EFFECT OF MATERNAL ALCOHOL CONSUMPTION ON

PLACENTAL BLOOD FLOW AND FETAL UPTAKE OF

ZINC, FOLATE, AND ANALOGS OF GLUCOSE AND

AMINO ACID

by

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ABSTRACT

The present study was undertaken to determine whether
retarded fetal growth caused by maternal alcohol consumption
prior to and during gestation is due to an interference with
the transfer of nutrients from the maternal circulation into
the fetus.

Female Sprague-Dawley rats were either given 20 percent
alcohol in drinking water and solid diet ad libitum (alcohol
group), or were pair-fed to the alcohol group (pair-fed group),
or were given water and solid diet ad libitum (ad libitum
control group) for four weeks. They were then mated and the
alcohol group was changed to 30 percent alcohol in water.
During the third week of gestation maternal plasma levels of
glucose, folic acid, and zinc were determined. On day 20
of gestation placental and fetal weights, the fetal uptake
of zinc, folic acid, and non-metabolizable glucose and amino
acid analogs, and the placental blood flow were determined.
The fetal uptake of the nutrients studied was investigated by
measuring fetal radioactivity 90 minutes after injecting the
dams into the femoral vein with either ^{3}H labeled folic acid
and methyl ($\propto$-D- U-^{14}C gluco) pyranoside, or zinc-65, or
2-amino(1-^{14}C) isobutyric acid. The radioactivity in the
maternal plasma and liver 90 minutes after injection was also
determined. Blood flow to the placentas as well as maternal
cardiac output and renal blood flow were measured using 15
micron diameter microspheres labeled with ^{57}Co. The
osmolality of the maternal plasma and maternal muscle water

content were determined in order to assess the degree of
dehydration, as alcohol in water was offered as the sole
source of fluids.

No differences in litter size existed between alcohol
and pair-fed or alcohol and ad libitum control groups,
however litter sizes of the ad libitum group were larger than
those of the pair-fed group. The 20 day old fetuses were
significantly smaller while the placentas were significantly
larger in the alcohol group than in either the pair-fed or
ad libitum controls. No differences were seen in maternal
plasma levels of glucose, folic acid, or zinc among the three
treatment groups. Cardiac output and blood flow to the
kidneys did not differ among the three treatment groups.
However blood flow to the placenta, whether expressed as
mL/min/g placenta or as mL/min/ entire placenta or as
% cardiac output was significantly reduced in the alcohol
group compared with the pair-fed and ad libitum control groups.
When expressed as mL/min/placenta differences in blood flow
were also seen between pair-fed and ad libitum control groups.
No significant differences were observed in the fetal uptake
of zinc or analogs of glucose and amino acid between the three
treatment groups. The folate taken up by the fetuses of the
alcohol treated dams was significantly higher than for pair-
fed but not ad libitum controls. No differences were observed
in maternal plasma or liver concentrations of any of these
compounds among the three groups 90 minutes after injection.
Plasma osmolality was increased by approximately 7 percent in

the alcohol group compared with pair-fed and _ad_ _libitum_
control groups. This indicates a moderate degree of
dehydration.

The present study demonstrates that the retarded growth
of the fetuses exposed to alcohol in utero is not due to an
impaired transfer of the nutrients tested from the maternal
circulation to the fetus; nor is it due to lower maternal
plasma levels of these nutrients. The significance of the
reduced placental blood flow in the alcohol exposed rats
on fetal growth and development is not clear. Thus the
growth-retarding effect of maternal alcohol ingestion may
still be due to a direct action of alcohol on the fetus.

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INTRODUCTION

Concern about the adverse effects of maternal alcohol consumption during pregnancy on offspring has been recorded since ancient times (1,2). However it has only been in the last decade that a relationship has been established between chronic maternal alcoholism and a specific pattern of anomalies in offspring of such women. This pattern of anomalies, termed fetal alcohol syndrome, is characterized by prenatal and postnatal growth retardation, mental deficiency, microcephaly, craniofacial anomalies, and other changes (3-5). Children with fetal alcohol syndrome are usually below the third percentile in height, weight, and head circumference, and do not appear to demonstrate catch-up growth. The average reported I.Q. is around 65 with a range from 16 to 106, indicating that most of the children were borderline to mildly retarded in comparison with Stanford Binet Intelligence Test 1972 norms (5).

It has been reported in the medical literature (5) that about 40% of babies born to heavy drinkers displayed characteristics of the fetal alcohol syndrome. By heavy drinkers is meant an intake of 90 mL (3 oz.) of absolute alcohol per day during pregnancy.

The extent to which these infants show fetal alcohol syndrome characteristics is probably determined by the amount and duration of exposure to alcohol in utero. Lower levels of alcohol consumption during pregnancy may induce a milder form of fetal damage (5). Little (6) demonstrated

that reduced birth weight due to alcohol comsumption is independent of maternal smoking habits, a recognized birth weight depressent.

Since the recognition of the fetal alcohol syndrome in children, numerous studies on the effects of maternal alcohol consumption on the progeny have been conducted in animals. It has been confirmed using rats that maternal consumption of alcohol during gestation results in retarded fetal and postnatal growth (7,8). Despite the fact that the dams did not receive alcohol after delivery, catch up growth was not observed during the first four weeks post-natally. These observations with animal models indicate that the retarded growth is not due to either caloric under-nutrition or to a specific nutrient deficiency as the nutrient and caloric intakes were similar in alcohol treated and pair-fed control animals. Maternal alcohol consumption might however cause retarded growth of the fetus by interfer-ing with the maternal-fetal transfer of nutrients across the placenta. Decreased placental nutrient availability, as a result of either reduced maternal plasma nutrient concentra-tion or a reduction in placental blood flow, may further contribute to growth retardation in the fetus.

To provide an explanation for alcohol induced growth retardation in the fetus, the present study was designed to determine the effect of maternal alcohol consumption on the availability of nutrients to the fetus in a suitable rat model. This was achieved through investigation of maternal

nutritional status, placental blood flow, and placental transfer of nutrients in rats consuming alcohol prior to and throughout gestation. In order to determine the nutritional status of the pregnant animals maternal plasma concentrations of glucose, folic acid, and zinc were measured. The blood flow to the placenta was determined with radioactive micro-spheres. Zinc, folic acid, and non-metabolizable glucose and amino acid analogs (methyl-α-D glucopyranoside and 2-amino isobutyrate) were selected to measure the transfer of nutrients from the maternal circulation to the fetus as these nutrients are known to have an effect on fetal growth and development (9,10,11).

LITERATURE REVIEW

<u>Human Studies</u>   a) Retrospective Studies

The connection between maternal alcohol consumption and faulty development of the offspring has been recognized since early Greek and Roman mythology. In Carthage the bridal couple was forbidden to consume wine on their wedding night in order that defective children might not be conceived (1). Similarly in the nineteenth century a report to the House of Commons of England by a select committee investigating drunkenness indicated that infants born to chronic alcoholic mothers acquired "a starved, shrivelled, and imperfect look" (2). In 1900 Sullivan reported increased abortion and stillbirth rates among chronic alcoholic women and an increased frequency of epilepsy in their surviving offspring (12). Since that period sporadic clinical reports have appeared suggesting an association between maternal alcoholism and serious abnormalities in the offspring (13,14), but none had substantial impact until the reports of Jones and Smith (3,15). Since then extensive investigation, both in humans and in animal models, has been carried out in this area.

In 1973 Jones and Smith (3,15) described a pattern of malformations among offspring of chronic alcoholic mothers and referred to this recognizable pattern of malformations as the fetal alcohol syndrome. These offspring of alcoholic mothers exhibited prenatal and postnatal growth deficiency as well as craniofacial abnormalities including microcephaly, short palpebral fissures, and maxillary hypoplasia. Joint

and cardiac anomalies were reported in over 50 percent of these children. Lack of postnatal growth response to high calorie feeding provided further evidence of the immutable nature of the deleterious prenatal effect of growth rate (15). Assessment of maternal nutritional status revealed no abnormalities aside from occasional iron deficiency (15). No pattern of malformation as described in fetal alcohol syndrome had been reported in offspring of iron deficient anemic women.

Intellectual development and motor performance was reported by Streissguth in twelve offspring of chronic alcoholic women (16). All except one of the children exhibited a borderline or retarded intelligence level. It was observed that children with the clear features of fetal alcohol syndrome were most retarded while those with lesser degrees of dysmorphology appeared less impaired. The same workers attempted to better characterize the fetal alcohol syndrome by a review of the charts of the National Institute of Neurological Disease and Strokes Perinatal Project (17). About 55,000 pregnant women were observed up to seven years postnatally and the performance and development of their offspring monitored. Alcohol consumption in the mother's clinical record was compared to child development. In 23 cases there was an indication of severe chronic alcoholism during and prior to pregnancy. Each of these cases was compared with control non-alcoholic women of equal socio-economic status, race, age, education, parity, and marital

status. Among the alcoholic group a 43 percent adverse outcome was observed, as compared with a 2 percent rate in the control group. Four of the alcohol exposed offspring died during the perinatal period and six exhibited dysmorphology consistent with the fetal alcohol syndrome. These offspring also displayed smaller growth parameters in the newborn nursery as well as at seven years of age. The validity of this work may be questioned on the grounds that chronic alcoholism of these mothers may have been complicated by certain adverse environmental conditions detrimental to the proper development of the offspring. Children of disorganized, multiproblem families are at greater risk in terms of psychological and intellectual development than are those from more stable family units. The I.Q. for children living with relatives or in foster homes averaged 84, whereas that of children living with their alcoholic mothers was 73 (17).

