exclusively in the cytosol fraction of liver (Lundquist, 1975).

$$(1)\quad CH_3CH_2OH + NAD^+ \underset{}{\overset{ADH}{\rightleftharpoons}} CH_3CHO + NADH + H^+$$

In this reaction, generally considered the rate limiting step in alcohol metabolism (Lundquist, 1970), ethanol is oxidized to acetaldehyde by the nicotinamide-adenine dinucleotide (NAD) - linked enzyme, alcohol dehydrogenase (ADH). ADH isoenzymes, with altered activities, have been

described in man (Ugarte et al., 1970) and mouse (Sheppard et al., 1968), and a fetal form different from the adult has been reported in mice (Krasner et al., 1974). With chronic alcohol administration, a second pathway, the hepatic microsomal ethanol oxidizing system (MEOS), is induced (Lieber and DeCarli, 1970).

$$(2)\ CH_3CH_2OH + NADPH + H^+ + O_2 \xrightarrow{MEOS} CH_3CHO + NADP^+ + H_2O$$

In vitro studies suggest that after chronic alcohol consumption, this system may account for up to 25 percent of adult alcohol metabolism (Lieber and DeCarli, 1970; 1972). Other studies, failing to find a correlation between MEOS induction and increased alcohol elimination, cast doubt on the significance of this pathway on in vivo metabolism (Roach, 1973; Mezey, 1976). Unlike the ADH pathway, the MEOS is not present in the fetal liver (Kato, 1966), and thus is excluded from the fetal metabolism of alcohol.

From the above, it can be seen that the embryo/fetus may be protected against the direct action of alcohol by both the maternal and fetal ADH pathways, as well as the maternal MEOS pathway. The relative importance of the maternal and fetal genetic constitution in the metabolism of alcohol, and thus the amount available for insult, can be resolved using a diallele cross. This method, described by Schmidt (1919) as "the method of complete intercrossing", involves making all possible matings between two or more strains of animals. In the case of three strains (A,B,C), there are nine mating combinations (AxA, AxB, AxC, BxA, BxB, BxC, CxA, CxB, CxC) resulting in six fetal genotypes (AA, BB, CC, AB or BA, AC or CA, BC or CB). By scoring the offspring for a phenotype such

as malformation or blood alcohol level, it should be possible to distinguish between a maternal effect, where the fetal phenotypes are associated with maternal genotype, and a fetal effect, where fetal phenotype is associated with the fetal genotype.

Alcohol induced alteration of the maternal and fetal metabolism may occur in a number of ways. Malabsorption of vitamins and minerals can result in deficiencies which theoretically could have adverse consequences on a developing organism (Lieber, 1975). Alcohol metabolism by ADH, equation (1), results in a reduction of the NAD/NADH ratio, thereby inhibiting NAD-linked reactions (Lieber, 1975; Lundquist, 1975). The consequence of this is not only the inhibition of alcohol metabolism, but also an inhibition of lactate metabolism, resulting in acidosis (Lieber, 1975). This alteration in the NAD/NADH ratio may be normalized by induction of the MEOS, equation (2), which provides $NADP^+$ for transhydroxylation with NADH, thus accelerating the ADH pathway by increasing the amount of available NAD (Veech et al., 1969). Induction of the MEOS also results in an apparent induction of the microsomal drug detoxifying system, which not only accelerates drug metabolism in the chronic alcoholic (Mezey, 1976), but also modifies hormone metabolism (Lieber and DeCarli, 1973).

Both mechanisms of teratogenesis may involve the same biological factors; the rate of alcohol metabolism by ADH and MEOS. In the case of teratogenesis by direct insult, an increased rate of metabolism would provide protection to the developing organism by decreasing the amount of teratogen available for insult. In the second mechanism, this same increased rate of metabolism would prove deleterious to the developing organism since the effects of altered metabolism, with the described

harmful side-effects, would also be increased. An examination of the role of alcohol metabolism as measured by ADH and MEOS activities would provide insight into both the mechanism and biological factors responsible for the malformations observed in this syndrome.

Experiments

The goal of the first experiment in this study was to establish an animal model of the fetal alcohol syndrome. At the same time, by using two strains of mice to manipulate the genetic variables, and several doses of chronic alcohol consumption to manipulate the environmental variable, the environmental and genetic factors responsible for the malformations were investigated.

The second experiment, which followed the first chronologically, utilized a diallele cross to determine the relative importance of the maternal and fetal genotypes in liability for malformation. Furthermore, in an effort to determine the mechanism of teratogenesis, and thereby gain insight into the critical biological factors, metabolic rates as determined by ADH activities in mothers and fetuses, and MEOS activities from the mothers, were investigated.

CHAPTER III

METHODOLOGY

Experiment 1

Animals:

CBA/J and C3H/1g^{Ml} mice (Mus musculus) obtained from the Medical Genetics breeding colony, University of British Columbia, were maintained on a 12 hour light cycle in the Zoology Vivarium, University of British Columbia. Animals were housed in standard clear polycarbonate cages, females in pairs and males singly, and allowed ad libitum access to Purina Laboratory Chow and tap water unless otherwise noted.

Diets

The liquid diets listed in TABLE 1, defined by the percent of ethanol derived calories (EDC) each contains (Freund, 1969; Freund and Walker, 1971), were fed to females ad libitum. The liquid preparation consisted of chocolate Metrecal (Mead Johnson Co., Evansville, Ind.) containing 0.95 cal per ml and Vitamin Diet Fortification Mixture (Nutritional Biochemicals, Cleveland, Ohio), 3 gm per liter. Diet 0 contained the basic liquid diet plus isocaloric amounts of sucrose (87 percent volume per volume and 3.5 cal per ml) for ethanol. Diet 15 consisted of the liquid diet plus 95 percent volume per volume ethyl alcohol (5.25 cal per ml),

TABLE 1

Composition of Diets

Diet (% EDC)	Ethanol (95% v/v)		Metrecal	
	Calories	ml/100	Calories	ml/100
	%		%	
Lab Chow	-	-	-	-
0	-	-	100	100.0
15	15	3.1	85	96.9
20	20	4.3	80	95.7
25	25	5.7	75	94.3
30	30	7.2	70	92.8
35	35	8.9	65	91.1

added such that the final preparation contained 15 percent EDC. Diet 20 contained 20 percent EDC, diet 25, 25 percent EDC, and so on. The liquid diets were prepared fresh daily and were the only source of calories. The preparation, composition, and documentation of nutritional adequacy of the liquid diets used were noted in detail previously (Walker and Freund, 1971); each treatment diet contained several times the minimum daily requirements of all nutrients, based on previous recommendations for mice (Walker and Zornetzer, 1974). Animals received the liquids from inverted 50-ml B-D plastic syringes with the needle end sealed by melting, through standard glass drinking tubes with 1.5 mm openings extending to approximately 3 cm above the cage floor. Daily fluid consumption and caloric intake was determined by measuring the amount of fluid left each day at between 10 AM and 12 noon, or 10 PM and 12 midnight. Caloric intake for control animals on Laboratory Chow was determined using metabolic cages.

