Early ethanol exposure in artificially-reared rats
by Beatrice S Fisher

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Psychology
Montana State University

Abstract:
The effects of early ethanol exposure on rat pup development and behavior, and later ethanol preference were examined. Artificial-rearing was used to determine the specific effects of alcohol apart from nutritional deficits. Reflex measures were used to assess the acute effects of alcohol on psychomotor development, and two-bottle preference tests were used to determine long-term effects of early alcohol expsoure on later alcohol preference.

Rat pups were found to be affected by ethanol exposure to varying degrees on the reflex tests. Growth rates were normal for all artificially-reared animals, regardless of whether or not they were exposed to alcohol. The effect of early ethanol exposure on later ethanol preference was not definitive, however results were suggestive of later increased preference for ethanol following early exposure.

Cataracts seen in artificial ly-reared animals were found to occur significantly more often in alcohol exposed animals than in controls.

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A thesis submitted in partial fulfillment
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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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TABLE OF CONTENTS

VITA....iv

ACKNOWLEDGMENTS....v

LIST OF FIGURES....viii

LIST OF TABLES....ix

ABSTRACT....x

INTRODUCTION....1

Artificial Rearing....2
Brain Development....3
Effects of Early Ethanol Exposure....4
Early Ethanol Exposure-Later Ethanol Preference....5

METHOD....10

Materials....10
Surgical Procedure....11
Daily Maintenance....15
Ethanol Exposure....19
Experiments 6-9....20
Reflex Measures....22
Brain Measures....22
Alcohol Testing....23

RESULTS....25

Growth Rates....25
Negative Geotaxis....27
Ethanol Preference....32
Brain Weight/Body Weight Ratios....37
Cataracts....37

TABLE OF CONTENTS-Continued

DISCUSSION....39

Growth....39
Postnatal Behavior & Gross Neurological Development....41
Cataracts....43
Ethanol Preference....44
Future Considerations....48

BIBLIOGRAPHY....50

LIST OF FIGURES

Figure		Page
1	Photographs of equipment and procedure used in artificially rearing rat pups	16
2	Growth rates for Groups 1-2-3	26
3	Growth rates for Groups 4-5	28
4	Negative Geotaxis results for Groups 4-5	29
5	Negative Geotaxis results for Groups 8-9	31
6	Ethanol Consumption--Groups 2-5 (Combined)	33
7	Water Consumption--Groups 2-5 (Combined)	34
8	Ethanol & Water Consumption--Group 5	36

LIST OF TABLES

Table		Page
1	Formula ingredients for artificial rearing diet	17
2	Daily Amounts of Formula used in artificial rearing	18

ABSTRACT

The effects of early ethanol exposure on rat pup development and behavior, and later ethanol preference were examined. Artificial-rearing was used to determine the specific effects of alcohol apart from nutritional deficits. Reflex measures were used to assess the acute effects of alcohol on psychomotor development, and two-bottle preference tests were used to determine long-term effects of early alcohol expsoure on later alcohol preference.

Rat pups were found to be affected by ethanol exposure to varying degrees on the reflex tests. Growth rates were normal for all artificially-reared animals, regardless of whether or not they were exposed to alcohol. The effect of early ethanol exposure on later ethanol preference was not definitive, however results were suggestive of later increased preference for ethanol following early exposure.

Cataracts seen in artificially-reared animals were found to occur significantly more often in alcohol exposed animals than in controls.

INTRODUCTION

In 1973 Jones and Smith first reported that a constellation of anomalies was often observed in infants born to mothers who consumed large amounts of alcohol. These researchers called the syndrome seen in such infants the Fetal Alcohol Syndrome (FAS).

The diagnosis of FAS is based on three major factors: (1) central nervous system dysfunction, (2) growth deficiencies, and (3) specific facial dysmorphology (Jones & Smith, 1973). Central nervous system dysfunction caused by early ethanol exposure is one of the primary concerns in FAS research. Basic reflex measures often define these functional deficits. Growth deficiency is another of the diagnostic symptoms in the research discussed here. Animal models have been used to study FAS. However, identifying the specific effects of alcohol apart from poor nutrition, motor deficits, feeding problems, inadequate maternal care and cross-fostering stress in animal studies is extremely difficult (Abel, 1983). Many methods have been used to expose rat fetuses and pups to alcohol. These include adding alcohol to a dam's drinking water as the only available fluid, putting alcohol in a liquid diet

for the dam, injecting alcohol into the dam as well as into the neonate, vapor inhalation and gavage (i.e. force feeding through a tube). Each of these techniques leads to one of two experimental complications. First, no way exists to measure how much alcohol the fetus is exposed to when the dam consumes alcohol; second alcohol interferes with lactation (Diaz & Samson, 1980), as well as pup's nursing ability (Riley, Bunis, & Greenfeld, 1984) and, therefore, pup nutrition. These factors of decreased lactation and poor nursing ability may both lead to nutritional deficits, apart from any effect of ethanol exposure.

Artificial Rearing

In 1969 Messer, Thoman, Terrasa, and Dallman introduced "artificial rearing" of rat pups. Hall modified this technique in 1975, Samson and Diaz (1982) further refined and developed it. Samson and Diaz (1982) and West, Hamre, and Pierce (1984) used this technique to examine the effects of alcohol exposure in rat pups.

Artificial rearing enables the investigator to determine exactly how much alcohol the neonate receives and it avoids many of the problems of nutritional complications such as decreased lactation and inadequate nursing. Since the pups are infused with a specific amount of formula each day nutrition levels are held constant between

experimental and control animals. The specific effect of alcohol on behavior and development may then be assessed.

Brain Development

The neonatal rat pup is an appropriate model for the third trimester of human development, particularly as it relates to brain development. The brain goes through several periods of rapid growth during development at which time it is extremely vulnerable to exogenous insult. In humans a rapid developmental period known as the "brain growth spurt" begins at mid-gestation, reaches a peak during the third trimester of pregnancy, and is complete by the third year of life. This period of rapid brain development occurs in virtually all mammals, but its time of occurrence is variable among species (Davison, 1977). The rat undergoes this "brain growth spurt" during the first fifteen postnatal days. The peak of the rat's brain growth spurt occurs at postnatal Days 6 through 9. This period in the rat closely corresponds to the third trimester of human fetal development.

In terms of whole brain weight, rats are born with approximately 12% total brain development, reaching 27% of development by postnatal day 9. Humans have approximately 12% brain development by gestational day 180 and at birth have 27% of their total brain development (Davison, 1977; Dobbing, 1968). This critical period for developing brain

includes crucial periods of myelination and synapse formation (Gottlieb, Keydar, & Epstein, 1977).

Effects of Early Ethanol Exposure

Many findings have indicated that prenatal or perinatal alcohol exposure result in similar developmental and behavioral consequences for humans and animals. In fact every form of teratogenesis in humans which is related to alcohol has been observed in animal models (Abel, 1984, p. 377).

Abel (1984) extensively reviewed the animal research on FAS, both behavioral and physiological. Some consistent findings with regard to large-dose prenatal alcohol exposure include:

1) Low birth weight which sometimes continues into adulthood (Abel & Dintcheff, 1978; Abel & Greizerstein, 1982; Martin, Martin, Sigman, & Radow, 1977; Philips & Stainbrook, 1976).

2) Delayed physical development. Eye opening and ear-flap uncurling are delayed in mice and rats (Martin, Martin, Sigman & Radow, 1978; Shaywitz, Griffieth, & Warshaw, 1979). Delayed puberty in female humans (Robe, Robe & Wilson, 1979) and in mice and rats have been reported (Boggon, Randal & Dodds, 1976; and Tittmar, 1977).

3) Hyperactivity. This is a very common and

consistent finding in both animals (Martin et al., 1978; Shaywitz et al., 1979) and humans (Landesman-Dwyer, 1982). Some very recent findings indicate that this hyperactivity is more often seen in male rats and that females may show a hypoactive response (Snoderegger, personal communication, November, 1985).

Similarly in humans, the three most consistent and salient behavioral manifestations of FAS are: 1) mental retardation, 2) hyperactivity and 3) poor or delayed motor development (Landesman-Dwyer, 1982). FAS children also are found to "present severe feeding problems" (Landesman-Dwyer, 1982, p. 133). The fact that they have low birth weights, poor suckling ability and are disinterested in food often results in a "failure to thrive" (Landesman-Dwyer, 1982, p. 133).

Early Ethanol Exposure--Later Ethanol Preference

Does early exposure to alcohol predispose an individual toward greater alcohol preference in later life? This question has received surprisingly little research attention (Abel, 1984). Several animal studies suggest an affirmative answer; however, the current evidence is far from conclusive. Bond and DiGuisto (1976b) found that offspring of alcohol-treated rats consumed greater amounts of 3% and 6% alcohol in adulthood. In this study, pregnant Wistar rats consumed a liquid diet containing sustagen and

ethanol. Controls were fed laboratory chow. Pups whose mothers were exposed to alcohol consumed significantly more ethanol in a preference test compared to controls at sixty days of age. It is not clear from this study whether increased consumption actually reflected a preference for alcohol or represented increased consumption of all fluids since the preference ratio was omitted (Abel, 1982). Pair feeding was not used as a control, so that nutritional effect also could not be ruled out. When dams were given Chablis wine as their only source of fluid during pregnancy and nursing, their offspring drank more wine than controls (Philips & Stainbrook, 1976). In this study, female hooded rats were given wine (N=3) or water (N=3) plus regular lab chow from Days 60-120, at which time they were mated. The dams continued with these diets during gestation and lactation. Their pups were tested at 170 days of age for alcohol preference. This preference test was done by placing two 90 ml bottles on the cages. One bottle contained water and the other Chablis wine. The wine group showed consistently larger daily fluid consumption for the five days of testing. The water consumption was the same for both groups, so the difference was in the amount of wine consumed. Abel (1982) and Abel and York (1979) failed to replicate these results, however, when they used a surrogate-fostering technique. A possible

explanation is that the pups in the Philips and Stainbrook study drank the alcohol (along with the dam) before weaning, whereas with surrogate fostering the pups would not have had this experience with wine. Another explanation is suggested by Galef and Henderson's (1972) work. These researchers found that rat pups prefer a diet which their mother received during lactation, even though the pups had never received this diet. Flavors supposedly are passed to the pups through the mother's milk (Galef & Henderson, 1972).