Adverse lifestyle, as well as contributing to postnatal intellectual developmental retardation of offspring, has been implicated as aggravating the prenatal effects of alcohol exposure (18). Elevated consumption of caffeine, nicotine, and narcotics as well as irregular maternal dietary and sleeping habits may compound the effects of alcohol on the fetus (15,18). Jones et al. (3) found no evidence of drug use by alcoholic mothers conceiving offspring characterized by fetal alcohol syndrome, and while most women smoked, they were reportedly not heavy smokers. However the accuracy of reported amounts and types of drugs consumed by outpatients

is questionable.

The initial documentation of the anomalies associated with fetal alcohol syndrome prompted a plethora of subsequent clinical studies in various countries (16-26). Large variation in the pattern of defects was reported, possibly linked to the degree and timing of alcohol consumption during pregnancy. Small widely spaced eyes, anteverted nostrils, and a carp-shaped mouth were common features characteristic of fetal alcohol syndrome.

Hypothalamic and pituitary function in four siblings exhibiting characteristics of fetal alcohol syndrome was investigated by Root et al. (19) to determine whether a hormone abnormality may account for the observed reduction in growth. Serum levels of human growth hormone, insulin, luteinizing hormone, and parathyroid hormone were all found within normal ranges in these siblings. It was inferred that postnatal growth retardation associated with fetal alcohol syndrome is not directly due to hormonal factors. Whether alcohol affects fetal hormone levels, or alternatively somatomedin function or hormone receptor development during gestation remains to be determined.

b) Prospective Studies

Although retrospective studies provide good evidence in support of the adverse effects of alcohol on the fetus, it is the prospective study which provides a more rigorous analysis. Subjects of a prospective study are assessed as to the severity and duration of alcoholism prior to delivery. The occurance

of differing patterns of drinking such as heavy episodic binges at certain times during gestation may be more readily determined in this type of study. Correlations may then be made between the outcome of pregnancy and extent or pattern of alcoholism in the mother. Prospective studies have been used to investigate the effect of dosage, duration, type of beverage consumed and the threshold amount of alcohol which elicits adverse effects in utero (20, 21, 23).

Mau and Netter (20) collected data on alcohol consumption from 5200 women at twenty German hospitals during the first trimester of pregnancy and compared alcohol intake to the well-being of the offspring. Women consuming alcohol gave birth to a larger number of offspring of a gestational age of less than 260 days, had more abortions, but showed no higher incidence of reduction in birth weight. Alcohol use, however, was evaluated subjectively as results were tabulated only as 'yes' (4.8 percent), or 'no' (95.2 percent). Thus under-reporting of actual levels of alcohol consumption may have influenced results.

A prospective study by Little (21) compared maternal alcohol consumption of 263 subjects before pregnancy, in early, and late pregnancy to birth weights of offspring. In order to control for smoking, which is related to reduced birth weight (22), similar proportions of smokers were selected for each category of pregnancy. Maternal age, height, parity, nicotine use, and alcohol consumption were variables entered into a regression equation which was computed for each of the three defined catagories of drinking

relative to pregnancy. Significant regression relationships were found between two of these defined periods and birth-weight. Daily ingestion of one ounce of absolute ethyl alcohol during the three month period prior to pregnancy was associated with a 91 g reduction in birth weight. The same amount of alcohol consumed during late pregnancy was assoc-iated with an average body weight decrease of 160 g. No other parameter of fetal health except birth weight was determined in this study and, since lower birth weights are not strictly related to teratogenic outcome, interpretation of these results must be made with caution. Furthermore, the relatively small sample size does not permit the robust analyses of more traditional statistical tests. However, this study takes into account some of the complicative factors such as smoking, age, height, and parity which hinder work in this area, and hence allows a better focus on the specific effects of alcohol in utero.

Kaminski et al. (23) have reported on the relationship between alcohol consumption among pregnant women and outcome of pregnancy from a prospective study of more than 9000 births. Mothers consuming more than 1.6 oz absolute ethyl alcohol per day were compared with those drinking less. Women consuming more than 1.6 oz per day were found to have more stillbirths and reduced infant birthweights as compared to the lighter drinking group. Actual differences between groups in birth-weight of offspring were relatively small even though they attained statistical significance. Placental weights, previously unreported in fetal alcohol syndrome, were found

significantly reduced in the heavier drinkers. No significant differences in the number of congenital malformations were reported between groups, nor was there any difference in mean gestational age. When risk factors including maternal age, parity, smoking, previous low birthweight of offspring, marital status, and metrorrhagia were taken into account through appropriate statistical techniques, differences between heavy and light drinkers persisted with regard to perinatal mortality, birthweight, and placental weight. Furthermore, types of beverages consumed were compared with outcome of pregnancy in those mothers drinking over 1.6 oz of alcohol per day. Infants born to women drinking only beer exhibited reductions in both mean birthweight and mean placental weight when compared with those who drank only wine. The actual differences in birthweight were again relatively small and while statistically significant may not be of clinical importance. Further findings showed that the type of beverage consumed was related to maternal socio-economic position, thus suggesting that environmental factors may contribute to the reduction in fetal growth.

c) Alcohol Induced Alteration in Maternal
   Metabolism as Contributing to Fetal
   Alcohol Syndrome.

Although extensive documentation of fetal alcohol syndrome, both in retrospective and prospective studies, has shown a significant association between maternal alcohol consumption and adverse outcome of pregnancy, these studies have

failed to ellucidate a mechanism of alcohol's mode of insult. Alcohol crosses the placenta and circulates through the fetal bloodstream (24). The odor of alcohol has been noted on the breath of a baby at birth which displayed characteristics of fetal alcohol syndrome. Ethyl alcohol determination on cord blood at birth revealed concentrations of as much as 150 mg per 100 mL (24). However, alcohol has not yet been shown to directly affect fetal metabolism. Because fetal development is dependent on the relationship between the mother and fetus, it can not be ruled out that alcohol's effect may be at a site other than the fetus.

A body of evidence is accumulating suggesting that alcohol may cause a persistent alteration in maternal metabolism or physiological state contributing to adverse fetal development (25, 28). These alterations are aggravated by duration and severity of alcoholism. Shruygin (25) has studied the effects of maternal alcoholism in 42 offspring of 19 mothers. Of 23 children born after their mothers had developed advanced alcoholism, 14 were mentally retarded, and many demonstrated signs of organic impairment of the central nervous system early in infancy. In contrast, 19 offspring born before their mothers had developed advanced stages of alcoholism demonstrated mainly vegetative, emotional, and behavioral disorders. These developed around ages 9 or 10 years and tended to remit following improvement in the social environment as a result of marriage or employment.

Majewski (26) has suggested a relationship between length of alcohol addiction in the mother and severity of

alcohol insult in utero. He reported that younger siblings are the most highly affected by maternal alcoholism due to the temporal progression of the disease. It was suggested that acetaldehyde, an alcohol metabolite, is responsible for alcohol's adverse effect. The extent of adverse effect may depend on the maternal acetaldehyde metabolism capacity, which is thought to be impaired with extended maternal alcohol consumption. Mean daily blood alcohol levels may increase with duration of the disease as a result of an increase in amounts of alcohol consumed. Thus the higher susceptibility of siblings whose mothers had been alcoholics for a longer time period may be explained simply as due to the elevated alcohol insult in utero. However this explanation is refuted by work documenting decreased birth weights in offspring of mothers who had abstained from alcohol consumption during pregnancy (27,28). Scheiner et al. (27) reported a case with congenital malformations as well as physical and intellectual developmental retardation associated with fetal alcohol syndrome. It was reported that the parents, although heavy drinkers for sixteen years had abstained one and a half years prior to conception. The possibility can not be ruled out that the mother did in fact drink while pregnant or that the child displayed not fetal alcohol syndrome but a suggestive phenotype with some other etiology.

Little et al. (28) have provided further evidence suggesting the existence of an alcohol susceptible maternal component, responsible at least partially, for the adverse

effects in utero. Infants born to women who had a history of alcoholism prior to conception, but had abstained during pregnancy, were compared to infants whose mothers drank heavily throughout the course of pregnancy and to infants of non-alcoholic controls. The three groups were matched for maternal education, smoking during pregnancy, race of offspring, and age of offspring at the time of the study. Mean birth weights of children born to abstinent alcoholics was determined as 258 g less than the mean birth weight of children born to controls. For the drinking alcoholic group the mean birthweight was 493 g below that of controls. The possibility was examined that the abstinent alcoholic women may have reported their drinking inaccurately, however it was noted that there would be no reason for them to do so purposely. In cases where the interviewer suspected inadvertent error, internal verifications were performed by listing the events in the subject's life during the time surrounding pregnancy, and crosschecking her recall during these events.