Diet Administration

To avoid weight loss and sickness, which might have resulted from an initial introduction to high ethanol doses, animals were introduced to the diets in stages. Virgin females, 60 to 100 days old were taken off Laboratory Chow and given diet 0. After ten days on this diet, they were introduced to ethanol by diet 15 or 20, which was given for ten days, followed by the next higher ethanol diet for ten days, until the predetermined number of females were in each diet group. The females were then maintained on their respective group diets for at least 30 days before the initial mating attempt. At this time, all females had been on liquid diets 80 days, and on their respective group diet from 30 to 80 days, depending on the diet group i.e., diet 0, 80 days; diet 15, 70 days;

diet 35, 30 days.

Matings and Pregnancy Management

Matings between animals of the same strain were initiated by introducing an estrous female into a males cage. To keep the males sober and the females intoxicated, mating time was restricted to 1.5 hours, at which time the mating pair was deprived of food and water. This was done to prevent confounding the results with male alcohol consumption, which has been reported to cause dominant lethal mutations (Badr and Badr, 1975; Klassen and Persaud, 1976). The presence of a copulation plug was taken as indicating day one of gestation. Females were kept on their respective diets throughout gestation, and on day 18, were weighed and then killed by cervical dislocation.

Fetal Examination

After opening the uterus, resorption sites, location and position of fetuses were recorded. Fetuses were removed by cutting the umbilical cords, and checked for life by noting colour, and probing until movement of either limbs or mouth was observed. Fetuses that failed to show any movement and were pale in colour were considered dead and counted with the resorptions. Fetuses were examined for external malformations of head, palate, limbs, digits, trunk and tail, and were sexed with the aid of a Wild dissecting microscope. The live fetuses were then weighted on a Mettler top loading balance and the weight recorded to one hundredth of a gram. One-third of the live animals were randomly assigned for Alizarin Red S Skeletan staining (Crary, 1962; Appendix A), and the remainder fixed in Bouin's solution for subsequent examination by free-hand razor sectioning (Wilson, 1965). Skeletal anomalies were determined

by observing prepared fetuses in gelatin filled petri dishes under a dissecting microscope. Soft tissue malformations were determined by observing 1-2 mm cross sectional slices placed in 70 percent ethanol in white porcelain spot plates under a dissecting microscope.

Determination of Blood Alcohol Levels

Blood samples were taken by retro-orbital bleed from females three days before the first mating attempt, and by either retro-orbital bleeding or cardiac puncture just prior to killing on day 18 of gestation. To maximize any variability in blood alcohol levels due to circadian fluctuations, random samples were collected over a daily 16 hour period, 10 AM to 2 AM. Using 20 microliter Drummond microcap glass blood collecting tubes, or a 3 cc tuberculin syringe armed with a 21 x 1.5 needle for cardiac puncture, 1 to 2 ml of blood was transferred into a heparin rinsed 16 x 125 mm screw capped culture tube and stored in the refrigerator 12 to 24 hours. At the time of assay, 0.1 ml of serum or plasma was diluted with 4.9 ml of a 0.9 percent solution of sodium chloride, and 0.1 ml of this sample was used with a Calbiochem 'Alcohol Stat-Pak' assay kit (Jones et al., 1970). The reaction was initiated by the addition of 0.1 ml prepared sample to 2.6 ml of prewarmed commercial reagent which contained 50 mM TRIS buffer (ph 8.8), .15 mM NAD, 13800 IU per liter ADH, and a trapping agent to remove acetaldehyde from the reaction mixture. After ten minutes of incubation at 30 degrees C, the molar equivalents of reduced NAD generated in the reaction mixture were measured in duplicate, against water, in quartz cuvettes with 1 cm light path, at 340 nm on a Beckman DU spectrophotometer. The blood alcohol levels in gm per 100 ml blood were determined by taking the numerical difference between the sample and blank (0.1 ml nine percent w/v sodium chloride in 2.6 ml

commercial reagent) absorbances; a method made possible by having the volume of reagent and sample fixed.

Data Analysis

Mean differences of measurements were tested at the 0.05 level of significance using a Type I Analysis of Variance (Sokal and Rohlf, 1969). Where significant differences were found, a Duncans Multiple Range Test was applied to determine the significant differences between means (Bliss, 1967). In the one measurement with a known expected frequency, a Chi-square test was utilized at the 0.05 level of significance.

Experiment 2

The methodology for Experiment 2 departed from that described for Experiment 1 as follows:

Animals

In addition to the CBA/J and C3H/1g^{M1} mice previously mentioned, C57BL/6J mice obtained from the Jackson Laboratories, Bar Harbor, Maine, were used.

Diet and Administration

To control environmental variables, all female mice were administered and maintained on a 20 percent EDC diet as previously described for Experiment 1.

Matings and Pregnancy Management

All possible matings between strains CBA/J, C3H/1g^{M1}, and C57BL/6J were made using a diallele cross. TABLE 2 illustrates the fetal genotypes produced in this cross.

TABLE 2

Fetal Genotypes Generated with a Diallel Cross

	Males		
Females	CBA	C3H	C57
CBA	CBA/CBA	CBA/C3H	CBA/C57
C3H	C3H/CBA	C3H/C3H	C3H/C57
C57	C57/CBA	C57/C3H	C57/C57

Fetal Examination

After external examination, the fetuses were decapitated, and one-half of the heads were prepared for skull skeletal staining as previously described, and the remaining half were fixed in Bouin's solution for brain examination by the freehand razor technique.

Determination of Blood Alcohol Levels

After decapitation, a mixture of blood and body fluids was drawn from the jugular area of the fetuses. Litters were pooled to obtain the required volume of blood, and were then analyzed, along with maternal samples, as described for Experiment 1.

Enzyme Preparations

Immediately after cervical dislocation, the maternal abdominal cavity was exposed and the liver quickly removed. The liver was washed in approximately 15 mls of an ice cold 1.15 percent potassium chloride (KCl) solution, weighed, chopped, and homogenized in four volumes of fresh ice cold KCl using an electric Virtis tissue homogenizer with a teflon pestle. This procedure was modified for the fetuses so that livers were held in ice cold 1.15 percnet KCl until the entire litter had been dissected (approximately 15 minutes), after which time the pooled livers were handled as a single sample. After a maximum of one week freezing, the homogenates were thawed and centrifuged in a Beckman preparative centrifuge at 4 degrees C, for 30 minutes at 10,000g. The supernatant was then adjusted to 50 mg per ml and used for the assay of alcohol dehydrogenase (ADH) activity, and the pellet was resuspended in ice cold 1.15 percent KCl to a concentration of 400 mg liver tissue per ml. This crude source of microsomes was then used to determine the maternal microsomal enzyme

oxidizing system (MEOS) activity.