A more conclusive study by Reyes, Garcia, and Jones (1984) which used pair-feeding and surrogate fostering techniques, showed that 45-day old male offspring of alcohol consuming dams exhibited an increased preference for 10% alcohol versus water. Data for female offspring, however, were inconsistent.

The literature available suggests the possibilty that early exposure to alcohol alters adult preference for alcohol. The results are not definitive, however, and further research is necessary. The methods described here are designed to answer the question of whether early exposure to ethanol alters adult preference for alcohol, as well as to look at the effects of early exposure to alcohol on physical and behavioral development.

The artificial-rearing method was used in this study

in order to allow precise control of nutrition and degree of alcohol exposure. Behavioral measures were taken thoughout development to assess acute and long term effects of early ethanol exposure, and relate these behavioral effects to those seen in FAS children. One major feature of FAS is apparent damage to the central nervous system which is reflected in psychomotor disabilities. The reflex measure in this study was the negative geotaxis which has been used by other researchers to determine psychomotor development. This measure has been used by Diaz (1982) and Shaywitz (1979) and was previously described by Altman and Sudarshan (1975).

The present studies have three specific goals. The first goal is to determine the role of alcohol without the complicating factor of nutritional deficits. Another goal is to study the effect of early ethanol exposure on physical development, activity, growth rates and brain development. The final goal is to determine whether or not early ethanol exposure modifies later ethanol preference.

Whereas these goals guided the direction of this research, the primary contribution of the work discussed here is in the development of the pup'n'cup (PNC) method in a new laboratory. A laboratory which is fully equipped to carry out the PNC procedures is now in place at

Montana State University. The set-up perfected in the course of this research is capable of artificially-rearing 20 rat pups at a time. The research room at the Animal Resource Center is climate controlled and provides an ideal environment to carry out the procedures used in the artificial rearing method. Immediate access to rat colonies and resource personnel adds to the desirability of this newly created laboratory.

METHOD

The technique of artificially-rearing rat pups by means of intragastric-cannula feeding was first developed by Messer, Thoman, Terrasa, and Dallman (1969), then modified by Hall (1975). Further refinement of diet and procedures were contributed by Diaz, Moore, Petracca, Schacher and Stamper (1982). The present study uses the most recent modifications of this technique.

Materials

The materials required for use in this procedure are approximately 20 cm of polyethelene tubing (PE-10) for each rat's intragastric cannula, a small curved steel wire (25 gauge) 10 cm long, and a piece of Silastic tubing (.012 inch I.D., .025 inch O.D., Dow Corning) 8 cm long. One end of the PE-10 tubing is flared with heat to create a slightly larger diameter (approximately .013 mm). This flared end secures a plastic disc onto the cannula. The plastic disc is made from a commerically available sandwich bag by using a paper punch. The disc is then poked lightly with a 23 gauge needle and the unflared end of the PE-10 tubing is threaded through the opening and the disc is very gently pushed down the length of the cannula up to the flared end of the tubing. The plastic

disc is then tested by gently tugging with the fingers; a secure disc is very important to the later proper functioning of the cannula. The PE-10 with the fitted plastic disc forms the completed intragastric cannula. The curved steel wire and the Silastic tubing are stored in a jar filled with corn oil until they are needed for surgery. The corn oil acts as a lubricant for the Silastic which is fit onto the wire and for the Silastic-plus-wire which is slid gently down the esophagus of the neonate (Samson & Diaz, 1982).

Surgical Procedure

After the cannulas are prepared (one for each pup plus extras) and the Silastic is slid onto the wire, the four day old rat pup is weighed and lightly anesthetized with methoxyflurane. The anesthetization consists of placing the pup in a small jar which has paper towels lining the bottom and with 2 ml of methoxyflurane placed under the toweling. The pup is left in this jar until he/she appears well anesthetized. The level of anesthetization is determined by the pup's breathing (rapid and shallow) and by a pale to slightly blue skin color. Pups at this age (4 days postnatal or 25 days gestational) are fairly resistant to methoxyflurane overdose. The use of an oxygen-enriched controlled dose of anesthetic has been desribed by Diaz (personel communication, 1985) and might

be an improvement over the current method. After the pup is anesthetized it is placed on its back and its mouth is gently opened. The Silastic-covered wire is removed from the corn oil and gently slid down the dorsal surface of the pup's esophagus. The Silastic extends over the entering end of the wire approximately 1-3 mm to ensure that the cutting tip is not exposed as it passes down the esophagus and into the stomach. This part of the surgery is quite critical and care must be taken to avoid entering the trachea. If a resistence is encountered within 2 cm the covered wire is removed and reinserted until it slides smoothly down the esophagus. A sufficient level of anesthesia is important to avoid the gag reflex, which can interfere with cannula implantation.

Once the covered wire has passed through the esophagus and into the stomach, the pup is shifted onto its right side. From this position the stomach is visible, because the pup has transparent skin, and the end of the covered wire can be seen as a slight bulge in the stomach area. At this point, the Silastic tubing covering the wire is slid gently away from the tip exposing the cutting edge of the wire. The wire is then pushed through the stomach, the peritoneal wall and the skin until 1 cm is exposed. Then forceps are used to grasp the exposed wire, and the Silastic is removed entirely from the mouth

end of the curved wire. The unflared end of the PE-10 cannula is then friction-fitted over the end of the wire exiting the pup's mouth. Once the cannula is secured onto the wire, the wire and cannula together are pulled down the esophagus and out the pup's abdomen until approximately 20 cm of the PE-10 cannula has exited the abdomen. The soft plastic disc on the cannula collapses as it travels down the esophagus and reopens in the stomach forming a seal which prevents formula from leaking out of the stomach.

The wire is removed from the cannula. A paperpunch size disc made of Tygon tubing (1/16 inch I.D., 1/8 inch O.D., 1/32 inch wall) is slid onto the cannula with the aid of a 20 gauge hypodermic needle. The Tygon disc is slid over the outside of the needle and the cannula is slid inside. The disc is then slid onto the cannula with forceps and up the length of the cannula to approximately 4 cm from the pup's abdomen. The wire used for surgery is then reinserted into the cannula which is now exiting the pup's stomach. The curved steel wire with its cutting tip is then passed through a fold of skin at the nape of the pup's neck. The slight pain the pup feels at this point is usually beneficial in overcoming the effects of anesthesia and actually helps the pup "wake up" and resume regular breathing. Finally, another Tygon disc is fit

onto the cannula with the 20 gauge needle and slid up the length of the cannula with forceps to approximately 2 cm from the first disc. The two discs, on either side of the pup's neck, act as a strain relief for the cannula and prevent undue force from being put on the wound in the stomach.

After the intragastric cannula is successfully implanted, the pup is placed in a plastic cup (grocery store liver cup) which contains a small amount of bedding, preferably the same type used in the maternal cage. The plastic cup (11 cm in diameter and 7.5 cm deep) is covered with a plastic lid which has many small holes. These holes are most effectively made with a quick touch from a hot soldering iron. This insures that the holes are a small size which prevents the pups from crawling out and drowning at a later date. Each plastic cup and pup is then placed inside another identical weighted cup which is floated in a warm water bath (37-38 deg. C). The cups are floated in this bath for the next 14 days and the temperature is gradually reduced to 25 deg. C as the pups acquire fur. The water bath provides a warm, humid environment and also ensures vestibular stimulation by the bobbing movement of the cups in the water (Samson & Diaz, 1982).

The photographs in Figure 1 depict the setup for artificially rearing rat pups. These photographs show the infusion pump, warm-water bath, and surgical procedures.

Daily Maintenance

The cannulas emerging from the pups' stomachs are run through holes in the lid of the plastic cups and connected to syringes which contain the milk formula These cannulas are connected to 10 cc plastic syringes (Becton-Dickenson) by a slightly larger tubing (PE-50) which is approximately 45 cm long. The PE-50 tubing is friction fitted over the pup's PE-10 cannula. This results in an airtight connection from the formula syringe to the pup's stomach. Syringes are placed on a modified infusion pump (Sage 954) which is controlled by a timed electrical circuit. Every 10 min the pump is turned on for 10 s and it infuses a predetermined amount of formula. The Sage 954 was used for Groups 1-5, while different pumps were used for the rest of the groups (see Experiments 6-10 for a description). The formula (Table 1) used is a modification of Messer's formula which has been developed by Diaz et al., (1982). After 23 hr each day the syringes are removed, cleaned thoroughly, and refilled with fresh formula. During this period, weighing, testing, and servicing are carried out. The servicing of pups consists of stimulating their anal/genital area by light stroking with