In summary, a review of the clinical studies reporting the effects of maternal alcohol consumption on the outcome of pregnancy indicate that this phenomenon, although noted historically, has only recently been extensively examined. Retrospective and prospective investigations have as yet failed to determine the mode of insult of alcohol, although the pattern of malformations characteristic of fetal alcohol syndrome have been well established. The extent of contribution of adverse environmental factors to fetal alcohol

syndrome is unknown. Duration of alcoholism relating to severity of fetal insult and appearance of fetal alcohol syndrome in offspring whose mothers stopped drinking prior to conception are both factors suggesting that the deleterious effect of alcohol may not be restricted directly to the fetus. Alternatively, alcohol may be deleterious to a susceptible component of maternal physiology or metabolism.

<u>Animal Studies</u>   a) Introduction

The advantage of the animal model in fetal alcohol
syndrome research is to allow investigation of the uncom-
pounded effect of alcohol on the unborn without complicative
environmental variables found in human studies. Early reports
evaluating the effects of maternal alcohol consumption on
fetal outcome in the fowl and the rat have appeared since
the turn of the century (29 - 34); however dubious method-
ology has often jeopardized the validity of these investiga-
tions. More recent studies employing isocalorically pair-
fed control groups, oral routes of alcohol administration,
and dosages similar to those encountered in the human alc-
oholic have been performed in rats, mice, and other species.
These improvements in experimental design have facilitated
the development of an animal model which closely approximates
fetal alcohol syndrome as manifest in the human.

b) Prenatal Effects of Alcohol Administration in
the Rat.

Sandor and Amels (35) made one of the initial signif-
icant contributions in the investigation of the effect of
alcohol on prenatal development in the rat. Alcohol was
administered through intravenous injection on days six,
seven, and eight of gestation in one group of animals and
days six and seven in another group. Dosages of 1.5 g per
kg body weight were administered to animals of each group.
Fetuses were removed at day nineteen of gestation by

ceasarian section and examined for dysmorphology. Of the
doses injected, alcohol in the concentration of 2 g per kg
body weight elicited twice the number of malformations as
did the lower concentration. Malformations observed included
skeletal structure anomalies, most apparent in the extrem-
ities and facial areas. Results furthermore indicated that
the two heavier dosages administered on days six and seven
were more deleterious than the three lighter doses given over
days six, seven, and eight although total amounts of alcohol
were identical. Intravenous injection does not represent
the normal mode of alcohol intake and factors such as
interference of normal nutrient absorption and rate of
increase of blood alcohol levels are not considered in this
study. However, results obtained show a similar pattern of
malformation in this rat model as seen in human fetal alcohol
syndrome.

Subsequent work involved the use of an oral route of
alcohol administration which is more analogous to the chronic
human alcoholic. Tze and Lee (36) fed pregnant rats 30 g
alcohol per 100 mL water as their only available drinking
fluid. A pair-feeding design was incorporated whereby, as
well as an alcohol fed and ad libitum control group, a third
group of rats received powdered diet and corn starch isocaloric
with that consumed by alcohol fed animals. The effect of
reduced caloric intake in the alcohol fed group was thus
accounted for by the existence of this pair-fed group. All
animals were mated one month after the initiation of this

dietary protocol and allowed to deliver at term. Gestation period did not vary among the three treatment groups. Only fifty percent of the alcohol dams known to have copulated delivered litters. In comparison, 88 and 91 percent of control and pair-fed groups, respectively, assessed as pregnant, produced litters. The average litter size of the alcohol treated mothers was significantly lower than that of pair-fed or control groups. Offspring of the alcohol fed group displayed microcephaly, cracked dry skin, reddening of areas of the head and body, and a generally shriveled appearance. Malformations reported by earlier authors (35) were not observed. A possible criticism of this model may stem from the fact that the pregnant rats were allowed to deliver at term and cannibalization may have affected the recorded litter size. Furthermore resorptions can not be determined. Sacrifice of the animals prior to delivery permits greater accuracy of assessment of litter size and number of fetal resorptions through determination of implantation sites at autopsy.

Studies using rat models of fetal alcohol syndrome do not consistently report associations between maternal alcohol exposure and fetal insult in spite of the improvements in design which have emerged over the last decade. Schwetz et al. (37) found no prenatal effects in rats given drinking fluid containing 12 to 20 percent alcohol. This level of alcohol consumption may have been insufficient to elicit the anomalies observed by other workers. Peak

blood alcohol levels were reported to be 40 mg per 100 mL, lower than values reported by other workers. Oisund et al. (38) also found no significant differences in body, brain, liver, kidney, or heart muscle weights between fetuses of control and alcohol exposed pregnant rats. Animals were fed 12 percent alcohol in their drinking water to cover 20-25 percent of their caloric intake. Alcohol exposure extended from three weeks prior to mating, during, and after pregnancy. Soya oil was used as an alcohol substitute in the pair-fed group animals. Again the observed absence of adverse effects of in utero alcohol exposure may be attributable to the low dosage level in this model. Similarly, Abel (39) and Abel and Greizerstein (40) showed that when pregnant rats were given alcohol by stomach tube through-out pregnancy, no abnormality in growth of offspring could be attributed to doses of 1 or 2 g per kg body weight, but following the administration of 4 or 6 g per kg, diminished birth weight, delayed postnatal growth, and greater postnatal mortality occurred in alcohol exposed offspring compared to control progeny. Generally, when sufficient dosage levels are administered to pregnant rats, studies report adverse fetal outcome. Henderson et al. (41) have also demonstrated that alcohol administration in a liquid diet to rats during pregnancy is associated with adverse effects on fetal via-bility and growth. Maternal blood alcohol levels were reported as 70-200 mg per 100 mL, substantially greater than those of Schwetz et al. (37), where no adverse effects

were noted. These studies suggest that a threshold level
of blood alcohol may be required to produce visible effects.

Although much interest in maternal alcohol consumption
has centered around the fetus, the placenta has recently
also become the focus of attention in the rat model of
fetal alcohol syndrome. Abel and Greizerstein (40) found
increased placental weights in alcohol exposed pregnant rats
when compared with controls, however differences were not
statistically significant. Wiener et al. (42), Skosyreva
(43), and Gordon et al.(44) have reported significantly
heavier placentas in alcohol exposed rats. Gordon et al.
(44) fed rats a liquid diet containing 26 percent alcohol
derived calories for two weeks prior to and during gestation.
Fetal weights did not vary between alcohol fed and control
groups suggesting that the increase in placental size found
on day 20 of gestation may exist as an adaptive mechanism
compensating for the deleterious effect of alcohol.

c) Prenatal Effects of Alcohol Administration
in the Mouse

The malformations observed in human fetal alcohol
subjects appear to be more easily elicited in the mouse
model of maternal alcohol consumption than in the rat model.
Kronick (45) employed a mouse model to study the effects of
acute alcohol exposure on the outcome of pregnancy. Alcohol
treatment consisted of an intraperitoneal injection of a
25 percent solution of ethyl alcohol at a does of 3 mL per

100 g body weight. The strength of this dose induced
hypnosis with animals remaining comatose for up to four hours
following treatment. One group of mice received injections
on the eighth and ninth day of gestation, another group on
the tenth and eleventh day of gestation, and a third group
on only one gestational day ranging from day seven through
day twelve. Control animals were injected with a saline
solution and matched with alcohol injected dams. Mice were
sacrificed on gestational day 18 and uterine contents
examined. Results indicated substantially elevated fetal
mortality rates in day eight and nine as well as day ten
and eleven alcohol exposed mice as compared to controls
injected with saline. Malformation frequency was increased
in alcohol-treated groups with the genesis of specific
malformations dependent on the time of injection during
gestation. Coloboma of the iris and ectrodactyly of the
forepaws appeared to have critical periods at days eight to
nine and at day ten, respectively, of gestation. The
intraperitoneal route of alcohol administration used in
this model is not consistent with the oral consumption
pattern as seen in human alcoholism, and may exist as a flaw
in methodology. A subsequent study by Chernoff (46) using
a mouse model employed an oral route of alcohol administra-
tion as well as pair-fed control groups.

Chernoff (46) fed alcohol through an all liquid diet to
two strains of mice. Alcohol treatment extended from thirty
days prior to mating until day 18 of gestation, at which time
females were sacrificed and uterine contents examined. One

strain of mice exhibited significantly more fetal malforma-
tions than the other, suggesting that the rate of alcohol
metabolism may differ between the two strains. The liver
alcohol dehydrogenase system, responsible for the metabolism
of alcohol, is known to be under genetic control in the
mouse (47). The fetal malformations included intrauterine
growth deficiency as evidenced by low fetal weight, incom-
plete ossification, neural and cardiac anomalies, and
skeletal dysmorphogenesis. The pattern of growth retarda-
tion together with ocular, neural, cardiac, and skeletal
anomalies were similar to that of the fetal alcohol syndrome
observed in humans.

Another animal model employing an orally administered
alcohol treatment and a pair-feeding design in mice was
developed by Randall and Taylor (48). Pregnant mice were
administered diets with 17, 25, or 35 percent of total daily
calories supplied by alcohol from day five to day ten of
gestation. Isocaloric substitution of sucrose for alcohol
was effected in the pair-fed group. Sacrifice of the gravid
dams was made on day 19 of gestation. Results showed that
in the 25 percent alcohol-calorie group, fifteen out of
sixteen litters had at least one malformed fetus. Systematic
external examination of the fetuses was followed by internal
examination under a dissecting microscope. Anomalies observ-
ed included malformations of the limbs, cardiovascular
abnormalities, urogenital hydronephrosis, and hydrocephaly.
As with the study of Chernoff (46), the anomalies found
in this mouse model are analagous with those of human offspring

displaying fetal alcohol syndrome.