Alcohol Dehydrogenase Assay

The ADH activity was determined according to the method of Bonnichsen and Brink (1955), which utilizes the linear generation of NADH in calculating the mean initial reaction velocity. The reagents, consisting of 10 mM glycine-sodium hydroxide buffer, pH 9.6, 72 mM ethanol and 0.1 ml liver supernatant were prewarmed in a 1 cm light path quartz cuvette to 30 degrees C, and the reaction was then initiated by the addition of 0.1 ml of 1.5 mM NAD, giving a total reaction volume of 3.3 ml. The generation of NADH was recorded at 0 and 3 minutes, in duplicate samples at 340 nm in a Gilford spectrophotometer. Values for a blank, prepared exactly as the sample except for the omission of ethanol, were subtracted from the sample values. The ADH activities were then calculated from the NADH millimolar absorptivity (the absorbance of 1 micromole per ml in a 1 cm light path) of 6.22 (Mezey et al., 1968), and expressed as nmoles per minute per gm liver for comparison with activities reported in the literature. Activity was also expressed in units corresponding to the change in absorption measured at 340 nm per minute per gm of liver protein. This unit corresponds to 161 nmoles of acetaldehyde formed per minute per gm liver protein (Lieber and DeCarli, 1970), and is useful when determining the total rate of liver ethanol metabolism. Protein content was measured using the method of Lowry et al. (1951).

Microsomal Ethanol Oxidizing System Assay

The MEOS activity was determined by the method of Lieber and DeCarli (1970) using the crude microsomal preparation. Ethanol oxidizing activity in this fraction has been reported to be comparable with that of

the corresponding isolated microsomes (Lieber and DeCarli, 1970), and has subsequently been used in mouse MEOS studies (Sze et al., 1976). The incubation mixture, consisting of 80 mM sodium phosphate buffer, pH 7.4, 50 mM ethanol, 0.3 mM NADPH, 5 mM magnesium chloride, and 20 mM nicotinamide, was placed in the main chamber of a 15 ml Warburg reaction vessel and preincubated in a circulating water bath at 37 degrees C. At zero time, 0.1 ml of the crude microsome preparation equivalent to 40 mg liver was added, and after further incubation at 37 degrees C for ten minutes, the reaction was stopped by the addition of 0.5 ml of 70 percent trichloracetic acid. The acetaldehyde produced in this ten minute period was then allowed to react with 0.4 ml of 1.5 mM semicarbazide in 17 mM potassium phosphate buffer, pH 7.0, which had previously been placed in the center well of the reaction vessel. After an overnight diffusion period at room temperature, the contents of the center well were collected, and the concentration of acetaldehyde bound to semicarbazide was determined in duplicate according to the method of Gupta and Robinson (1966). In this procedure, a 0.2 ml aliquot from the center well is diluted to 1 ml and measured at 224 nm against a blank containing an equivalent amount of semicarbazide solution. Under these conditions, 0.1 umole acetaldehyde gave an absorbance of 0.33, thereby allowing expression of the MEOS activity in units corresponding to nanamoles of acetaldehyde produced per minute during the initial linear phase of the reaction. Protein was measured by the method of Lowry et al. (1951).

CHAPTER IV

RESULTS

Experiment 1

The effects of varying amounts of EDC on maternal caloric intake, liver weight and blood alcohol levels are shown in TABLE 3. Daily caloric intake averaged over a ten day period was not significantly different within strains (see Appendix B for ANOVA tables on all analyses). Liver weights, taken at day 18 of gestation and expressed as grams per 100 grams body weight, were not significantly different within strains, nor were blood alcohol levels taken three days prior to mating and on day 18 of gestation. Maternal blood alcohol levels on day 18 of gestation were significantly different within strains on different diets (CBA: $15 < 20$ $25 < 30$; C3H: $< 20 < 25 < 30 < 35$); increasing the EDC significantly increased the blood alcohol level. Maternal blood alcohol levels were also significantly different between strains of those diets where comparisons were possible, with the CBA having a higher alcohol level than the C3H (Diets 20, 25, and 30, C3H $<$ CBA).

TABLE 4 shows the effects of variable amounts of EDC on implantations and resorptions. On Diet 30, CBA females had no visable resorption sites and carried no offspring to day 18 gestation even though they gained weight early in pregnancy. The same was true for C3H females on Diet 35. Because of this embryolethal effect, no fetal data could be collected for CBA on Diet 30, or C3H on Diet 35. Average implantations per litter,

TABLE 3

Effect of Diets on Caloric Intake, Liver Weight, and Blood Alcohol Levels in CBA and C3H Females

Strain	Diet	Daily Cal Intake	Liver Wt. Per 100 Gm	Blood Alcohol* 1	Blood Alcohol* 2
	(% EDC)	mean (SEM)	mean (SEM)	mean (SEM)	mean (SEM)
CBA	Lab Chow	14.8 (0.47)	6.70 (0.16)	0	0
	0	15.6 (0.48)	6.27 (0.24)	0	0
(n = 10 females for each diet)	15	15.7 (0.60)	6.16 (0.31)	74 (2.4)	73 (2.0)
	20	15.5 (0.52)	6.40 (0.24)	122 (5.0)	131 (2.3)
	25	16.1 (0.52)	6.38 (0.28)	177 (2.3)	175 (3.0)
	30	16.8 (0.53)	6.86 (0.24)	311 (10.9)	315 (6.7)
C3H	Lab Chow	15.4 (1.21)	5.64 (0.24)	0	0
	0	16.0 (0.54)	5.68 (0.28)	0	0
(n = 10 females for each diet)	20	16.1 (0.48)	5.72 (0.27)	83 (4.1)	87 (2.1)
	25	16.4 (0.62)	5.55 (0.26)	142 (15.0)	146 (5.8)
	30	16.3 (0.62)	5.72 (0.25)	279 (9.2)	278 (3.9)
	35	16.1 (0.52)	5.67 (0.29)	372 (14.2)	373 (6.9)

* Measurement 1, taken three days prior to mating, and measurement two, taken at day 18 gestation, are expressed as mg ethanol per 100 ml blood.