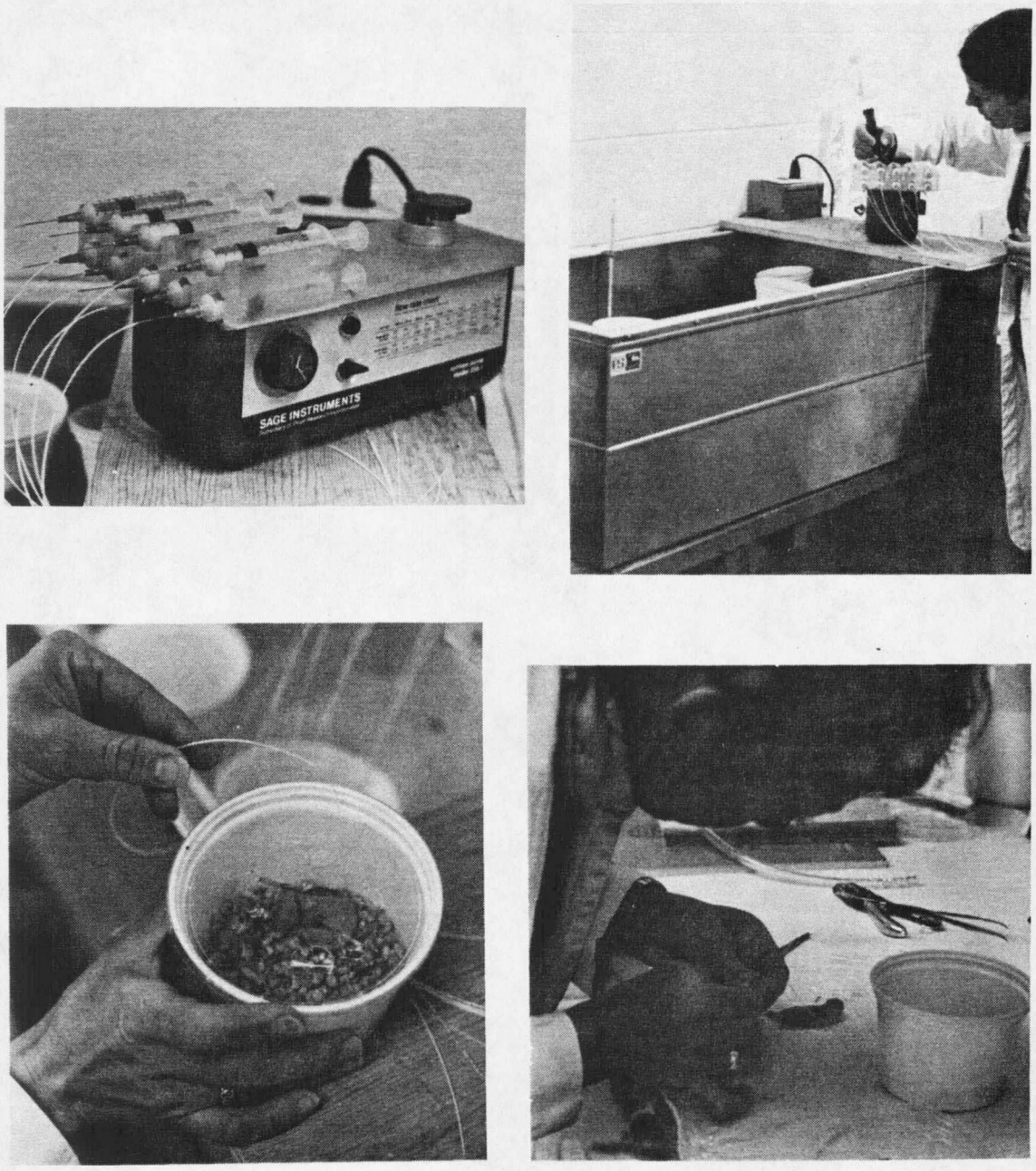

Figure 1. This figure shows the setup for artificial rearing. Top left shows the infusion pump with syringes. Top right shows the water bath used to float the pups in cups. Bottom left is a newly incannulated pup. Bottom right shows a pup during surgery (cannula implantation).

Table 1. Formula used in artificial rearing.

Milk Formula
(Messer, et al. 1969)
(Modified by Diaz, 1984)

	ml/500 ml
Evaporated Milk (Carnation velvetized)	375 ml
*Distilled Water with Dexoycholic Acid	85 ml
Mineral Solution**	5 ml
Vitamins (Poly Vi-Sol, Mead Johnson Infant Drops	5 ml
Corn Oil (Mazola)	30 ml
dl-Methionine (Sigma #M-9500)	0.20 g
l-Tryptophan (Sigma #T-0254)	0.25 g
Riboflavin	0.005 g
Casein (Hydrolysate, Sigma #C-0626) Enzymatic for milk	15.0 g

**Mineral solution gm/10ml water (distilled)

Fe II Gluconate (Alfa Prod)	0.0207 g
Cu Gluconate (Alfa Prod)	0.0105 g
Zn Gluconate (Alfa Prod)	0.0160 g

* Deoxycholic Acid Conc. (Sigma #D-6750, Sodium Salt 7-Deoxycholic Acid.....0.1% (or 0.2g /200 ml)

a soft tissue to stimulate urination and defecation. This is normally accomplished by the dam's licking in the home cage. It seems to be an essential factor in normal digestion and elimination. Each pup's cannula is flushed with distilled water or, if the pup appears bloated, with a deoxycholic acid solution. Deoxycholic acid has been found to alleviate the bloating in artificially-reared pups (Diaz, et al. 1981). Apparently, mother-reared pups ingest this salt by licking the mother's feces in the maternal cage (Diaz, personal communication, November, 1984).

The strain relief discs are adjusted at this time to accomodate growth. Servicing is carried out at the same time each day on a soft heated surface so that the pup does not experience stress from the cold. Behavioral tests of righting, negative geotaxis and cliff avoidance are also done at this time. The amount of formula is increased daily according to the pups'growth rate (see Table 2).

Table 2. Daily Amounts of Formula (ml/23 hr)

Days of Age (Postnatal)	Formula Amount	Days of Age (Postnatal)	Formula Amount
4	2.0	11	7.6
5	2.6	12	8.6
6	3.0	13	9.0
7	3.6	14	9.0
8	4.6	15	10.0
9	5.6	16	10.0
10	6.6	17-18	10.0

Note. Lab chow is added to diet on Days 17-18.

Ethanol Exposure

Female Holtzman rats were mated with experienced male Holtzman rats at the Montana State University Animal Resource Center and then individually housed until parturition. Four days after parturition, 10 male pups which were randomly selected from two to three litters were fitted with intragastic cannulas and artifically reared until postnatal Day 18, at which time they were sacrificed (Group 1) or weaned to lab chow and put in individual cages (Groups 2-5). The 10 pups in each group were weight-matched and then randomly assigned to either the control (formula only) or alcohol group (formula plus ethanol). Five groups of animals were artifically reared and the experimental subjects were exposed to varying amounts of alcohol on various days. The concentrations and periods of exposure were: Group 1 exposed to 3% (v/v) on days 6 through 9. Group 2 exposed to 5% on Day 6, 1% on

Day 7, and 5% on Days 8 and 9. Group 3 exposed to 3% on Days 4 through 7. Groups 4 and 5 exposed to 3% on Days 6 through 9. The amount of formula given each day is shown in Table 2.

Experiments 6-9

The method described above was the same for the second set of experiments except for the pumps used to artificially rear the animals, the number and sex of pups used, and the amount and days of ethanol exposure. The pumps used for Groups 6-9 are described here briefly. Because of many problems associated with adapting the Sage 954 to push 10 syringes, a pump was designed specifically for this purpose. These pumps (2) were designed and built by Gordon Williams and John Rompel of the Technical Services Shop at Montana State University. These specially designed pumps have the internal circuitry necessary to control all parameters of diet delivery. The pumps may be set to control the percentage of time they pump during an 18.2 min cycle (from 0.78% to 100%) This percentage of feed time in the 18.2 min feed cycle is continually repeated over the 23-hr feeding period. The final hour is used for testing and servicing pups. A Hurst model LAS stepping motor activated by digital timing circuits determines the distance forward the syringe push plate is moved during each on-cycle. With a 10 ml Becton-Dickenson

Plastipak syringe the push plate moves forward 6.14 mm for each ml pumped. The setting for the push plate has a range (in the continuously on mode) of 0.1 to 10 mm/min. The amounts of formula these pumps can deliver in each 23 hr period ranges from less than 0.2 ml to more than 10 ml. Larger syringes would allow greater volumes. Utilizing these pumps in Experiments 6-9 greatly increased our ability to feed predictable amounts of formula. Compared to commercially available pumps (e.g., Harvard 5000) these pumps are much less expensive (about $1000/unit), much smaller, and extremely easy to use. The high degree of reliability and accuracy of the pumps has been a tremendous improvement in the artificial rearing of rat pups in our laboratory.

Groups 6-9 were treated in most respects the same as Groups 1-5; however, all subjects were sacrificed on or before Day 18. In this second set of experiments there were 20 animals per group and both males and females were used. Groups 6-9 were also weight-matched and randomly assigned either to the experimental or the control condition. The experimental animals received 4% ethanol on Days 5 through 9 (postnatal). Data on pups in Groups 6 and 7 are not included here since most of these pups were sacrificed between Days 7-12 (postnatal), and the criterion for including subjects in the negative geotaxis was

survival to Day 12.

Reflex Measures

All groups (1-9) were tested for negative geotaxis reflex during the artificial-rearing period. In this test the pup is placed head down on a 30-degree inclined plane. The reflex is measured by timing how long it takes the pup to turn from a head down to a head up position (approximately 180-degrees). The pups are given a 60 s time limit to complete this response. If the subject takes 60 s or longer to complete the response he receives a score of 60. The test apparatus is made by cutting a cardboard box at the appropriate (30-degree) angle and covering the surface with a soft cloth to prevent slipping.