Lack of prenatal effects of alcohol in mice has also been reported (37, 49), possibly due, as seen in the rat model, to an insufficient level of exposure to alcohol. Determination of the level of alcohol exposure is a current methodological problem in animal models of fetal alcohol syndrome. Comparisons of alcohol intake between man and animals are difficult, plagued by problems relating to differing alcohol metabolizing rates. Measurement of ingested ethyl alcohol expressed as g per kg of body weight per day is used by some workers, however serum alcohol levels presently are most widely accepted. The use of serum alcohol levels as indicators of alcohol intake obviates the difficulties acquired because of differing alcohol pathways and metabolic rates among animal species.

The preceding studies indicate that using a suitable animal model of maternal alcohol exposure, the pattern of malformation seen in offspring born to humans are better approximated in the mouse than in the rat. However, clear cut adverse effects including prenatal growth retardation are observed in the rat, and the use of certain experimental protocols makes this larger animal a more attractive experimental subject.

d) Prenatal Effects of Alcohol Exposure in
   Other Animals

Although most of fetal alcohol syndrome research using

animal models has focused on rats and mice, other species
have also been the subject of investigation. Simulating
blood alcohol levels which would be normal in the human
alcoholic, Sandor and Elias (50) studied the effects of
alcohol in chick embryos in early stages of development. In
eggs injected with alcohol embryos exhibited higher mortality
rates and weight loss towards the end of incubation as com-
pared with controls. However, extrapolation from the develop-
ment of the chick embryo to that of the embryo in utero is
tenuous, hence in most subsequent work mammals were used as
experimental subjects.

Dexter et al. (51) fed miniature Sinclair swine alcohol
solutions orally both prior to and during gestation. Miniature
swine consume large quantities of alcohol despite adequate
diet and constantly available water, allowing investigation
into the effects of prenatal effects of alcohol consumption
in the presence of adequate nutrition and without disturb-
ing an established behavior. Results showed that consumption
by pregnant swine of alcohol at levels equivalent to one
quart of whiskey per day in the human resulted in a decrease
in litter size and birth weight, as well as increased rates
of occurance of morphological anomalies and postnatal mort-
ality. Anomalies observed were similar to those seen in
human fetal alcohol syndrome subjects.

Ellis and Pick (52) used a beagle model to study the
effects of maternal alcohol consumption on the fetus.
Alcohol was administered through gastric intubation at

six different dosage levels in an attempt to determine a
threshold level for onset of adverse effects. With increas-
ing dosage a higher occurance of fetal mortality and mal-
formation was observed, with no animal in the group exposed
to alcohol at the greatest dosage level delivering live born
offspring. Blood alcohol levels of this group reached 255 mg
per 100 mL. Animals consuming the lowest of the dosages,
1.4 g ethyl alcohol per kg body weight, displayed no signi-
ficantly increased rate of malformation or stillbirth,
however progressively higher dosages were found detrimental
to fetal welfare. Extrapolating these results to the human
subject, if a woman weighing 60 kg were to consume 6 oz of
100 proof alcohol per day during gestation, the wellbeing of
her fetus may be at risk. The use of the beagle in fetal
alcohol syndrome research is advantageous in that a close
similarity in alcohol pharmacokinetics exists between man
and the dog, however restrictions in group size inherent in
using an animal of this size may limit the usefulness of
certain statistical treatments.

Ho et al. (53) fed alcohol to pregnant monkeys and
hamsters in order to determine its distribution in the
maternal and fetal blood, organs and central nervous system.
The distribution of alcohol in the placenta and amniotic
fluid was also studied. Ethanol labeled with ^{14}C was inject-
ed intravenously at doses ranging from 0.5 to 1.5 g per kg
body weight following which animals were killed by bleeding
at fixed time intervals. Radioautography of the brain, fetal,
and placental sections was performed, and alcohol determinations

made on homogenates of these tissues. Results indicated
that during late pregnancy both the placenta and amniotic
fluid contained high concentrations of alcohol compared with
distribution to other tissues. Concentrations of unmeta-
bolized alcohol in the hamster fetus was higher in later
pregnancy than prior to placental development, suggesting
that the placenta was not a barrier to alcohol. Alcohol
metabolism in the monkey was slower in the fetus than in the
mother, as indicated by higher alcohol concentrations in
fetal tissues 90 minutes after injection of the dose. Fetal
alcohol dehydrogenase levels are reported to be reduced when
compared with maternal levels, possibly accounting for this
higher fetal accumulation of alcohol (54). The ^{14}C labeled
alcohol in the monkey fetus was localized in the liver,
pancreas, kidney, lung, and heart muscle with a high concent-
ration in the cerebellum. The hippocampus, putamen, and
geniculate body were also sites of accumulation. No observed
malformation in alcohol exposed fetuses was reported in this
study.

     e) Comparison of Prenatal Alcohol Exposure and
        Prenatal Malnutrition on Body Composition
        in Offspring.

The mechanism by which maternal alcohol consumption
affects fetal growth remains unclear. Studies reporting
absence of association between alcohol consumption during
pregnancy and adverse outcome suggest that inadequate
nutrition is at least in part responsible for the deleterious

effect in utero (37,38). However differences in body com-
position of offspring exposed to alcohol in utero as compared
to food restricted pair-fed offspring (40,41) suggest that
these growth retarding effects are not similar. Reductions
in brain, heart, kidney, and liver weights were observed in
fetuses exposed to alcohol in utero (41). Moreover, signif-
icantly elevated total tissue protein concentrations were
found in 20 day fetuses in a rat model of alcohol exposure
in utero (41). These results are contrary to observations
in offspring of malnourished rats where lean body mass is
generally reduced or unaffected (55).

Abel and Greizerstein (40) have also described metabolic
changes in growth retarded rat fetuses as a result of alcohol
exposure in utero. In the fetuses whole body sodium content
was significantly elevated in the alcohol treated group. No
differences were noted in whole body zinc, calcium, or
magnesium content of fetuses between alcohol and food
restricted pair-fed groups. Elevated whole body sodium
content is not characteristic of the malnourished rat (56).
Furthermore, no decrease in fat content of the alcohol
exposed fetus, as is found in the fetuses of malnourished
rats, was reported in this study (40). These results suggest
that malnutrition is not solely responsible for the effects
associated with in utero exposure to alcohol.

  f) Comparison of Growth and Development in
   Offspring of Alcohol-Fed Versus
   Malnourished Animals.

Although differences in body composition have been reported between fetuses of alcohol exposed and malnourished rats (40,41), the similarity in the pattern of growth and developmental retardation in each case suggests that these effects may share a common etiology. Studies investigating the effects of maternal protein deficiency on neonatal growth show striking similarities to those on the adverse effect of alcohol. Young born to protein restricted mothers but cross fostered to control mothers at birth exhibit a persistent retardation in growth, which is not corrected by reducing litter size (55). Delayed appearance of ossification centers and retarded skeletal growth were observed with little indication of catch up growth. Leichter and Lee (7), Lee and Leichter (8), and Detering et al. (57) have reported similar findings in alcohol exposed offspring. In addition to retarded growth, offspring of animals receiving alcohol during pregnancy also exhibit a retardation in the rate of skeletal maturation, despite the fact that mothers did not receive alcohol after delivery (7). Catch up was not observed either in growth or skeletal maturation during the first four weeks postnatally. Furthermore, culling the litter size from eight to three of alcohol exposed offspring to account for the possibility of postnatal malnutrition had no statistically significant stimulatory effect on any of the parameters of physical growth or maturation measured (8). A reduction in brain weight, crown rump length and tail length were observed at three weeks post partum in rat pups exposed to alcohol in utero but weaned to control dams compared with pups of

equivalent age from pair-fed dams (57).

These studies indicate that the postnatal growth retard-ation observed in fetal alcohol syndrome animal models is similar to that ascribable to maternal protein malnutrition. This is contrary to indications based on body composition determinations. The observed postnatal growth retardation appears to be of different origin because of reports suggest-ing that, while in protein malnourished rats growth hormone synthesis is impaired (55), no growth hormone abnormalities have been observed in humans displaying fetal alcohol syndrome (58). Rosso and Kava (59) have suggested that the mechanism of fetal insult from malnutrition results from a decreased maternal-fetal transfer of nutrients. Although the use of pair-feeding designs in animal models of fetal alcohol syndrome has indicated that maternal food insuffi-ciency is not entirely responsible for the retarded fetal growth, alcohol may adversely affect the supply of nutrients to the fetus, thus mimicking the effect of malnutrition. This may explain the similarities observed in modes of action of these growth retarding factors.

MATERIALS AND METHODS

a) Preliminary Treatment of Animals

One hundred and twenty virgin Sprague-Dawley rats (Biobreeding Laboratories, Queb. Canada) weighing 200-225 g were individually housed in screen bottom cages. The living environment was maintained at 21°C with a twelve hour light-dark cycle. After a one week stabilization period animals were assigned at random to one of three dietary treatment groups. Group 1 (alcohol) received 10 percent ethyl alcohol (v/v) in drinking water and a nutritionally adequate diet (Purina Rat Chow) ad libitum. After one week the ethyl alcohol content of the drinking water was increased to 20 percent. Group 2 (pair-fed) was administered amounts of nutritionally adequate diet (Purina Rat Chow) equal to that consumed by the group 1 animals during the previous 24 hours, with alcohol isocalorically substituted with corn starch. Drinking water was provided to the pair-fed group ad libitum. Group 3 (ad libitum control) was allowed the nutritionally adequate diet and drinking water ad libitum. After four weeks on this regimen all animals were bred overnight to male Sprague-Dawley rats. The presence of sperm in the vaginal washings established day 1 of pregnancy. At that time the alcohol content of the alcohol group drinking water was increased to 30 percent. Maternal body weights were determined weekly and food and alcohol consumption was determined daily throughout the experiment.

b) Determination of Plasma Alcohol, Glucose,

Folic Acid, and Zinc.