TABLE 4

Effect of Diets on Implantation and Resorption in CBA and C3H Females

Strain	Diet	Number Implants	Average Implants	Resorptions Number	%
	(% EDC)		mean (SEM)	mean (SEM)	
CBA	Lab Chow	48	4.8 (0.51)	0.1 (0)	2
	0	56	5.6 (0.50)	0 (0)	0
(n = 10 litters for each diet)	15	40	4.0 (0.44)	2.3 (0.47)	58
	20	53	5.3 (0.56)	3.5 (0.67)	66
	25	52	5.2 (0.49)	3.8 (0.62)	73
C3H	Lab Chow	110	11.0 (0.91)	0.8 (0.32)	7
	0	73	7.3 (0.63)	0 (0)	0
(n = 10 litters for each diet)	20	68	6.8 (0.71)	0 (0)	0
	25	65	6.5 (0.37)	1.6 (0.37)	25
	30	61	6.1 (0.67)	4.4 (0.52)	72

measured as live and dead births plus resorption sites, did not differ significantly between CBA females, however a significant difference was found between C3H females on Lab Chow and those on liquid diets (Lab Cho > 0=20=25 > 30). There were significantly more implants per litter in the Diet 0, 20, and 25 females than in Diet 30 suggesting that the liquid diet as well as high blood alcohol concentrations effect implantation in this strain. In CBA females, the average number of resorptions per litter was significantly less in fetuses from the Lab Chow and Diet 0 compared to those from the Ethanol containing diets (Lab Chow=0 < 15=20=25). Similarily, in the C3H, the Lab Chow, Diet 0, 20, and 25 females had significantly fewer resorptions than females on Diet 30 (Lab Chow=0=20=25 < 30). These data demonstrating increased percentage of resorption with increasing EDC are illustrated in Figure 1.

Fetal measurements of live births, sex, average weight per litter, and percent with one or more abnormalities are shown in TABLE 5. The sex ratio was not significantly different from the expected 1:1 ratio for all the offspring examined. Average CBA fetal weights were significantly greater in the Lab Chow and Diet 0 controls compared to Diet 15 fetuses which were significantly heavier than fetuses from Diets 20 and 25 (Lab Chow=0>15>20=25). In the C3H, Diet 0 fetuses were significantly heavier than Lab Cow fetuses, possibly a reflection of the smaller litter size in the latter (0>Lab Chow>20>25=30). Diet 20 fetuses were lighter than those from females on Lab Chow, but heavier than those on Diets 25 and 30. The percent of fetuses with at least one abnormality increased with increasing EDC in both strains. The most common skeletal abnormality observed was an incomplete or apparently missing occipital bone (TABLE 6).

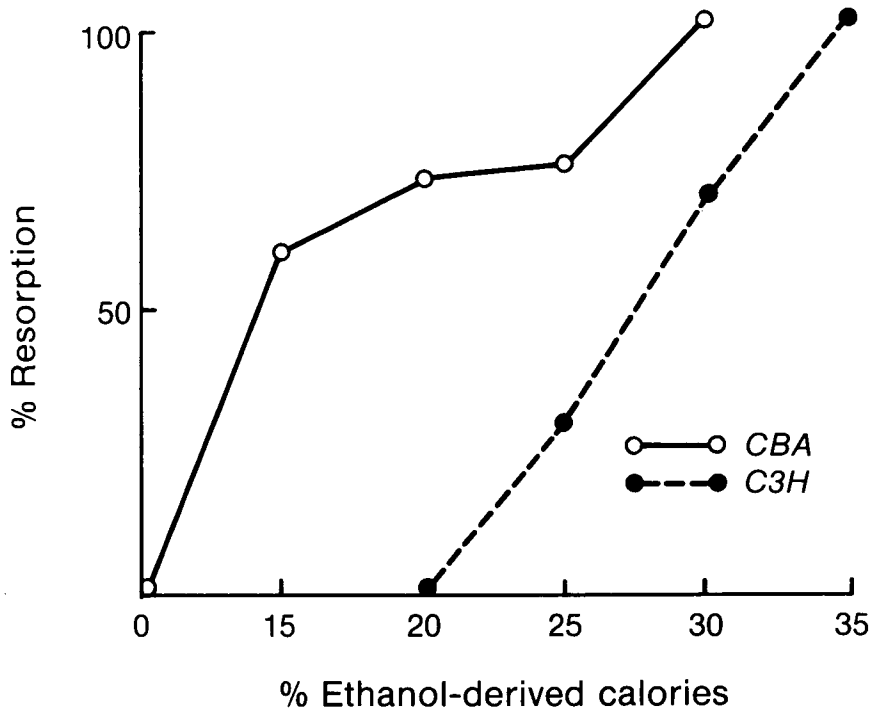

Figure 1. Dose Response Curve of Resorption Rate

TABLE 5

Effect of Diets on Live Births, Sex, Fetal Weights and Fetal Abnormalities in CBA and C3H Females

Strain	Diet	Live Births	Sex		Fetal Wt. (gms)	Abnormal
	(% EDC)		F	M	mean (SEM)	(%)
CBA	Lab Chow	47	24	23	0.97 (0.005)	2
	0	56	25	31	0.95 (0.025)	0
(n = 10 litters for each diet)	15	17	7	10	0.64 (0.040)	59
	20	18	10	8	0.51 (0.053)	100
	25	14	7	7	0.51 (0.081)	100
C3H	Lab Chow	102	61	41	1.14 (0.087)	2
	0	73	35	38	1.27 (0.018)	3
(n = 10 litters for each diet)	20	68	31	37	0.77 (0.040)	79
	25	49	25	24	0.52 (0.012)	100
	30	17	7	10	0.58 (0.056)	100

TABLE 6

Types and Frequency of Skeletal Anomalies

Strain	Diet	Total Fetuses Examined	Occiput	Sternum	Ribs	Total Abnormal
	(% EDC)					%
CBA	Lab Chow	15	1	0	0	7
	0	18	0	0	0	0
	15	6	6	3	0	100
	20	6	6	6	4	100
	25	5	5	5	3	100
C3H	Lab Chow	35	2	0	0	6
	0	24	1	0	0	4
	20	22	18	6	5	82
	25	16	16	7	9	100
	30	6	6	4	6	100

This was found in both strains at the lowest alcohol containing diet. With the higher EDC, apparently missing sternebra and rib anomalies, including fusion and misalignment were produced. Examination of soft tissues revealed a high percentage of brain anomalies for both strains at the lowest EDC diets (TABLE 7). These anomalies included dilated or immature cerebral ventricles and absence of the corpus callosum. Cardiac anomalies including ventricular septal defects and hemopericardium, were observed at the lowest EDC diet, and the percentage rose with increasing EDC. Open-lids at birth were found in 100 percent of the CBA in the higher two treatment diets, but could not be evaluated in the C3H since this strain is genetically open-lidded at birth. Exencephaly and gastroschisis were observed in both strains at higher EDC.

To summarize the results of Experiment 1, maternal blood alcohol levels, percent resoprtion, and percent of abnormal offspring increased with increasing EDC while average fetal weight decreased. Furthermore, a difference in strain response was found in the percent resorption and maternal blood alcohol levels measured on the same diet.

Experiment 2

TABLE 8 shows the number of implants and resorptions by fetal genotypes in the diallele cross, Diet 0. TABLE 9 shows the same information for Diet 20. The average number of resorptions per litter for the three strains used was not significantly different between fetal geneotypes on Diet 0, but on Diet 20, a significant difference was found (CBA > C3H-C57). This difference is attributable to the contribution of the CBA maternal genotype.