Initially three other reflex measures were taken along with the negative geotaxis measure, including righting, free-fall righting and cliff avoidance. These tests were dropped after the first batch of pups, because no consistent results were seen, and two of these tests seemed either to be traumatic to the pups or strained the cannulas. The negative geotaxis measure has been used by other researchers (Samson & Diaz, 1982; Shaywitz et al., 1979) and has been reported by Altman and Sudarshan (1975) to be an indication of psychomotor development.

Brain Measures

The first group of artificially-reared pups (Group 1)

was sacrificed on Day 19 (postnatal) with an overdose of Nembutal and cardiac perfused with 6% buffered formalin, for 10 min using a 23 gauge needle. After the perfusion the subjects' brains and internal organs were removed and placed in a 6% buffered formalin solution for five days. The brains were removed from this solution, blotted with paper towels, weighed, and measured. The weighing was done on a Satorius 1212MP balance. The measurements of the cerebral and cerebellar dimensions were made using Starret calipers. The overall cerebral length was measured from frontal pole to anterior surface of cerebellum, and the cerebral width was measured at the maximum point across both hemispheres. The width and depth of the cerebellum were also measured. These procedures, as well as the measurements taken, were based on the protocol described by Samson and Diaz (1982).

Alcohol Testing

Preliminary single-bottle test. Initial ethanol tests were conducted on Group 2 following a 19 hr water deprivation. Animals were offered either 10% ethanol (Day 35) or 5% ethanol (Day 36). Intake was measured at 15 min intervals for 1 hr. This test was replaced with a more detailed 2-bottle preference test.

Short duration, two-bottle preference test. Over a three-day period subjects in Groups 2-5 were

deprived of water daily for 18 hr, after which they were offered two bottles, one containing water and the other one of three ethanol solutions (1%, 5%, 10%). Order of ethanol concentrations and positions of the bottles were randomized, and intake volumes were measured at 60 min. The subjects were rehydrated for 6 hr between tests. This method of preference testing was used for Groups 2 and 3 (combined), 4, and 5.

Long duration preference testing (water versus 5% ethanol only.)Based on the results of these preference tests, a new method of prefernce testing was begun. These last preference tests were conducted on Group 5. No deprivation was used and the initial test duration was 24 hr followed by a 17 hr test. Animals were tested in sial test cages (as opposed to home cages in the previous tests) and computer-assisted data collection was utilized. The subjects in Group 5 were tested for five days using the 5% ethanol versus water choice. The final three days of testing utilized a 5 hr test period which began 1 hr before the onset of the dark cycle, and included a 21 hr water deprivation.

RESULTS

The statistical tests used were: Independant *t*-tests, F_{max}, and χ^2. These tests were run with MSUStat software (Lund, 1983). A repeated measures analysis of variance was used for the long-duration preference tests. This analysis was done with the Bio-Medical Data Processing 2-V program (Dixon, 1983).

Growth rates

Figure 2 shows the growth curves for Groups 1, 2, and 3 combined. This figure shows the mean daily weights for all pups surviving to day 18 in Groups 1, 2, and 3 (N=7 controls, N=10 experimentals). There was no difference between the experimental and control pups in growth rate. Groups 1, 2, and 3 shown in Figure 2 had highly similar growth rates both within groups and between groups. The experimental subjects shown in Figure 2 had been exposed to varying amounts of alcohol on various postnatal days. These initial three groups were used to determine reasonable dose levels and days of exposure. The experimental and control pups grew at nearly identical rates.

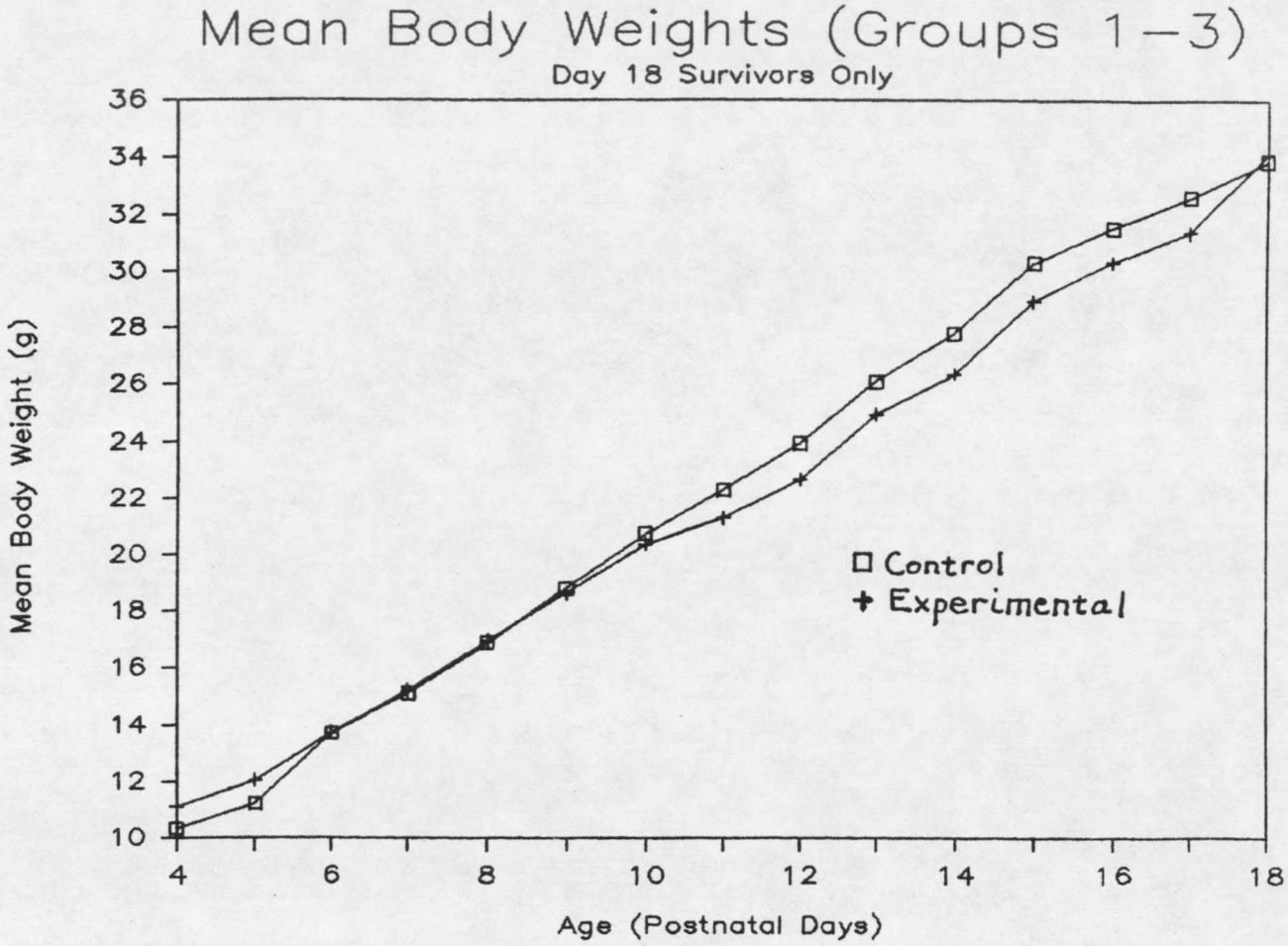

Figure 2. Mean body weights for Groups 1, 2, and 3.

Figure 3 shows the mean daily weight of experimental (N=7) and control (N=6) subjects from Groups 4 and 5. Again the growth rates are similar for control and experimental animals. These experimental pups (Groups 4 and 5) were exposed to 3% (v/v) ethanol on Days 6-9 (postnatal). In all nine groups, there were no differences betweeen the growth rates of ethanol-exposed and control animals. The variances were very small. Groups 8 and 9 are not included here because there were too few animals that survived to Day 18. The growth rates for these two groups showed no differences between the ethanol-exposed pups and the controls. Growth rates for Groups 8 and 9 were nearly identical to the growth rates shown in Figures 2 and Figure 3.

Negative Geotaxis

Figure 4 shows the group mean latency on the negative geotaxis response for groups 4 and 5 (combined). The experimental subjects (n=6) were exposed to 3% (v/v) ethanol on days 6-9. The negative geotaxis response data for Groups 1, 2, and 3 have not been included here because these groups were exposed to varying amounts of ethanol on various days. As can be seen in Figure 4, the experimental pups had a somewhat longer latency on this reponse than did the controls for most days of alcohol exposure (postnatal Days 7-10). However, only on Day 9 was there a