Blood samples (300-400 uL) for the determination of maternal plasma alcohol, glucose, folic acid, and zinc were taken from the tip of the tail early in the morning during the third week of gestation. The blood samples were collected into heparinized hematocrit tubes, centrifuged at 1500 rpm for 5 min., and the plasma was placed in small plastic tubes. The plasma was kept frozen at -5° C. until the time of assay.

Plasma alcohol concentrations were determined by the alcohol dehydrogenase method (Sigma Chemical Company, Saint Louis, Missouri, Technical Bulletin No. 331-UV). Glucose determinations were performed using the glucose oxidase method (Sigma Chemical Company, Technical Bulletin No. 510). Folic acid was measured by the radioassay from Bio-Rad Laboratories, Richmond, California, (Quanta-Count Folate, Bulletin No. 4201), and zinc was determined by atomic absorption spectrophotometry (Perkin-Elmer Atomic Absorption Spectrophotometer, Model 603).

c) Placental Blood Flow Determination

Cardiac output and blood flow to the placenta and other organs were measured with radioactive microspheres as described by McDevitt and Nies (60) and Bruce (61). On day 20 of gestation the rats were anesthetized by intraperitoneal injection of 10 mg per 100 g body weight of Inactin

(Promonta, Hamburg, Germany). A cannula (PE 10, Intramedic, Clay Adams Co., Parsippany, N.J.) was then inserted into the left femoral artery and attached to a withdrawal pump (Harvard Apparatus, South Natick, Mass.) to draw a reference sample for calculation of the blood flow rate. A second cannula (PE 50, Intramedic, Clay Adams Co., Parsippany, N.J.) was introduced into the left ventricle via the right carotid artery for injection of the microspheres. The ventricular location was determined by observing the characteristic pressure waves using a pressure transducer and chart recorder (Harvard Apparatus, Millis, Mass.) connected to the cannula of the right carotid artery. Both cannulas were filled with heparinized saline.

Microspheres (New England Nuclear, Boston, Mass.) labeled with ^{57}Co and with a mean diameter of 15 microns were suspended in 6 percent dextran and mixed thoroughly prior to injection into the left ventricle. About 40,000 microspheres in a total volume of 0.2 mL were injected over a period of 20 seconds. Simultaneously the reference sample of blood from the femoral artery was withdrawn at a rate of 0.445 ml/minute for 90 seconds.

Following the collection of the reference sample the rat was killed by incision of the heart. Fetuses, placentas, heart, kidneys, and a muscle sample were immediately removed from the carcass, lightly blotted, and weighed. Only litters of four fetuses or more were included in the study. The position of the carotid cannula was confirmed at this time by dissection of the heart. All tissues including the

reference blood sample were assayed for radioactivity in

a gamma counter (Picker Nuclear, North Haven, Conn. Model

600-120). Cardiac output and blood flow to placentas and

organs were calculated using the following formulae:

$$\text{Cardiac Output (mL/min)} = \frac{\text{CPM injected X reference sample withdrawal rate (mL/min)}}{\text{reference sample CPM}}$$

$$\text{Organ Blood Flow (mL/min)} = \frac{\text{reference sample withdrawal rate (mL/min) X organ CPM}}{\text{reference sample CPM}}$$

To investigate the possibility that the level of alcohol

exposure in this study may result in dehydration, the water

content of the muscle sample was determined by heating to

constant weight in a vacuum oven at 70°C. Using a sample of

arterial blood collected prior to sacrifice of the animal,

another parameter of dehydration, plasma osmolality, was

determined using an osmometer (Precision System Inc., Newton,

Mass.).

d) Fetal Uptake of Folic Acid, Zinc, and
Analogs of Glucose and Amino Acid

To determine the fetal uptake of folic acid, zinc, and

analogs of glucose and amino acid, rats on day 20 of gestation

were anesthetized with ether and injected with either: 1 μCi

methyl (α- D$[U^{14} - C]$gluco) pyranoside and 10 uCi 3', 5',

7, 9 -^{3}H folic acid, or 1 μCi 2 - amino (1-^{14}C) isobutyric

acid, or 8 μCi ^{65}Zn in HCl solution, per 100 g body weight.

All compounds were injected into the left femoral vein.

Ninety minutes after the injection the fetuses and maternal

liver were removed, weighed, and blood was obtained from the

dam by heart puncture. The decision to use the 90 minute time point after labeled compound injection was based on a preliminary study which indicated that the concentration of label in the fetus was higher at 90 minutes than at either 40 or 60 minutes. Radioactivity was determined in plasma and liver of the dam and in four fetuses located in positions 1 and 3 of each uterine horn counting from the terminal end. Litters with eight fetuses or more were included in the study. To measure the radioactivity of tritium and ^{14}C the liver and fetuses were homogenized in 9 parts of distilled water and centrifuged at 2,000 rpm for 10 min. Aliquots of the supernatant fractions (0.5 mL) were transfered to glass scintillation vials containing 10 mL of scintillation fluid (ASC, Amersham, Arlington Heights, Ill.) and the radio-activity was counted in a Picker Nuclear Liquimat Scintillation Counter. Corrections for quenching were made by the channels ratio method. In the case of ^{65}Zn the fetuses, livers and plasma were placed in test tubes and radioactivity was counted using a Picker Nuclear Autowell II Gamma Spectrometer. All the radioactive compounds were purchased from Amersham, Arlington Heights, Ill.

e) Statistical Analysis of Results

Results were examined statistically by analysis of variance, using the Midas Computer Package (62). In the case of significant differences, Sheffe's intervals (0.9 and 0.95) were used with paired comparisons to identify which

group was responsible for the difference. A level of signifi-
cance of $P \leqslant 0.05$ was used for all tests.

RESULTS

a) Food Intake and Maternal Body Weights

Table 1 shows the average daily food and alcohol consumption and weekly body weights of rats given alcohol, pair-fed, or ad libitum fed prior to pregnancy. Aside from the first week of this period body weights of ad libitum control animals were significantly greater ($p < 0.05$) than those of either alcohol group or pair-fed group animals. This is reflected in the reduced food consumption of these latter two groups. Average ethyl alcohol consumption during this period by alcohol group animals amounted to approximately 2.5 mL per day.

Table 2 shows the daily caloric intakes of the three treatment groups prior to pregnancy. Total caloric intakes of the alcohol and pair-fed groups were about 80 percent of that of the ad libitum controls. The absence of variability in amounts of food consumed by the pair-fed group is reflective of the pair-feeding design where all animals of this group consumed all of the food given. Prior to pregnancy alcohol provided 25 to 30 percent of the calories of the alcohol group.

Table 3 shows the average daily food and alcohol consumption and weekly body weights of rats given alcohol, pair-fed, or ad libitum fed during pregnancy. Significantly reduced body weights are observed in alcohol ($p < 0.0001$) and in pair-fed ($p < 0.001$) group animals when compared with ad libitum controls during each of the three weeks of gestation. The absence of statistically significant differences in body

weights among the three groups at day one of gestation may reflect that the fact that since mating occurred over a two week period, a large variability in body weights within each group may have developed. No significant differences were noted in body weights during gestation between alcohol group and pair-fed group animals. The average consumption of ethyl alcohol by the alcohol group was 4.68 ± 0.05 mL (mean $\pm$ standard error) per day during this period.

Daily caloric intakes of the three treatment groups during pregnancy are displayed in Table 4. During gestation, alcohol provided about 34 percent of the calories in the alcohol group. Total caloric intakes of the alcohol and pair-fed groups were 71 percent of that of ad libitum controls during the first week of gestation and 91% during the last two weeks of gestation. This is reflected in the reduced weight gain of these two groups over ad libitum controls as seen in Table 3, even though there appears to be no significant difference in caloric intake during the latter part of gestation.

b) Litter Size, Fetal Body Weights, and
   Placental Weights.

Litter size, fetal body weights, and placental weights of rats on day 20 of gestation are shown in Table 5. Although no differences were observed in litter size among alcohol exposed animals when compared with those of either pair-fed or ad libitum control groups, a significant reduction in

TABLE 1

Daily food and alcohol consumption and weekly body weights of rats given alcohol, pair-fed, or ad libitum fed prior to pregnancy. Means ± standard deviation.