TABLE 10 shows the results of Diet 0 on the number of live fetuses available for examination, average fetal weight per litter, and number

TABLE 7

Types and Frequency of Soft Tissue Anomalies

Strain	Diet	Total Fetuses Examined	Dilated Brain Ventricles	Cardiac	Eyes Open	Gastro-schisis	Exence-phaly	Total Abnormal
	(% EDC)							%
CBA	Lab Chow	32	0	0	0	0	0	0
	0	38	0	0	0	0	0	0
	15	11	4	2	0	0	0	36
	20	12	12	12	12	3	0	100
	25	9	9	8	9	1	3	100
C3H	Lab Chow	67	0	0	-	0	0	0
	0	49	1	0	-	0	0	2
	20	46	36	18	-	3	4	78
	25	33	33	25	-	11	10	100
	30	11	11	10	-	9	9	100

TABLE 8

Implants (I), Resorptions (R) and Percent Resorptions (%R) by Fetal Genotype; Diet 0

Females		Males		
		CBA	C3H	C57
CBA	I	110	112	110
	R	4	4	3
	%R	4	4	3
C3H	I	186	175	173
	R	6	5	5
	%R	3	3	3
C57	I	184	191	182
	R	6	8	8
	%R	3	4	4

TABLE 9

Implants (I), Resorptions (R) and Percent Resorptions (%R) by Fetal Genotype; Diet 20

Females		Males		
		CBA	C3H	C57
CBA	I	101	108	108
	R	63	80	77
	%R	62	74	71
C3H	I	172	157	179
	R	7	7	8
	%R	4	4	4
C57	I	179	186	180
	R	7	9	6
	%R	4	5	3

TABLE 10

Live Fetuses (LF), Weight (W), Abnormalities (A)*
And Percent Abnormal (%A) by Fetal Genotype; Diet 0

Females		Males		
		CBA	C3H	C57
CBA	LF	106	108	107
	W**	0.96 (0.02)	0.98 (0.02)	0.98 (0.02)
	A	1	2	2
	%A	1	2	2
C3H	LF	180	170	168
	W	1.01 (0.02)	1.02 (0.03)	0.90 (0.02)
	A	4	5	3
	%A	2	3	2
C57	LF	178	183	174
	W	0.99 (0.02)	0.98 (0.02)	1.00 (0.01)
	A	2	2	2
	%A	1	1	1

* See text for explanation.

** Weight in grams is shown as mean (SEM) per fetal genotype group; n = 20 litters.

TABLE 11

Life Fetuses (LF), Weight (W), Abnormalities (A), Percent Abnormal (%A) and Blood Alcohol Levels (BAL) by Fetal Genotype; Diet 20

Females		Males		
		CBA	C3H	C57
CBA	LF	38	28	31
	W*	0.42 (0.03)	0.44 (0.03)	0.47 (0.03)
	A	38	28	31
	%A	100	100	100
	BAL**	140 (1.4)	139 (2.2)	138 (2.2)
C3H	LF	165	150	171
	W	0.76 (0.02)	0.75 (0.01)	0.75 (0.02)
	A	126	124	138
	%A	76	83	81
	BAL	95 (2.3)	98 (2.4)	95 (4.6)
C57	LF	172	177	174
	W	1.01 (0.01)	1.05 (0.03)	0.99 (0.01)
	A	70	71	70
	%A	41	40	40
	BAL	45 (2.6)	43 (2.0)	43 (2.1)

* Weight in grams is shown as mean (SEM) per fetal genotype group; n = 20 litters.

** Blood alcohol level in mg per 100 ml blood is shown as mean (SEM) per fetal genotype group; n = 20 litters.

and percent of fetuses with an abnormality of incomplete or apparently missing occipital bone, or dilated brain ventricle. TABLE 11 gives the same data for diet 20, and also includes the average fetal blood alcohol level for each fetal genotype. On Diet 0, there was no significant difference in the average fetal weight per litter between fetal genotypes, and the percent of abnormal offspring remained below 3 percent. On Diet 20, there was a significant difference in average fetal weight per litter, with the major contribution being due to a maternal effect (C57>C3H>CBA). The percent of abnormal offspring also demonstrated this maternal effect, as did the average fetal blood alcohol levels where the CBA was significantly greater than the C3H which was significantly greater than the C57 (CBA>C3H>C57).

TABLE 12 shows the average fetal alcohol dehydrogenase (ADH) activity for Diets 0 and 20. On both diets there was a significant difference between fetal genotypes with major contributions by both the maternal and paternal genotype (C57>C3H>CBA). A significant difference was found between treatments when maternal genotype and diet were used as the major source of variation. In this case, the average activity on Diet 20 was significantly greater than that of Diet 0.

Measurements of maternal liver weight, alcohol blood levels, ADH activity, and microsomal ethanol oxidizing system (MEOS) activity for the diallele cross are shown by strain for Diets 0 and 20 in TABLE 13. There was no significant difference in liver weight per 100 gm animal within strains on the different diets, however there was a significant difference between the strains (CBA>C3H>C57). Average maternal blood alcohol levels were significantly different between strains (CBA> C3H> C57), and were in the same direction as observed in the fetuses (TABLE 11).

TABLE 12

ADH Activity (micromoles/min/gm liver) by Fetal Genotype and Diet

Females	Diet	Males		
		CBA	C3H	C57
		mean (SEM)	mean (SEM)	mean (SEM)
CBA	0	0.86 (0.040)	1.02 (0.031)	1.49 (0.058)
	20	0.94 (0.009)	1.12 (0.040)	1.52 (0.027)
C3H	0	1.09 (0.054)	1.12 (0.054)	1.58 (0.045)
	20	1.13 (0.040)	1.18 (0.042)	1.98 (0.045)
C57	0	1.54 (0.031)	1.52 (0.040)	2.21 (0.045)
	20	1.74 (0.031)	1.93 (0.058)	2.37 (0.139)

TABLE 13

Measurements of Maternal Liver Weight, Alcohol Blood Levels, ADH and MEOS Activities in Diallele Cross

Strain	Diet	Liver Wt.[1]	Blood Alcohol[2]	ADH[3]	MEOS[4]
	(%EDC)	mean (SEM)	mean (SEM)	mean (SEM)	mean (SEM)
CBA	0	6.40 (0.66)	- -	1.58 (0.04)	2.84 (0.22)
	20	6.40 (0.64)	135 (12.4)	1.54 (0.03)	10.64 (0.34)
C3H	0	5.55 (0.80)	- -	1.86 (0.04)	9.54 (0.51)
	20	5.71 (0.66)	96 (12.6)	1.83 (0.04)	11.11 (0.41)
C57	0	5.14 (0.53)	- -	3.12 (0.05)	8.60 (0.49)
	20	5.13 (0.73)	43 (6.8)	3.17 (0.04)	9.81 (0.44)

1 expressed as gm per 100 gm body weight (n = 60 per diet)

2 expressed as mg per 100 ml blood (n = 60 per diet)

3 expressed as mmoles per min per gm liver protein (n = 10 per diet)

4 expressed as nmoles per min per mg liver protein (n = 10 per diet)

ADH activities were also significantly different between strains (C57> C3H>CBA) and once again, followed the direction observed in the fetuses (TABLE 12). However, in the adults, a significant difference was not noted between the diet groups. MEOS activity had a significant interaction between strain and diet. This may be interpreted as showing that the MEOS activity in CBA mice is affected more by ethanol in the diet than is the MEOS activity in C3H and C57 mice.