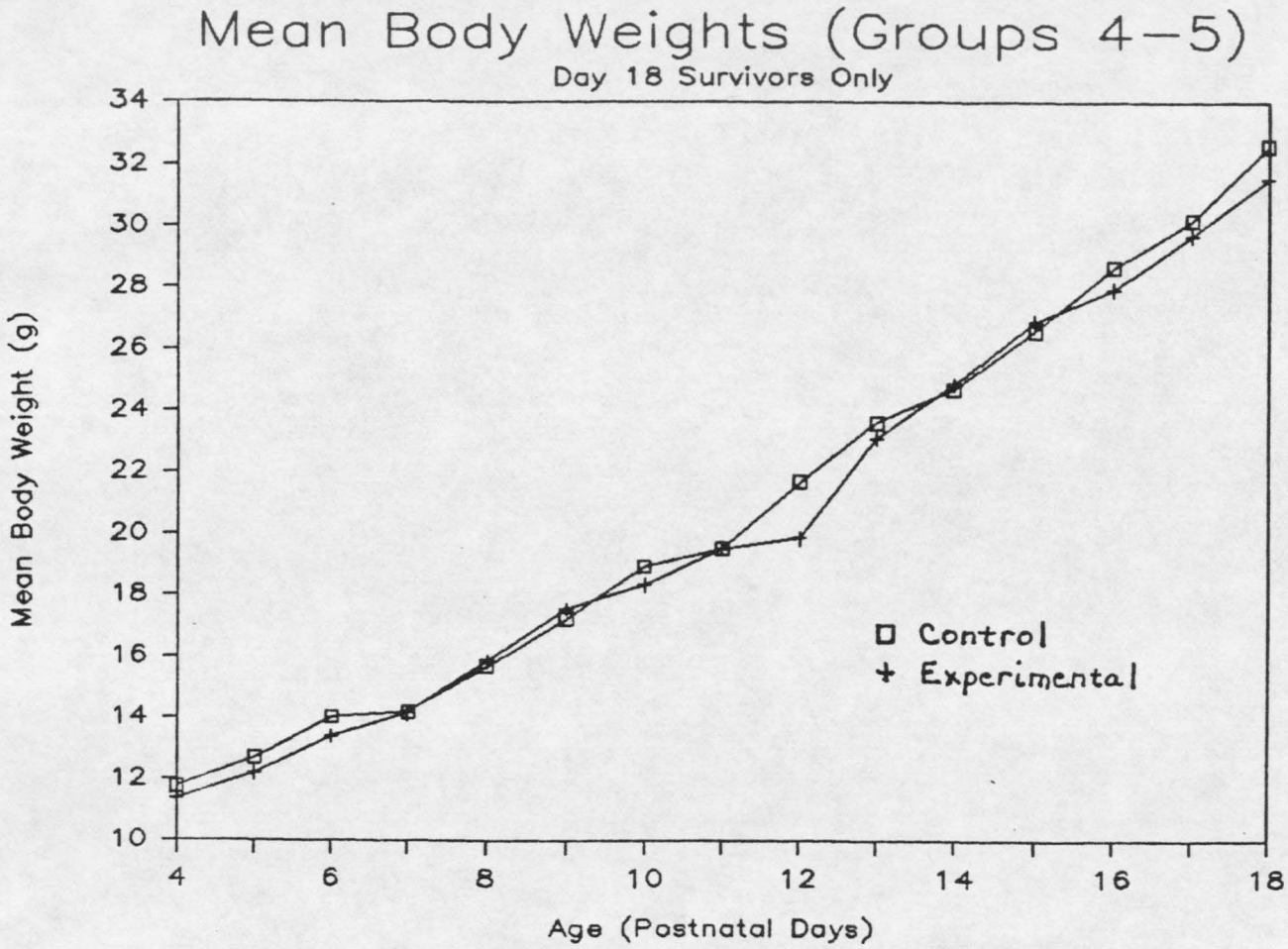

Figure 3. Mean body weights for Groups 4 and 5.

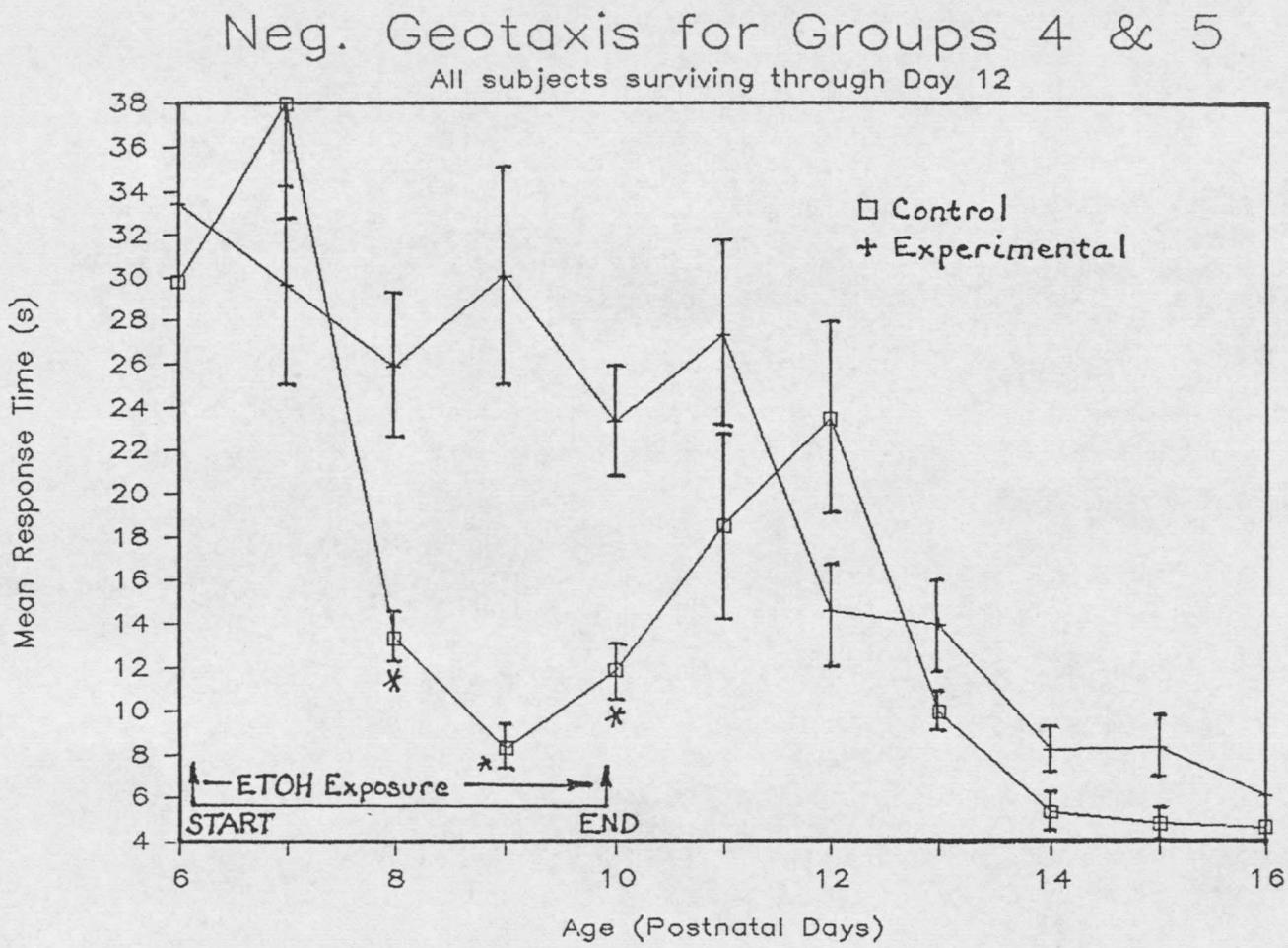

Figure 4. Negative Geotaxis Reflex measures for Groups 4 and 5.
*Indicates statistically significant F_{max} ($p < .05$).

statistically significant difference in latency, $t(13) = 2.234$, $p < .05$.

An F_{max} anlaysis of group variance shows that the variances of the experimental group was significantly greater than that of the control group on Days 8, 9, and 10. On Day 8 the variance resulted in an $F_{max}(5, 8) = 32.26$ $p < .03$. On Day 9 analysis showed an $F_{max}(5, 8) = 45.45$ $p < .01$; and also on Day 10 $F_{max}(5, 8) = 17.24$, $p < .01$.

Figure 5 shows the mean reponse latency for Groups 8 and 9. The experimental rats in these two groups had been exposed to 4% (v/v) ethanol on Days 5-9 postnatal. The experimental subjects showed a somewhat longer latency to respond than the controls on all days of alcohol exposure. Only two of these days, 6 and 11, had significantly different means. A t-test of Day 6 resulted in a $t(14) = 2.97$, $p < .01$; and for Day 11, $t(14) = 2.21$, $p < .04$. An F_{max} analysis of the group variances showed no significant differences on any test days. The higher dose of ethanol (4%) likely affected more subjects to a higher degree than the 3% exposure, and therefore the variability was not as great as that seen in Groups 4 and 5.