	Alcohol Group			Pair-fed Group			Ad libitum fed Group	
	Food Consumed (g)	Alcohol C'med. (mL)	Body Weight (g)	Food Consumed (g)	Starch C'med (g)	Body Weight (g)	Food Consumed (g)	Body Weight (g)
Number of Rats	(13)	(13)	(33)	(12)	(12)	(27)	(12)	(27)
Pre-pregnancy days 1-7	12.1 ±2.1	2.0 ±0.3	236.5[a] ±13.6	12.1 ±0.0	2.5 ±0.0	233.1[a] ±8.00	16.9 ±4.1	241.7[a] ±11.6
8-14	10.3 ±2.2	2.5 ±0.5	242.0[a] ±15.9	10.3 ±0.0	3.4 ±0.0	244.1[a] ±8.74	16.8 ±2.6	256.2[b] ±16.0
15-21	10.0 ±2.8	2.8 ±0.4	252.7[a] ±16.9	10.0 ±0.0	3.7 ±0.0	248.3[a] ±7.77	16.6 ±3.1	267.9[b] ±19.4
22-28	10.0 ±2.5	2.7 ±0.3	260.4[a] ±20.2	10.0 ±0.0	3.6 ±0.0	259.0[a] ±9.04	18.2 ±2.6	275.4[b] ±20.5

a, b, = figures in the same row not sharing the same letter are significantly different at $p < 0.05$.

TABLE 2

Daily caloric intakes of rats given alcohol, pair-fed, or *ad libitum* fed prior to pregnancy. Means ± standard deviation

	Alcohol Group		Pair-Fed Group		Ad Libitum fed Group
	Food Consumed	Alcohol Consumed	Food Consumed	Starch Consumed	Food Consumed
		(Calories)			
Number of Rats:	(13)	(13)	(12)	(12)	(12)
Pre-pregnancy days					
1-7	51.4 ±9.0	10.0 ±2.1	51.4 ±0.0	10.0 ±0.0	71.8 ±17.5
8-14	43.8 ±9.3	13.6 ±3.5	43.8 ±0.0	13.6 ±0.0	71.4 ±11.1
15-21	42.5 ±11.9	14.8 ±2.8	42.5 ±0.0	14.8 ±0.0	70.6 ±13.1
22-28	42.5 ±10.6	14.4 ±2.1	42.5 ±0.0	14.4 ±0.0	77.4 ±11.0

TABLE 3

Daily food and alcohol consumption and weekly body weights of rats given alcohol, pair-fed, or <u>ad libitum</u> fed during pregnancy. Means $\pm$ standard deviation.

| | Alcohol Group | | | Pair-fed Group | | | <u>Ad Libitum</u> fed Group | |
	Food Consumed (g)	Alcohol C'med (mL)	Body Weight (g)	Food Consumed (g)	Starch C'med (g)	Body Weight (g)	Food Consumed (g)	Body Weight (g)
Number of Rats:	(11)	(11)	(34)	(10)	(10)	(32)	(6)	(29)
Days of Gestation								
1	–	–	269.2^a ±20.3	–	–	267.3^a ±17.0	–	275.0^a ±24.6
2-7	9.66 ±2.0	4.05 ±0.7	273.8^a ±22.4	9.6 ±0.0	5.27 ±0.0	276.3^a ±23.1	20.5 ±6.7	297.0^b ±25.5
8-14	11.6 ±1.8	4.7 ±0.7	298.4^a ±26.8	11.6 ±0.0	6.09 ±0.0	299.1^a ±22.8	19.0 ±7.2	326.9^b ±28.9
15-20	12.3 ±2.2	5.3 ±1.0	331.6^a ±28.8	12.3 ±0.0	6.90 ±0.0	339.6^a ±29.4	20.5 ±12	388.1^b ±38.2

a, b, = figures in the same row not sharing the same letter
        are significantly different at $p < 0.05$.

TABLE 4

Daily caloric intakes of rats given alcohol, pair-fed, or <u>ad libitum</u> fed during preganancy, Means ± standard deviation.

	Alcohol Group		Pair-fed Group		Ad libitum fed Group
	Food Consumed	Alcohol Consumed	Food Consumed	Starch Consumed	Food Consumed
			(Calories)		
Number of Rats:	(11)	(11)	(10)	(10)	(6)
Day of gestation 2-7	41.1 +8.5	21.1 +5.2	41.1 +0.0	21.1 +0.0	87.1 +28.5
8-14	49.3 +7.6	24.4 +4.9	49.3 +0.0	24.4 +0.0	80.8 +30.6
15-20	52.3 +9.3	27.6 +7.0	52.3 +0.0	27.6 +0.0	87.1 +51.0

litter size (p < 0.01) was found in the pair-fed over the ad libitum group. The fetal and placental weights were not affected by their location on the uterine horns.

No evidence of fetal malformation was observed in any of the three treatment groups, however fetuses of alcohol exposed animals exhibited reddening of the skin and a generally more shrivelled appearance than those of either pair-fed or ad libitum control groups. Fetal body weights on day 20 of gestation were significantly reduced in the alcohol group when compared with either the pair-fed (p<0.0001) or ad libitum control (p < 0.0001) groups. Furthermore, fetal body weights of the pair-fed group were also significantly lower than that of the ad libitum controls (p < 0.05), possibly reflective of the reduced caloric intake of the former group. By contrast, placentas of the alcohol group were substantially heavier (p < 0.001) and placentas of the pair-fed group lighter (p. < 0.05) relative to those of ad libitum controls.

Animals were occasionally inadvertently omitted at weekly weighing during the pregestational or gestational periods hence were excluded from the treatment group mean body weight. This is reflected in differences in the number of rats or litters between Tables 1 through 5, where prior to day 20 of gestation, actual sample size may be larger than tabulated.

TABLE 5

Litter size, fe tal body weights, and placental weights of rats at day 20 gestation given alcohol, pair-fed, or _ad libitum_ -fed. Means $\pm$ standard error.

	Alcohol Group	Pair-fed Group	Ad libitum Group
Litter size	$11.3 \pm 0.32^{a,b}$	10.8 ± 0.41^{b}	12.3 ± 0.43^{a}
Number of litters	35	33	26
Fetal body weight (g)	2.01 ± 0.03^{a}	2.33 ± 0.04^{b}	2.51 ± 0.08^{c}
Number of litters	35	33	26
Placental weight (g)	0.614 ± 0.02^{a}	0.416 ± 0.01^{b}	0.485 ± 0.02^{c}
Number of litters	11	12	10

a,b,c,= figures in the same row not sharing the
same letter are significantly different at $p < 0.05$

c) Alcohol and Nutrient Concentrations

in Maternal Plasma

Maternal plasma concentrations of ethyl alcohol, glucose, folic acid, and zinc during week three of gestation are shown in Table 6. Average plasma alcohol levels during days 15-17 of gestation of the alcohol group were 76 ± 6.6 mg per 100 mL (mean $\pm$ standard error). Maternal plasma concentrations of glucose, folic acid, and zinc did not differ significantly among the three treatment groups, and were within expected ranges in all cases (9,63,64).

d) Placental Blood Flow

The data on placental blood flow, maternal renal blood flow, and maternal cardiac output is presented in Table 7. Blood flow to the placenta, whether expressed as mL/min per placenta, mL/min per g placenta, or as percent of cardiac output, was significantly reduced in the alcohol group when compared with either pair-fed ($p<0.05$, $p<0.001$, $p<0.05$, respectively) or ad libitum control ($p<0.001$, $p<0.001$, $p<0.05$) groups. Furthermore, when expressed as mL/min per placenta a significantly decreased blood flow was observed in pair-fed animals in comparison with ad libitum controls ($p<0.05$), however this decrease was not statistically significant when flow was expressed as mL/min per g placenta due to the differences in placental weights between groups. No significant differences were seen in blood flow to the

kidney among the three treatment groups, nor in the maternal
cardiac output. It is interesting to note the substantially
elevated standard error measurement of renal blood flow in the
alcohol group compared with that of pair-fed or ad libitum
control groups.

e) Fetal Uptake of Nutrients

Fetal uptake of 2-amino isobutyrate, methyl-α-D
glucopyranoside, folic acid, and zinc at day 20 of gestation
for alcohol, pair-fed, and ad libitum fed groups is shown in
Table 8. In the case of 2 amino isobutyrate, methyl-α-D
glucopyranoside and zinc no significant differences in fetal
uptake were observed over the 90 minute incubation period
among the three treatment groups. Trends exist in the cases
of 2-amino isobutyrate and zinc favouring a reduced fetal up-
take by the alcohol group over pair-fed and ad libitum groups
however statistical significance is not achieved. The folate
taken up by the fetuses of the alcohol group was significantly
higher ($p<0.05$) than that of the pair-fed, but not ad libitum
fed controls. The position of the fetus on the uterine horn
appeared to have no effect on the rate of uptake of any of
the compounds studied.

f) Plasma and Liver Concentrations of Injected
   Compounds and Maternal Plasma Osmolality and
   Muscle Dry Weight.

As indicated in Table 9, no significant differences were
observed in the concentrations of the four radioactive compounds

TABLE 6

Ethyl alcohol, glucose, folic acid, and zinc concentrations in maternal
plasma during week three of gestation in rats given alcohol, pair-fed or
ad libitum fed.   Means ± standard error.

	Alcohol Group	Pair-fed Group	Ad Libitum fed Group
Ethyl alcohol (mg/100 mL)	76± 6.6 ($\bar{1}$1)[1]	–	–
Glucose (mg/100 mL)	99.8±2.9[a] (14)	96.5±3.0 (14)	104.7± 2.3 (12)
Folic acid (ng/mL)	46.5±4.2 (7)	37.3±3.6 (6)	47.9±7.1 (7)
Zinc (ppb)	349±69.8 (8)	445±20.8 (8)	402± 83.4 (8)

a, No statistically significant differences
   were observed between treatment groups.