To summarize the results of Experiment 2, blood alcohol levels, percent resorptions, and percent abnormal fetuses were greatest in fetuses from CBA females, and least in fetuses from C57 females, regardless of paternal genotype. Maternal and fetal ADH levels went in the opposite direction with the C57 greatest; a difference between control and treatment activities was only demonstrated in the fetal activities. Maternal MEOS activities had an interaction effect between strain and treatment, with the CBA showing the greatest increase with ethanol treatment. A summary of the significant sources of variation in measurements determined by analysis of variance in both experiments is shown in TABLE 14.

TABLE 14

Significant Sources of Variation in
Measurements Determined by Analysis of Variance

	Source of Variation			
Measure	Diet	Maternal	Paternal	Strain x Diet
Maternal				
Blood Alcohol	X	X		
Implants	X			
Resorptions	X	X		
Liver Wt.		X		
ADH		X		
MEOS	X	X		X
Fetal				
Weight	X	X		
Blood Alcohol	X	X		
ADH	X	X	X	

CHAPTER V

DISCUSSION

The primary goal of this study was to establish a mouse model of the fetal alcohol syndrome, and thereby provide a tool for further investigations. Using the Metrecal-ethanol diet of Freund (1969), female mice consumed intoxicating levels of alcohol without apparent ill-effects on caloric intake or liver weight (TABLE 3), indicating the absence of dietary malnutrition. With increasing EDC diets, maternal blood alcohol levels increased from 0 to 373 mg per 100 ml blood. The fact that levels did not appreciably vary over a 16 hour testing period or between testing days at least 21 days apart indicates that the daily fluctuation was minimal, a finding previously reported by Freund (1969). In CBA and C3H strains of mice, increasing the EDC increased both the resorption and malformation rates (TABLES 4 and 5). Malformations, which included both the skeletal system and soft tissues, exhibited a specific dose-response pattern irrespective of fetal sex (TABLES 6 and 7). Most common was a missing or reduced supraoccipital bone which occurred at a high frequency from the lowest EDC diet in both strains. Brain anomalies, including dilated ventricles and agenesis of the corpus callosum were also found from the lowest EDC diets, at a slightly lower frequency than the occipital abnormality. With increasing EDC, cardiac anomalies of ventricular septal defects and hemopericardium, missing sternebra and vertebral centra, and fused and malaligned ribs were noted along with the brain and skull anomalies. In addition, at the highest EDC diets gastroschisis and

exencephaly were frequently observed. Open eye-lids at birth, observed from Diets 20 and 25 in the CBA, could not be evaluated in the C3H since this strain is genetically open-lidded at birth.

Similarities between the mouse and human fetal alcohol syndrome are shown in TABLE 15. They include prenatal growth deficiency as evidenced by low fetal weight and incomplete ossification; skeletal, neural, cardiac and occular anomalies; and prenatal wastage. Urogenital defects reported in the human syndrome (Tenbrink and Buchin, 1975; Goetzman et al., 1975) and in the mouse studies of Kronick (1976) and Randall (1977) were not observed in this study, possibly because of a strain difference in response to inducing such malformations, or more likely because of the examination procedure used which excluded detection of most urogenital anomalies with the exception of obvious hydronephrosis. Perinatal death, while not observed directly, was indicated by exencephaly and low fetal weights. Because of experimental protocol, performance could not be evaluated. From the similarities found, it appears that given conditions consistent with human maternal alcoholism, it is possible to demonstrate a mouse model of the fetal alcohol syndrome.

The features of the mouse syndrome exhibited a dose-response effect ranging from embryolethality at high EDC diets to neural malformations at the lowest dose tested. This same effect was observed for resorption and malformation rates (TABLES 4 and 5), as well as fetal weights. This finding of a dose-response effect in the absence of dietary malnutrition supports the notion that an environmental factor, the amount of maternal chronic alcohol consumption prior to and during gestation, significantly contributes to the anomalies observed in the syndrome.

By holding the EDC constant while varying the maternal genotype, a

TABLE 15

Similarities Between the Human and Mouse Fetal Alcohol Syndrome

	Man	Mouse
1. Growth Deficiency	X	X
2. Skeletal Anomalies	X	X
3. Neural Anomalies	X	X
4. Cardiac Anomalies	X	X
5. Occular Anomalies	X	X
6. Prenatal Wastage	X	X
7. Perinatal Death	X	X
8. Low Performance	X	?

genetic factor was observed operating in the syndrome. Given the same diet, CBA females had higher blood alcohol levels, lower fetal weights, and increased rates of malformation and resorption when compared to the C3H females (TABLES 3, 4, and 5). This difference in dose-response, illustrated for resorptions in Figure 1, is indicative of a genetic difference in liability for embryo/fetal malformation, with the CBA being the more liable.

The association between EDC, maternal blood alcohol levels, and anomalous offspring suggest that the critical factor influencing abnormal development is the amount of prenatal alcohol exposure, as measured by maternal blood alcohol levels. It should be noted that the lowest dose of 43 mg per 100 ml blood was well below the 100 mg per 100 ml blood used for a diagnosis of human alcoholism (Criteria Committee, 1972). Since the full expression of malformations in the fetal alcohol syndrome has been reported only in the offspring of chronic alcoholic women, the possibility exists that a milder form of the syndrome involving only neural malformations may be found in the offspring of women who have been moderate to heavy drinkers during pregnancy. A determination of the lowest dose required to produce neural malformations would be extremely valuable from a clinical point of view.