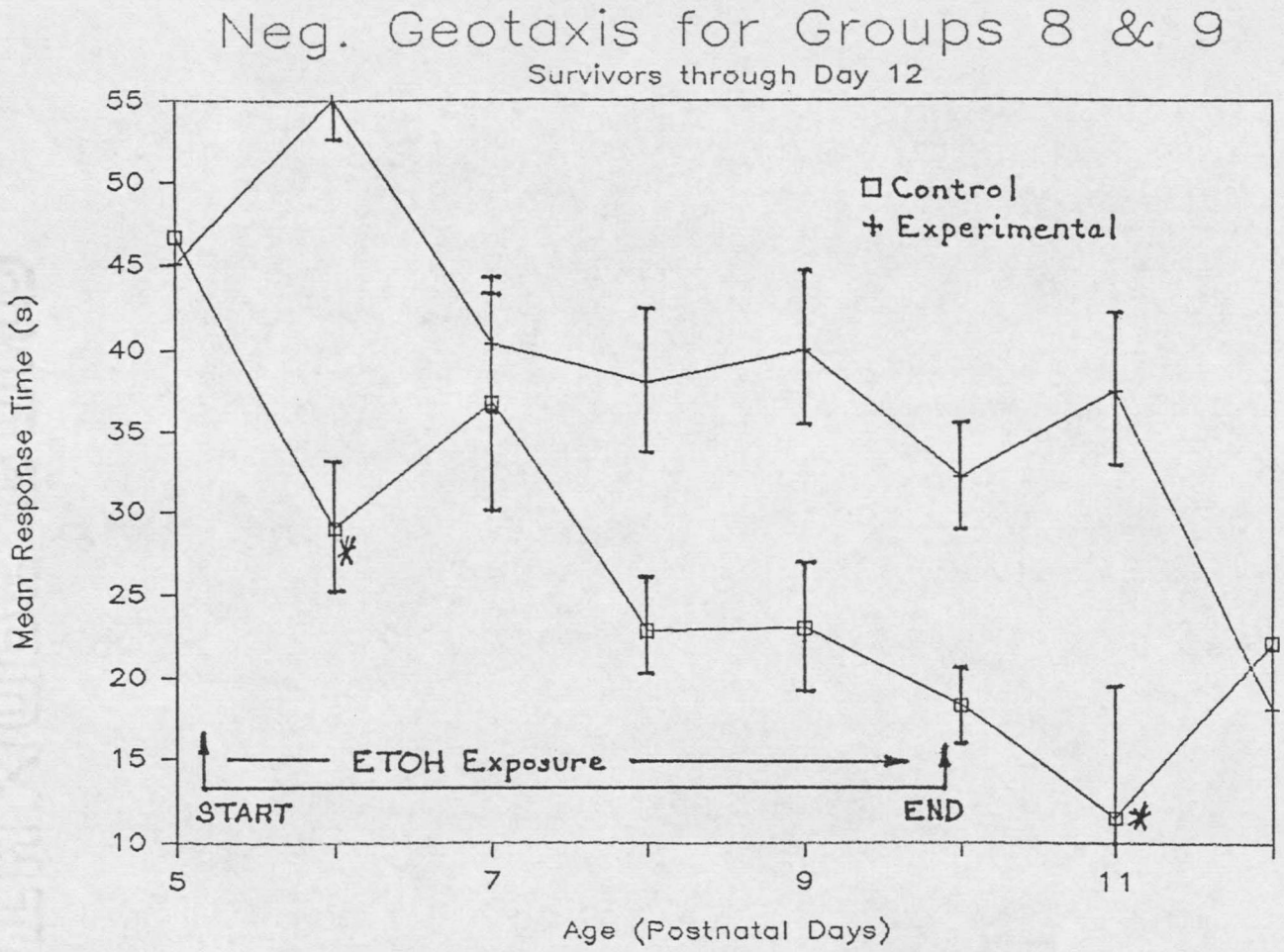

Figure 5. Negative Geotaxis Reflex measures for Groups 8 and 9.
* Indicates significant group mean differences, t-test, ($p < .05$).

Ethanol Preference

Figures 6 and 7 show the amounts of ethanol and water, respectively, consumed during one-hour preference tests by Groups 2-5 (combined). The subjects were water-deprived for 18 hr, then offered water versus one of three solutions of ethanol (1%, 5%, or 10% v/v). Order of presentation of solutions and position of bottles were ramdomly determined.

Figure 6 shows that the experimental subjects drank somewhat more ethanol than the controls at all concentrations offered. The only significant difference, however, was the 10% concentration, $t(25) = 2.17$, $p < .04$. There were no significant differences of the water consumption rates.

All of these one hr two-bottle preference tests were carried out in the pup's home cage. These tests were given to pups from Groups 2-3 (combined), Group 4, and Group 5. We also tested Group 2 in a single-bottle test. After 19 hr of water deprivation the subjects were given either 10% ethanol (Day 35 postnatal) or 5% ethanol (Day 36 postnatal). At both concentrations, ethanol-exposed subjects drank approximately twice as much ethanol as controls during the first 30 min but this difference appeared to decrease by 1 hr.

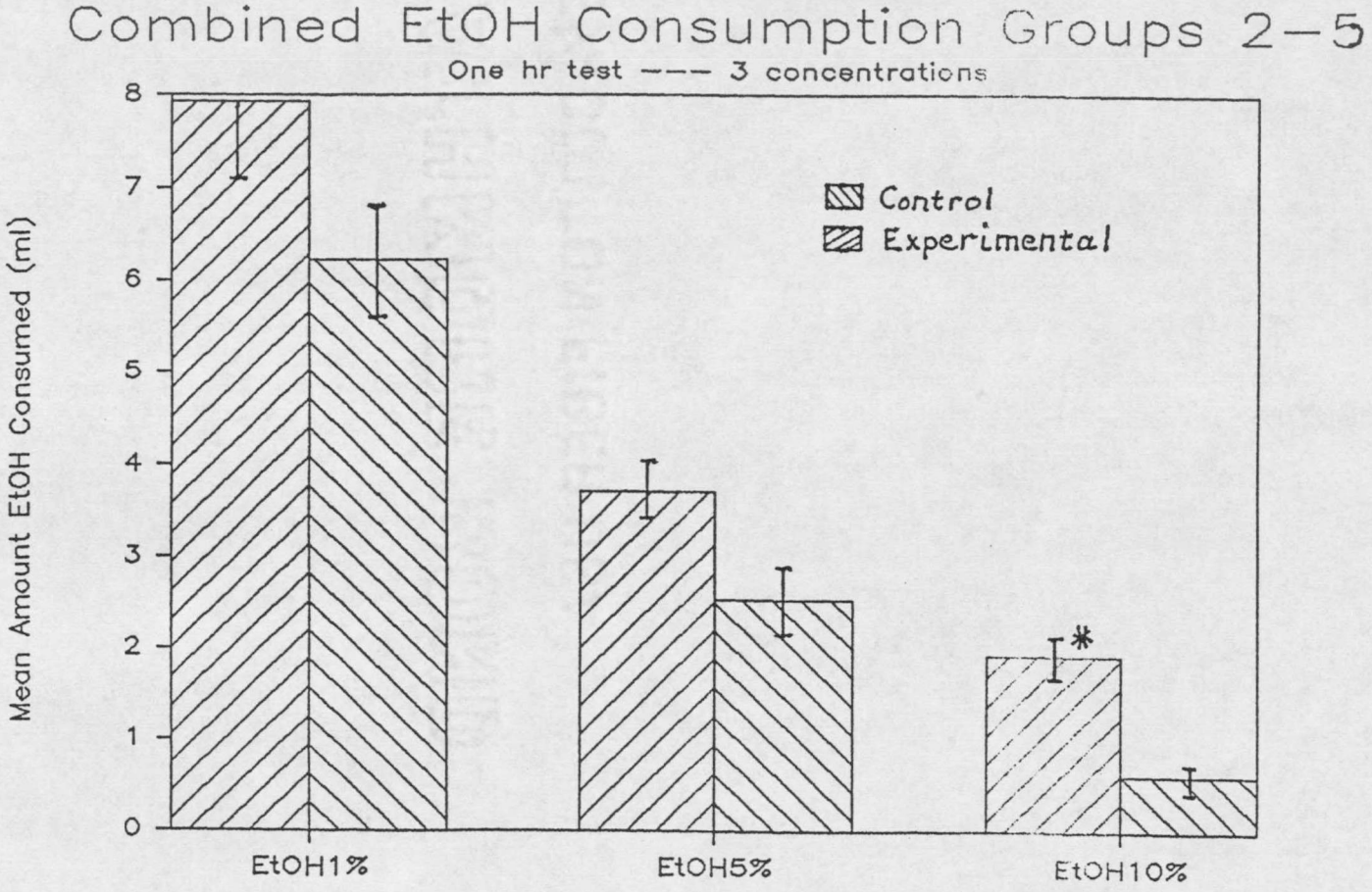

Figure 6. Ethanol consumption for Groups 2, 3, 4, and 5. Three concentrations were presented with water; 1% ethanol (v/v), 5% ethanol (v/v) and 10% ethanol (v/v).
*Indicates significant group mean difference, t-test ($p < .05$).