1= figures in brackets
   refer to number of
   rats.

TABLE 7

Blood flow to placentas and to the maternal kidneys, and cardiac output on day 20 gestation of rats given alcohol, pair-fed, or <u>ad libitum</u> fed. Means ± standard error.

	Alcohol Group	Pair-fed Group	<u>Ad libitum</u> Group
Number of litters	11	12	10
Placental blood flow: mL/min/placenta	0.170 ± 0.02^a	0.260 ± 0.03^b	0.357 ± 0.03^c
mL/min/ g placenta	0.227 ± 0.03^a	0.623 ± 0.07^b	0.743 ± 0.07^b
% of cardiac output	0.270 ± 0.03^a	0.399 ± 0.05^b	0.460 ± 0.04^b
Renal blood flow: % of cardiac output	13.2 ± 1.6^a	10.9 ± 0.61^a	11.6 ± 0.36^a
Maternal cardiac output: mL/min/100 g body weight	20.5 ± 1.3^a	20.8 ± 0.98^a	20.4 ± 1.0^a

a,b,c= figures in the same row not sharing the same letter are significantly different at p < 0.05.

in the maternal plasma or liver 90 minutes after injection of
2-amino isobutyrate, methyl-α-D glucopyranoside, folic acid,
and zinc among the three treatment groups.

Table 10 shows two parameters of dehydration, plasma
osmolality and muscle dry weight for alcohol, pair-fed, and
ad libitum fed groups. There is a statistically significant
increase in both plasma osmolality and muscle dry weight in
alcohol exposed animals when compared with either pair-fed
($p < 0.001$, $p < 0.05$, respectively) or ad libitum control ($p < 0.001$,
$p < 0.05$, respectively) groups. This represents a moderate
degree of dehydration (about 7 percent) in the alcohol group.

TABLE 8

Fetal uptake of 2-amino isobutyrate, methyl-α-D glucopyranoside, folic acid, and zinc from the maternal circulation at day 20 of gestation. Mean $\pm$ standard error.

Compound injected	Alcohol group	Pair-fed group	Ad libitum controls
2-Amino (1-^{14}C) isobutyric acid			
DPM/g fetal body weight	23,814 $\pm$ 1444	26,615 $\pm$ 3007	27,679 $\pm$ 656
	(7)[1]	(5)	(5)
Methyl (α-D-[U-^{14}C]gluco)pyranoside			
DPM/g fetal body weight	10,914 $\pm$ 453	9,840 $\pm$ 357	9,414 $\pm$ 574
	(11)	(10)	(6)
[3',5',7,9-^{3}H] Folic acid, K salt			
DPM/g fetal body weight	72,406 $\pm$ 3634[2]	61,509 $\pm$ 1838[2]	62,601 $\pm$ 4669
	(11)	(10)	(6)
Zinc-65			
CPM/g fetal body weight	1,796 $\pm$ 177	1,873 $\pm$ 309	2,601 $\pm$ 298
	(6)	(6)	(5)

[1]Figures in parentheses indicate the number of litters.

[2]Significant at $p < 0.05$.

TABLE 9

Concentration of 2-amino isobutyrate, methyl-α-D glucopyranoside, folic acid, and zinc in maternal plasma and liver 90 minutes after injection of the radioactive compounds.   Means $\pm$ standard error.

Group	Alcohol	Pair-fed	Ad Libitum	Alcohol	Pair-fed	Ad Libitum
		Liver			Plasma	
Compound injected:						
2-Amino isobutyrate (DPM/g)	62100[a] +7310	79100 +2940	62800 +4380	20200 +1250	17400 +600	17800 +1030
Number of litters	7	5	5	7	5	5
Methyl − α -D glucopyranoside (DPM/g)	34700 +1870	36500 +2100	31400 +7540	49500 +2940	50600 +1340	51600 +3540
Numbers of litters	6	5	2	10	9	6
Folic acid (DPM/g)	493100 +41600	396500 +30800	527500 +45300	151400 +13200	153800 +5910	147600 +5530
Number of litters	6	5	2	11	9	6
Zinc chloride (CPM/g)	14800 +1350	22200 +4040	14400 +1160	1300 +136	1320 +255	960 +126
Number of litters	6	5	4	6	6	4

a = there were no statistically significant differences
    between treatment groups.

TABLE 10

Maternal plasma osmolality and muscle dry weight (% of wet weight) in
alcohol, pair-fed, and ad libitum fed rats. Means ± standard error.

Group	Plasma osmolality (mosm./L)	Muscle dry weight (% of wet weight)
Alcohol	302.4 ± 4.59^a	$25.68 \pm .58^a$
Number of litters	10	11
Pair-fed	280.0 ± 2.30^b	23.99 ± 13^b
Number of litters	9	12
Ad libitum	278.8 ± 2.05^b	24.57 ± 22^b
Number of litters	9	10

a, b = figures in the same column not
       sharing the same letter are signif-
       icantly different at $p < 0.05$

DISCUSSION

The findings of this study indicate that prenatal alcohol exposure is deleterious to fetal development in the rat. Although no obvious malformations were observed in the 20 day gestation fetuses in any of the three groups of rats, birth weights of the offspring of rats consuming alcohol prior to and throughout pregnancy were significantly decreased over rats fed either isocalorically or ad libitum. These findings are consistent with past work using a rat model in fetal alcohol syndrome research (7,8,41). The daily alcohol consumption, and subsequent blood alcohol levels, of the alcohol group dams was similar to that of other studies in animals where decreased birth weights were observed (7,8,41). The blood alcohol levels of alcohol treated dams in the present study (76 mg/100 mL) are however less than in blood from mothers of infants displaying fetal alcohol syndrome, taken shortly after birth of the infant (130 mg/100 mL) (24). The minimum amount of alcohol exposure required to affect fetal wellbeing remains to be determined.

The absence of differences in maternal body weights between alcohol and pair-fed groups at day 20 of gestation is reflective of the isocaloric feeding design of the experiment. Both alcohol and pair-fed groups also exhibit a significant reduction in maternal body weight when compared with ad libitum group animals, as might be expected by the lower caloric intake of the former groups. However the differences in fetal body weights among the three groups at day 20 of gestation

suggest that the caloric restriction, although a contributing factor, cannot be entirely responsible for the growth retardation in alcohol exposed fetuses. Other factors must exist to account for this reduction in fetal weight between the two isocalorically fed groups.

To investigate whether alcohol consumption before and during gestation had an effect on the nutritional status of the mother, plasma concentrations of certain nutrients were determined. The absence of any difference in plasma levels of glucose, folic acid, or zinc during the third week of gestation suggests that maternal metabolism of these nutrients is not affected by alcohol consumption. Furthermore, the concentration in liver or plasma of folic acid, zinc, and analogs of glucose and amino acid 90 minutes after injection was not significantly different among the three groups. These findings, together with the absence of difference in maternal body weights of alcohol treated and pair-fed animals on day 20 of gestation support the notion that alcohol exposure at the present level and duration does not affect maternal nutritional status. This is contrary to past work where in the non pregnant rat, alcohol exposure has resulted in reduced plasma levels of folic acid, and thiamin (65). The possibility cannot be excluded that maternal status of certain nutrients which were not measured in the present study were sufficiently altered by alcohol consumption to affect adequate fetal supply, however it is likely that any deficiency would manifest itself in an observable reduction in maternal body weight. The

decreased birth weights of the offspring of rats exposed to alcohol in utero over those fed isocalorically in this study can not therefore be readily explained through an alcohol induced alteration of maternal nutrient utilization or plasma concentration.

To further investigate the possibility that alcohol interferes with the adequacy of nutrient supply to the developing fetus, the hemodynamic status of the mother was studied at day 20 of gestation. The use of radioactive microspheres for estimation of cardiac output and organ blood flow is a widely accepted procedure (60,61,66). The fractional distribution among body tissues of a ventricularly introduced pulse of radioactive microspheres is proportional to the percentage of the cardiac output reaching those tissues. Creation of an artificial organ of known blood flow by means of reference sampling of blood at a fixed rate at the time of the microsphere pulse permits calculation of cardiac output. Thus the blood flow to any tissue can be determined, as both the cardiac output and the percentage of the cardiac output reaching the tissue are known. In measuring placental blood flow with microspheres there is some question as to the efficiency with which small (15 micron) microspheres are trapped in the placental channels. However, Bjellin et al.(67) have demonstrated that 15 micron microspheres give a better estimate of placental blood flow than do 50 micron microspheres, due to the tendency of the latter to axial streaming in blood vessels. The figures reported presently (21 mL/ min/ 100 g body weight for cardiac output and 11.6% of cardiac output

for kidney flow) are in agreement with those of Bruce (61) and Sapirstein et al. (68) of 26 and 22 mL/ min/ 100 g for cardiac output, respectively, and 8.62% for kidney blood flow(61). The placental blood flow value reported by Bruce (61) of 1.21 mL/min/g is greater than that of the ad libitum control group in the present study (0.743 mL/min/g). However blood flow determinations by Bruce were performed on day 22 of gestation as is reflected in average fetal weights of 4.21 g when compared with average fetal weights of 2.51 g in the ad libitum control group at day 20 of gestation in the present study.