The relative importance of the maternal and fetal genotypes in the syndrome was determined using a diallele cross. Fetal insult was directly associated with the maternal genotype, and was independent of fetal genotype (TABLE 11). As before, the maternal genotype determined maternal blood alcohol levels, and hence the degree of fetal malformation. By graphing the percent abnormal fetuses vs. maternal blood alcohol levels (Figure 2), it is readily apparent that the percent of abnormal young is

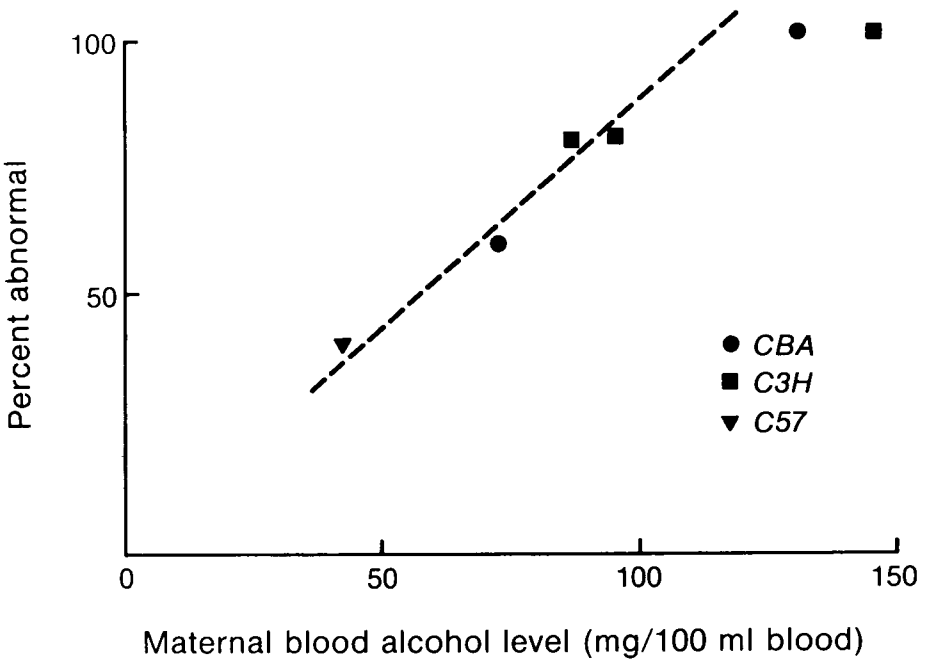

Figure 2. Percent Abnormal Fetuses for Varying Blood Alcohol Levels

similar regardless of maternal strain, however, the diet or amount of dietary alcohol required to reach a specific blood alcohol is strain dependent. This finding implies that the quantitative blood alcohol level is a more sensitive indicator of the maternal alcoholic state than the amount of alcohol in the diet, since the latter can be influenced by biological factors that are ultimately under genetic control.

The finding of a direct association between maternal genotype, blood alcohol level, and degree of fetal insult sheds some light on the results of the Boston City Hospital study that failed to find a correlation between the amount of alcohol consumed during pregnancy and the degree of fetal insult (Ouellette, 1977). It could well be that two females consuming an equal amount of alcohol would have very different blood alcohol levels, and therefore offspring with varying degrees of the syndrome. Measurement of maternal blood alcohol levels at different times throughout gestation would be of value in testing this hypothesis from the mouse model, and would be of value from a clinical point of view, since if true, the amount of maternal alcohol consumption would be only one of two factors determining risk to the offspring.

Efforts to determine the factors involved in the maternal alcohol clearance rate in this study were inconclusive; a result commonly reported in the literature (Mezey, 1976). Hepatic alcohol dehydrogenase (ADH) activity differed in the three strains tested, with female C57 having the highest activities and the CBA having the lowest (TABLE 13). This finding, which is consistent with other studies on strain differences in mouse ADH activity (Sze et al., 1976; Belkap et al., 1972; Sheppard et al., 1968) suggest that blood alcohol levels after chronic alcohol intake are in the opposite direction of ADH activity. Whether these results are meaningful

or just coincidental because of the number of strains used is unanswered by this study, however, a further investigation would be of value. Fetal ADH acitivities were influenced by maternal and paternal genotype, as well as by diet (TABLE 12). However, since fetal blood alcohol levels were dependent on maternal genotype rather than fetal ADH activity, it can be assumed that fetal metabolism of alcohol is not of significance in this syndrome. Induction of the fetal ADH system reported previously by Sze et al. (1976), was also observed in this study, however the biological significance of this induction is not known.

An alternate pathway of alcohol metabolism, the microsomal ethanol oxidizing system (MEOS), has been shown to be induced in rats following chronic alcohol administration (Lieber and DeCarli, 1972). This finding, which has been confirmed in mice (Sze et al., 1976; Lieber and DeCarli, 1974), was also observed in the present study. In the non-induced state, the three strains had differing activities with the CBA females being the lowest (TABLE 13). This suggests a genetic component operating within the system, an observation also found in DBA and C57 mice (Sze et al., 1976). When comparing induced with non-induced activities, an interaction between strain and diet occurs, indicating the degree of induction was much greater in CBA females than in females of the other two strains. Since the CBA females also had the greatest blood alcohol levels, this finding supports previous reports that the role of the MEOS in vivo may be minimal (Roach, 1973). However, the fact that the activity is greatly increased in the CBA suggests that perhaps the induction may play another role in the fetal alcohol syndrome, namely, an alteration of maternal metabolism that could influence normal prenatal development. Unfortunately, the characteristics of this system have not been fully elucidated, and an

investigation into the effects of induction on hormone metabolism, known to be affected by induced microsomal detoxifying systems (Lieber and DeCarli, 1973), would be of great value.

To determine the theoretical total oxidation of ethanol per animal, the enzyme activities were converted to nanomoles of acetaldehyde produced per min per mg liver protein. By assuming the total liver protein was proportional to liver weight in all three strains, it can be seen from TABLE 16 that the theoretical rate of ethanol metabolism could not account for the difference in blood alcohol levels since the CBA females had the highest metabolic rate and the highest blood alcohol levels. Clearly, another factor or set of factors must play a major role in determining ethanol clearance rates, which from this study, appear to be under at least partial genetic control. An understanding of this mechanism could lead to a simple test for ethanol clearance rate, and thereby be of value in counselling for risk in this syndrome.

The results from this study suggest that maternal chronic alcoholism exerts teratogenic insult by two mechanisms. The positive association between fetal blood alcohol levels and rate of malformation support the mechanism of direct action on the embryo/fetus. As prenatal exposure to alcohol increases, anomalies of the syndrome increase. At the same time, induction of the MEOS is associated with increased rates of fetal malformation, suggesting modification of maternal metabolism as a second mechanism of teratogenesis. A detailed study of these possible mechanisms would be of help in elucidating the pathogenesis of this syndrome.