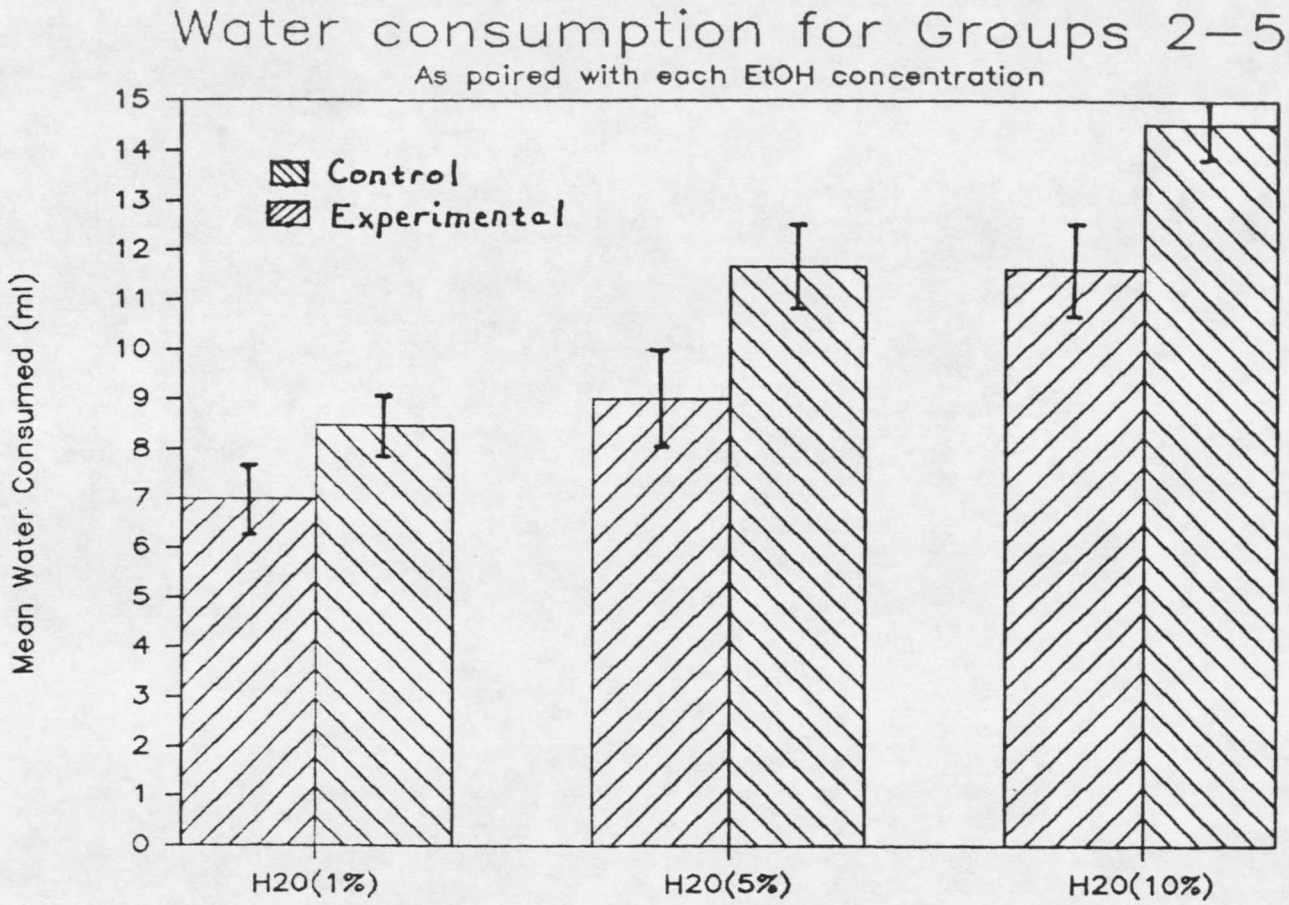

Figure 7. Water Consumption for Groups 2, 3, 4, and 5. The amount of water consumed with each ethanol concentration (1%, 5%, and 10%) is shown.

Figure 8 shows the amount of ethanol and water consumed in three 5 hr tests by subjects in Group 5. Subjects were deprived of water for 21 hr then given two bottles - one containing water and the other containing 5% (v/v) ethanol solution. The experimental animals drank more ethanol than the controls on each of the three test days. However, an analysis of variance repeated measures design (BMPD-2V; Dixon, 1983) did not show any significant differences between the two groups. There was only a significant overall effect of days, $F(2, 8) = 5.040$, $p < .04$. The small number of subjects (n=3) and the relatively large variances may explain why the means showed no main effect or interaction. Group 5 was also given one 24-hr test and one 17-hr test. On the 24-hr test pups in the control group drank a mean of 6.67 ml ($\pm$ 5.50 ml) ethanol, whereas pups in the experimental group drank a mean of 17 ml ($\pm$2.64 ml) ethanol. On the 17-hr test the controls consumed 13.33 ml ($\pm$8.73 ml) ethanol whereas the experimentals consumed 18.67 ml($\pm$3.05 ml) ethanol. Total fluid consumption was 33 ml for both groups on the 24 hr test. Total fluid consumption on the 17-hr test was 28 ml for controls compared to 35 ml for the experimentals. Both groups appeared to increase their consumption of ethanol with successive days of testing.

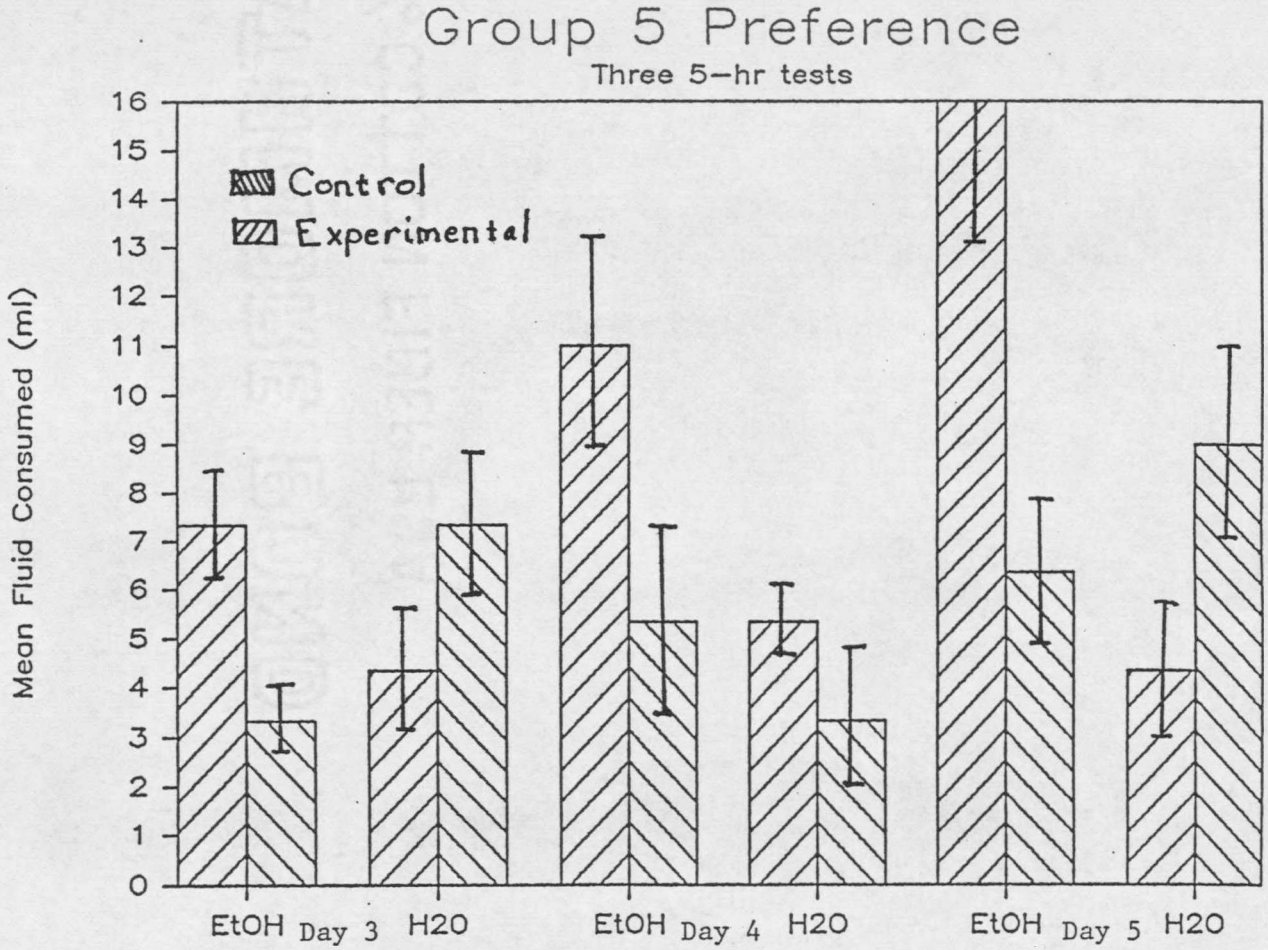

Figure 8. Ethanol and water consumption for Group 5 in three 5-hr tests. Water was paired with a 5% ethanol (v/v) solution. Position of solutions was randomly determined.

Brain Weight/Body Weight Ratios

The brain/body weight ratio was 0.033 (SEM = ±0.001) for eight experimental pups and it was .039 (SEM = ±0.001) for five control animals. These subjects came from two separate groups of animals. Of the eight experimental subjects, four were from Group 1 which was exposed to 3% (v/v) ethanol for Days 6 through 9 (postnatal). The other four experimental animals in this group came from Group 9, which was exposed to 4% ethanol on Days 5-9 (postnatal). The brain measurements were from animals 18 days old (Group 1) and 10 days old (Group 9). The differences in ages of these subjects makes the brain weight/body weight ratio a more appropriate measure for comparison than simple brain weight. A *t*-test comparing control and experimental group means is significant, $t(11) = 2.59$, $p < .02$.

Cataracts

A total of 58 animals in Groups 1-10 survived to eye opening. No consistent difference in time of eye opening was observed between the experimentals and controls. However, casual observation suggested that artificially-reared animals opened their eyes somewhat earlier than mother-reared animals.

Cataracts were observed in 23 of the artificially-reared animals. The cloudiness observed was confirmed to be cataracts by a pathologist, Dr. Larry Stackhouse, of

the Department of Veterinary Research at Montana State University. Of the 23 animals in which cataracts were observed, 19 were ethanol-exposed and 4 were control animals. Using a chi-square analysis this treatment difference in the frequency of cataracts in the experimental animals is higly significant, χ^2 (1, $\underline{N}$ = 58) = 9.042, $\underline{p}$< .003).

DISCUSSION

Artificial-rearing appears to be a suitable method for determining the effects of early ethanol exposure on neonatal rat reflex behavior as well as later ethanol preference. While artificial rearing is not without limitations and drawbacks, it solves many of the nutritional complications often inherent in developmental alcohol research. In particular, the level of nutrition and alcohol exposure can both be determined more precisely using this method than with maternal exposure or acute neonatal exposure during mother-rearing.

Growth

The growth rates of all groups of artificially-reared animals were similar, and pups that were exposed to alcohol did not differ in their growth rates when compared to controls. Growth of the artificially-reared animals compared quite favorably with normally-reared controls, and by Day 21 (postnatal) their weights were within normal limits based on data from Charles River Breeding Laboratories, Inc.

The fact that the subjects exposed to ethanol as neonates showed the same growth rate as controls is inter-

esting because growth deficiencies are a fairly consistent finding in human FAS children (Landesman-Dwyer, 1982; J. Opitz, personal communication, May, 1985). Perhaps rat growth is affected differently by perinatal ethanol exposure and cannot be compared to human growth rates. Other explanations for this difference may be the time of ethanol exposure; i.e., our subjects received ethanol during a fairly limited time of development (Days 4-9 postnatal), which closely corresponds to the third trimester of human gestation. It is likely that human FAS children are exposed to varying amounts of ethanol over a longer developmental period.