Maternal cardiac output and renal blood flow expressed as % cardiac output were not affected by alcohol consumption or by pair-feeding when compared with ad libitum fed controls. Sapirstein et al.(68), using 86 Rb to measure blood flow, also found no differences in cardiac output between non pregnant alcohol exposed and control rats fed ad libitum. Cardiac output has been found reduced in alcohol treated non pregnant rats, but only when narcotizing dosages were administered (69). Increases in blood flow rates to brain, heart, kidneys, and lung as well as decreases in blood flow rates to spleen, muscle, and kidneys have been reported in the literature (68,70). The large standard error measurement in the renal blood flow of alcohol exposed animals in the present study, in comparison with that of either pair-fed or ad libitum control groups, is consistent with the variability of findings reported in the literature.

The present study demonstrates a marked reduction in blood flow to the placentas in the alcohol treated rats, whether expressed in mL/ min/ placenta, or as percent of cardiac output. As the weight of each placenta was significantly greater in the alcohol treated animals than in controls, the blood flow per unit of placental weight is even more markedly reduced by alcohol consumption. The relative proportions of placental weight derived from maternal or fetal components were not determined, so it can not be stated that blood flow per unit of maternal placental tissue is reduced. Nevertheless, the total blood flow to the placenta is decreased, implying a reduced delivery of nutrients and essential metabolites.

The reason for the increased placental weight in the alcohol group is not readily apparent. Other factors which retard fetal growth, such as uterine artery ligation and calorie malnutrition, are accompanied by reduced placental weight (59, 71). In accordance, it is noted that in the present study the calorie restricted group displayed significantly smaller placentas than either alcohol treated or ad libitum control groups. The heavier placentas of the alcohol group are not due to increased fluid retention as the percent dry weight is the same as in the control group (72). Wiener et al. (42) and Skosyreva (43) have also reported increased placental weights in alcohol-exposed rats similar to the present results. Gordon et al. (44) also reported heavier placentas in alcohol treated rats and showed that it is due to hyperplasia. Histological examination may

cast some light on the nature of these findings. It is possible that the increased placental size may exist as a result of mechanisms which compensate for the reduced blood flow to this organ in the rat chronically exposed to alcohol.

The reduction in placental fraction of cardiac output in alcohol treated rats reported here contrasts with recent work by Rosso and Kava (59). These workers reported a decreased placental blood flow in food restricted rats, without observing a change in the fraction of cardiac output reaching this organ. If calorie restriction existed as the cause of fetal growth failure in alcohol treated pregnant animals, a similar pattern of hemodynamic alterations would be expected in food restricted rats. In the present work, no differences exist in cardiac output between alcohol treated and ad libitum controls, however Rosso and Kava (59) note a 50 percent reduction in cardiac output in rats fed a calorie restricted diet (50 percent of controls) as compared with control group animals. Thus the mechanism of blood flow reduction in the present study exists through a redistribution of a normal cardiac output, whereas in the malnourished rat, the distribution of blood flow remains constant but the expansion of blood volume during late gestation fails to occur, resulting in a lowered cardiac output. This lends further evidence in support that the action of alcohol on the fetus in utero is different from that of maternal malnutrition.

As a marginally food restricted group, the pair-fed group of the present study may be compared with the malnourished rat. The reduction in placental blood flow of pair-fed over ad

libitum controls in the present study is similar to the results
of Rosso and Kava (59), however, in contrast, neither the
fraction of cardiac output reaching the placenta, nor the
cardiac outputs differ between pair-fed and ad libitum groups.
This may be due to the more severe nature of the calorie
restriction (50 percent as compared with about 20 percent in
pair-fed group) of malnourished animals (59) over those pair-
fed in the present study.

In these studies the placental availability of nutrients
may be impaired as a result of the significant and substantial
reduction in the supply of blood to transfer areas of the
villous membrane. This reduction in blood supply appears to
originate through a redistribution of cardiac output. It is
thus possible that if placental nutrient availability is
restricted, that fetal growth retardation may occur secondary
to a reduction in fetal nutrient uptake. Reduction of plac-
ental blood flow has been associated with reduced placental
transfer of nutrients. Nitzan et al. (71) found reductions
in placental transfer of aminoisobutyric acid and deoxy-
glucose in intrauterine growth restricted (IUGR) rats, where
ligation of a uterine artery was performed. However the acute
nature of this reduction in placental blood flow may not allow
compensatory mechanisms to restore normal nutrient transfer.
Alcohol in the present study was administered prior to and
throughout gestation, thus sufficient time may have elapsed
to allow development of such mechanisms. Furthermore, uterine
artery litagation may cause a larger placental blood flow
restriction than that ascribable to alcohol, beyond the limits

of these mechanisms. It can therefore be argued that a reduction in placental nutrient transfer need not necessarily accompany a reduction in placental blood flow as effected by chronic alcohol consumption.

As a measure of fetal uptake of nutrients, the placental transfer of radiolabeled zinc, folate, and analogs of glucose and amino acid were studied in rats exposed to alcohol during gestation. Metabolite analogs are used because they are handled by the transport systems of the compounds they represent as would the compounds themselves, however they are not degraded in vivo thus preventing the production of radio-labeled byproducts. The 90 minute time point after injection of the radiolabeled compounds was selected by comparison with uptake rates at 40 and 60 minutes after injection. A proportionally increased fetal uptake was observed at 90 minutes over each of the alternate time points, indicating that even if back-flow of the injected compounds was occurring the net flux was from mother to fetus. This period was thus chosen to maximize the amount of radiolabel incorporated into the fetus compared with the amount injected into the mother.

Except in the case of folic acid, no differences in fetal uptake rates of the compounds studied were seen in alcohol exposed fetuses when compared with pair-fed or ad libitum control groups. It is uncertain why an increase in fetal uptake of folic acid in alcohol group animals occurred when compared with the pair-fed group. Possibly as a result of a deficiency at an earlier stage of gestation a 'catch up' mechanism is in effect. The absence of a reduction in fetal

uptake of the compounds studied suggests that the growth
retardation seen in offspring of animals chronically exposed
to alcohol is not as a result of inadequate placental nutrient
transfer at least at day 20 of gestation. This supports the
concept that compensatory mechanisms to restore adequate
nutrient transfer may arise, as suggested by the increase in
placental size, in animals chronically exposed to alcohol
where a reduction in placental blood flow has occurred.

The results of the present study are in contrast to those
of Lin and Maddatu (73) who found a reduced transfer of aminoi-
sobutyrate to fetuses of rats administered alcohol from day
6 to 21 of gestation. The ingestion of an alcohol solution
in the present study for at least one month prior to mating
may have allowed sufficient time for a compensating effect
to have occurred. Furthermore, no increase in placental size
was noted by Lin and Maddatu (73). According to Rider (74),
adaptation to alcohol intake seems to occur as demonstrated
by the poorer reproductive performance of the dams placed on
alcohol on day one of pregnancy as compared with that of the
previously alcohol adapted dams, and in the decreased survival
of the offspring. Henderson et al. (75) has also demonstrated
in rats that chronic and acute alcohol administration reduces
placental ^{14}C-valine uptake. However, these authors used an
in vitro preparation which may account for the differences
in results.

If maternal alcohol consumption does not interfere with
the fetal uptake of nutrients, to explain the observed growth
retardation in utero, alcohol must exert its deleterious

effect in some alternate manner. Brown et al. (76) demon-
strated that exposure to alcohol retards growth and differ-
entiation in cultured rat embryos, suggesting that alcohol
can exert a direct effect on fetal growth, without the con-
founding factor of nutrition. It is possible that the fetus
may be unable to utilize nutrients once they have been trans-
fered into the fetal circulation. Rawat (77) reported that
alcohol consumption by pregnant rats resulted in a significant
inhibition in the rate of $(U-^{14}C)$-leucine incorporation into
fetal cardiac proteins. Decreased cardiac total RNA and un-
changed total DNA in alcohol exposed neonates has also been
reported by Henderson and Schenker (78). Thus evidence exists
in support of alcohol affecting growth in utero by some mech-
anism other than through reducing placental transfer of
nutrients.

In these studies alcohol in water was offered as the sole
source of fluids. As alcohol has a diuretic action and no
other source of water was available to the animals, plasma
osmolality and muscle water content were measured as indi-
cators of dehydration. Both parameters indicated that a
moderate degree of dehydration (7 percent) had occurred, but
the significance of this for the measurement of placental
blood flow, fetal nutrient uptake, and overall fetal devel-
opment remains unclear.

In summary, maternal nutritional status appears to be
unaffected by alcohol treatment under the conditions of this
experiment. The observed reduction in placental blood flow
on day 20 of gestation in rats chronically exposed to alcohol

may reduce placental transfer, hence fetal uptake, of certain nutrients resulting in a retardation of fetal growth and development. However, if such a reduction in fetal uptake occurs, nutrients other than those studied presently must be involved as no decrease in the rate of fetal uptake was observed for zinc, folate, or analogs of glucose or amino acid. Alternatively, compensatory mechanisms, perhaps reflected in the increase in placental weight emerging as a result of long term alcohol exposure, may restore normal transfer of all nutrients to the fetus, regardless of the reduction in placental blood flow. Thus the growth retarding effect of maternal alcohol ingestion may still be due to a direct action of alcohol on the fetus, either affecting primary fetal metabolism or fetal nutrient utilization.

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