In conclusion, the results of this study, while answering several basic questions about the fetal alcohol syndrome, have also posed several questions that require further investigation. Having established a mouse

TABLE 16

Theoretical Rate of Total Ethanol
Oxidation in CBA, C57 and C3H Mice

Strain	ADH*	MEOS*	Total Activity Times Liver Wt.	Total Activity per 100 gm Animal
CBA	3.14	10.64	13.78 x 6.40	88.20
C57	6.45	9.81	16.26 x 5.14	83.58
C3H	3.76	11.11	14.87 x 5.63	83.73

* Expressed as nmoles acetaldehyde produced per min per mg liver protein.

model for the human syndrome, it was possible to demonstrate that the degree of fetal insult was dependent on the maternal blood alcohol levels. In turn, these levels are determined by the interaction of an environmental and biological component. At levels exceeding 131 mg alcohol per 100 ml blood, all viable offspring exhibited features of the syndrome. The lowest blood alcohol level compatible with normal offspring has yet to be determined. The factors involved in maternal alcohol elimination appear to be under genetic control, but what they are and how they act is unknown at this time. What is clear is that the MEOS activity is greatly induced in the strain showing the greatest liability to the syndrome, raising the possibility that altered maternal metabolism may make secondary contributions to the syndrome. Regarding the clincial aspects of the syndrome, all indications from this study suggest that the risk to the offspring is not a function of amount of maternal alcohol consumption, but rather a function of the amount of maternal consumption required to increase blood alcohol levels above a particular critical value. This presents difficulties in counselling for the syndrome, since the same amount of alcohol consumption by two women may result in differing blood alcohol levels, and hence, different risks to the offspring. Until the time when a simple quantitative test is available, the best means for risk determination will have to rely on repeated blood alcohol measurements throughout pregnancy. While this method may be used to alert the physician of a high risk infant, it is limited in its applicability to the general population. Therefore, until investigations with the animal model clearly elucidate risk factors for the offspring, it is strongly suggested that women contemplating pregnancy be advised to abstain from alcoholic beverages both before and during pregnancy.

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APPENDIX A

Alizerin Red Staining Procedure for Skeletons

Fetuses are placed in the following solutions:

1. 95% ethanol for 24 hours
2. 1% KOH until animals are clear and pink in color (24 hours)
3. 1% KOH and alizarin red stain (1 or 2 drops) for 12-24 hours
4. glycerin and KOH (1:9) for 48 hours
5. glycerin and KOH (1:3) for 48 to 96 hours
6. glycerin and KOH (1:1) for 48 hours
7. store in glycerin

APPENDIX B

Analyses of Variance Tables

ANOVA for Caloric Intake from TABLE 3

Source of Variation	d.f.	Sums of Squares for Stains	
		CBA	C3H
Diets	5	22.15 (NS)*	6.15 (NS)
Error	54	147.10	272.70
Total	59	169.25	278.85

* In this and all other ANOVA Tables, (NS) indicates not significant, and (S) indicates significant at the 0.05 level.

ANOVA for Maternal Liver Weight from TABLE 3

Source of Variation	d.f.	Sums of Squares for Stains	
		CBA	C3H
Diets	5	3.51 (NS)	0.20 (NS)
Error	54	33.83	38.55
Total	59	37.34	38.75

ANOVA for Maternal Blood Alcohol Levels by Strain from TABLE 3

Source of Variation	d.f.	Sums of Squares for Strains	
		CBA	C3H
Subgroups	7	632606 (S)	1031733 (S)
Day (A)	1	149 (NS)	263 (NS)
Diet (B)	3	632103 (S)	1031328 (S)
A x B	3	354 (NS)	141 (NS)
Error	65	19826	12170
Total	79	652431	1043903

ANOVA for Maternal Blood Alcohol Levels Between Strains from TABLE 3

Source of Variation	d.f.	Sums of Squares for Diets		
		20	25	30
Strain	1	10341 (S)	4322 (S)	6808 (S)
Error	18	923	3837	5407
Total	19	11264	8159	12215

ANOVA for Implants from TABLE 4

Source of Variation	d.f.	Sums of Squares for Strains	
		CBA	C3H
Diet	4	15.28 (NS)	157.32 (S)
Error	45	113.70	231.10
Total	49	128.98	388.42

ANOVA for Resorptions from TABLE 4

Source of Variation	d.f.	Sums of Squares for Stains	
		CBA	C3H
Diet	4	131.72 (S)	133.12 (S)
Error	45	97.10	46.40
Total	49	228.82	179.52

ANOVA for Fetal Weights from TABLE 5

CBA

Source of Variation	d.f.	Sums of Squares
Diet	4	5.724 (S)
Error	147	4.514
Total	151	10.238

C3H

Source of Variation	d.f.	Sums of Squares
Diet	4	24.59 (S)
Error	304	21.651
Total	308	46.24

ANOVA for Resorptions from Diallele Cross, TABLES 8 and 9

Source of Variation	d.f.	Sums of Squares for Diets	
		0	20
Subgroups	8	1.211 (NS)	444.10 (NS)
Paternal (A)	2	0.011 (NS)	3.23 (NS)
Maternal (B)	2	1.011 (NS)	435.60 (S)
A x B	4	0.189 (NS)	5.27 (NS)
Error	163	40.450	610.80
Total	179	41.661	1054.90

ANOVA for Fetal Weights from Diallele Cross, TABLES 10 and 11

Source of Variation	d.f.	Sums of Squares for Diets	
		0	20
Subgroup	8	0.049 (NS)	14.636 (S)
Paternal (A)	2	0.007 (NS)	0.012 (NS)
Maternal (B)	2	0.036 (NS)	14.594 (S)
A x B	4	0.006 (NS)	0.030 (NS)
Error	163	1.220	3.704
Total	179	1.269	18.340

ANOVA for Fetal Blood Alcohol Levels from TABLE 11

Source of Variation	d.f.	Sums of Squares
Subgroups	8	68220.7 (S)
Paternal (A)	2	7.6 (NS)
Maternal (B)	2	68178.3 (S)
A x B	4	34.8 (NS)
Error	28	1179.2
Total	44	69399.3

ANOVA for Fetal ADH Activity by Diet from TABLE 12

Source of Variation	d.f.	Sums of Squares for Diets	
		0	20
Subgroup	8	6.701 (S)	9.442 (S)
Paternal (A)	2	3.260 (S)	3.902 (S)
Maternal (B)	2	3.328 (S)	5.302 (S)
A X B	4	0.121 (NS)	0.238 (NS)
Error	28	0.387	0.640
Total	44	7.088	10.482

ANOVA for Fetal ADH Activity
by Maternal Genotype from TABLE 12

Source of Variation	d.f.	Sums of Squares
Subgroups	5	9.9848 (S)
Maternal (A)	2	9.1331 (S)
Diet (B)	1	0.7971 (S)
A x B	2	0.0546 (NS)
Error	79	0.7126
Total	89	10.6974

ANOVA for Maternal Blood Alcohol
Levels from Diallele Cross, TABLE 13

Source of Variation	d.f.	Sums of Squares
Strain	2	256255 (S)
Error	177	21294
Total	179	277550

ANOVA for Maternal Liver Weight, ADH and
MEOS Activity from Diallele Cross, TABLE 13

Source of Variation	d.f.	Sums of Squares for Measurements		
		Weight	ADH	MEOS
Subgroup	5	99.22 (S)	27.939 (S)	458.3 (S)
Strain (A)	2	97.46 (S)	27.932 (S)	134.4 (S)
Diet (B)	1	0.25 (NS)	0.002 (NS)	186.7 (S)
A x B	2	0.51 (NS)	0.005 (NS)	137.2 (S)
Error	49	161.02	0.968	91.8
Total	59	259.25	28.907	550.0