Much of the available human research has looked at alcohol insult which occurred over the nine months of gestation. Therefore, it may be that time of exposure is a contributing factor to the growth deficiencies seen in human FAS children. Nutritional factors may also play a contributing role in human studies, whereas artificial rearing holds nutrition constant. For whatever reasons, the alcohol-exposed animals in the present studies grew at a rate which was nearly identical to that of the controls. This has also been found by other researchers using the artificial rearing technique (Diaz & Samson, 1980; West et al., 1984).

Although we did not find any growth deficiencies in

terms of body weight, a significantly smaller brain/body weight ratio was seen in the experimental animals when compared to controls. This smaller brain/body weight ratio was seen in Day 10 postnatal animals from Group 9 as well as in Day 18 postnatal animals when these groups were pooled. Samson and Diaz (1982) have reported similar results.

Postnatal Behavior and Gross Neurological Development

Initially with the first group of animals (Group 1) we recorded righting ability, cliff avoidance and negative geotaxis. Only the negative geotaxis measure showed consistent effects of acute ethanol by increasing reversal latencies during treatment days and for one or two days after cessation of the ethanol treatment.

Experimental animals in Groups 4 and 5 showed a somewhat longer mean latency on the negative geotaxis measure on three of the four days of alcohol exposure and one day after the end of the alcohol treatment. The individual response to alcohol was highly variable. Some subjects were obviously affected (falling over; inability to stand) while others appeared to be minimally affected. The mean latency differences approached statistical significance on one treatment day only; however, the group variances were significantly different on the last three days of the four-day exposure period. This increased

amount of variability in the experimental groups has been noted by Riley and Meyer (1984):

> "Not every offspring is expected to be equally affected. In fact differences between pups in the same litter in terms of degree of effect may be striking. Some may be grossly affected while others appear completely normal." (p. 100)

The reasons for the highly variable response to ethanol exposure are not readily apparent. It may be that individual offspring differ in the degree of liver development. The subjects with less developed livers may be less able to metabolize the ethanol, and thus be more behaviorally affected.

The result of ethanol exposure in Groups 8 and 9 was similar to that of Groups 4 and 5 (i.e., on most days of alcohol exposure the experimental animals had longer latencies to complete the negative geotaxis than the controls). Groups 8 and 9 were exposed to a higher dose of ethanol than groups 4 and 5 (4% vs. 3%); also the period of exposure was one day longer (5 days vs 4 days). The result of this higher dose and longer exposure was a more severe withdrawal effect indicated by tremors, twitching, hypersensivity to touch and poor coordination; as well as a higher mortality. This higher dose and longer-exposure withdrawal effect was seen on Day 11 (see Figure 5), and was noted in daily observation during servicing. However, as in the earlier groups, daily group mean differences

were only significant on days 6 and 11. Moreover, we did not see the dramatic differences in variability between groups. It is likely that this higher dose of ethanol (4%) affected more subjects to a higher degree than the lower (3%) dose of ethanol. Mortality at 4% was greater than with the 3% exposure, therefore pups which were more affected by the ethanol died, and did not contribute to the data.

Cataracts

An interesting aspect of this research is a serendipitous finding. The animals that survived to eye opening (Days 11-14) were observed to occasionally have cataracts. Initially, we saw these cataracts in ethanol animals only (Groups 1-7). The appearance of cataracts has apparently not been mentioned in any of the research published on artificial-rearing methods. Our findings on the frequency of these cataracts in alcohol-exposed animals indicate that alcohol exposure appears to be a contributing factor. It may be that we see the cataracts appearing more often in experimental (alcohol-exposed) subjects than in controls because we used Sprague-Dawley albino rats. Albino rats may be more genetically predisposed to intraocular malformations than other strains, or the cataracts may simply be more noticeable because of the very light (pink) color of their eyes.

There is at least one article, however, which suggested that in humans prenatal alcohol exposure causes various intraocular malformations including cataracts (Kerstin, 1981). While it may be that the cataracts are partly a result of nutritional deficiencies of the diet (Diaz, personal communication, May, 1985), our findings suggest at least that alcohol may be interacting with dietary deficiencies in the production of cataracts.

Ethanol Preference

Preference testing of artificially-reared subjects was carried out to determine if early exposure to ethanol might increase later preference for ethanol. Research on this question is surprisingly limited and inconclusive (Abel, 1984).

Initial ethanol-consumption tests seemed to indicate that experimental subjects drank ethanol differently than controls. If pups were water deprived for 19 hr and then offered a single bottle containing 10% ethanol (Group 2, Day 35 postnatal) or 5% ethanol (Day 36 postnatal), the ethanol-exposed animals drank approximately twice as much ethanol as the controls during the first 30 min, but this difference decreased by 1 hr.

A more detailed two-bottle preference test was then conducted. This two-bottle test consisted of pairing water with one of three ethanol solutions (1%, 5%, or 10%)

and after 18 hr of water deprivation offering one of the combinations (ethanol vs. water) to the subjects in their home cage. The ethanol-exposed subjects drank more ethanol at each concentration (1%, 5%, and 10%) than did the controls. However, the only difference which approached statistical significance was for the 10% concentration. The results of this preference testing seem to suggest that early exposure to ethanol may cause an increase in later preference for ethanol.

Using the subjects in Group 5, a final series of preference tests was conducted. The results also suggested a difference between early ethanol-exposed animals and controls. All test days showed higher mean consumption of ethanol by experimental subjects. Because of the small number of subjects (n=3) in the control and experimental groups (n=3), the data can only be seen as suggestive and perhaps worthy of future investigation. An interesting finding from a group x solution x days (2 x 2 x 3) analysis of variance (repeated measure design) of this data indicated a significant effect of days $F(2) = 5.04$, $p < .04$. The subjects in both groups drank more ethanol across successive test days. An acquisition of preference has been found by others. For instance Veale and Meyers (1969) reported increased alcohol preference following repeated experiences with alcohol. They systematically

increased ethanol concentrations from 3% to 30% in 11 tests and reported that rats drank two to three times as much alcohol in the seventh sequence as in the first. Does early exposure to ethanol change this rate of intake acquisition? Our data suggest this possibility but overall group mean differences were not statistically significant nor was the group x days interaction. Nevertheless, this would be an interesting topic for future research.

A review of the negative geotaxis data from Groups 4 and 5 indicates that one animal who appeared to be quite affected on the days of ethanol exposure also showed a fairly high preference for 5% ethanol. This subject received a score of 60 s (longest possible latency) on three of the four days of alcohol exposure. On later preference tests he drank five times as much ethanol as he did water. The results of the three five-hour preference tests for this subject were: 11 ml ethanol, 7 ml water; 20 ml ethanol, 5 ml water; and 26 ml ethanol, 5 ml water. Only one other subject in this group had a score of 60 s for three of the four days of alcohol exposure. This subject died during the transition from formula to regular lab chow (cause unkown). Although one subject is hardly definitive, a possibly important fact is that this subject appeared to be quite behaviorally affected during acute alcohol exposure and later showed a very strong preference

for 5% ethanol over water. This may suggest a persistently increased alcohol insenstivity or actual preference. Thus, longitudinal studies of subjects might be useful in the future. Subjects which are quite behaviorally affected from early ethanol exposure as measured by psychomotor reflex tests, may show a different rate of acquisition of preference for alcohol in later preference studies. It is possible that this different sensitivity to ethanol could be related to alcohol abuse seen in humans.

The present work would seem to support that of others who suggest that there is a relationship between early ethanol exposure and later increased preference for ethanol (Bond & DiGuisto, 1976; Phillips & Stainbrook, 1976; Reyes, Garcia, & Jones, 1984). Artificial rearing may also be seen as an acceptable method for investigating the effects of early ethanol exposure.

Whether preference for ethanol is actually increased or taste sensitivity is decreased is a question which this research has not answered. The question is certainly important and should be investigated in the near future. The role of opiates in alcohol preference (Samson and Doyle, 1985) as well as in taste sensitivity (Lynch and Libby, 1983) suggests an interesting area of study. The indication that fairly low doses of ethanol (3%v/v) early in development may have long-term consequences on later

ethanol preferences might be useful in a number of ways. Alcohol addiction has been reported to be quite high on some American Indian reservations (Department of Health & Environmental Sciences, Helena Montana). This high rate of alcoholism is apparently resistant to change by social programs, education and increased awareness. The causes most often given for this problem are social in nature. However, since many in this population may be addicted to alcohol, it is possible that a fairly large number of the offspring have been exposed to alcohol prenatally. If prenatal exposure to alcohol affects later preference for alcohol, this rather high addiction rate may be partly explained as a consequence of early exposure and resulting physiological consequences. It may be interesting to approach this extremely high addiction rate for American Indians from an early exposure, physiological point of view.

Future Considerations

This research indicates that the artificial-rearing method of raising rat pups is a very useful method for early ethanol-exposure research. The results, especially from the later preference studies seem promising enough to justify more research on this question. Design of actual preference studies should take careful consideration of concentrations, length of tests, and number of tests.

Looking at alcohol preferences under varying environmental conditions would seem to be a worthwhile approach. These conditions might be home cage versus test cage, deprivation and social conditions (single or group testing), and a variety of other stress factors. The inclusion of some of these other factors (different environments, social factors) may more appropriately model human alcohol consumption and shed some light on the factors involved in acquisition of alcohol preferences.

Further investigation of the etiology and later recovery of cataracts seen in these artificially-reared animals would also be indicated from the research presented here.

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