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THE EFFECT OF MATERNAL ETHANOL INHALATION DURING PREGNANCY ON THE CRANIOFACIAL DEVELOPMENT OF THE A/J MOUSE

by

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Abstract

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Jones et al. (1973) have described a syndrome affecting children born to chronically alcoholic mothers. In addition to a variety of general signs, one of the most specific features was a retruded, underdeveloped maxilla. To date, however, no specific studies have been conducted to characterize precisely the nature of the attendant facial signs.

A study was performed to evaluate the effect of ethanol on the midfacial growth of 117 mice whose dams had been exposed to an ethanol vapor during five days of gestation; fifty-two mice served as controls. The effect of ethanol on the incidence of clefting was also investigated in a separate sample of 164 experimental and 199 control fetuses.

Data were collected in three categories:

(1) General appearance, physical condition, and body weight

(2) Roentgenographic cephalometric measurements of the craniofacial complex

(3) Incidence of clefting

In general, the experimental animals did not reveal any

gross distortion or malformation due to ethanol. No significant effects of ethanol on body weights were seen. As demonstrated by analysis of covariance, an isolated reduction in maxillary advancement was seen in mice exposed to ethanol prenatally--the maxillary incisors and molars were retruded and the length of the snout was decreased. No change in overall cranial height or cranial base length was noted. There was no increase in the incidence of clefting.

Anecdotal reports of the symptoms of children born to alcoholic mothers indicate a retruded and hypoplastic maxilla. The results of the present investigation support those findings. In light of contemporarily received opinion regarding the unique anatomical configuration of the septum, it is postulated that ethanol may have caused a selective growth retardation of the cartilaginous nasal septum.

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INTRODUCTION

The causes of obvious craniofacial malformations, specifically those related to growth retardation, have been the subject of serious research investigations for many years. Maternal ethanol intake has been suggested as a cause of growth inhibition in developing fetuses and newborn since the early 1900's. It has recently been associated with a syndrome, described by Jones et al. (1973), of which maxillary growth inhibition represents one of the prominent features.

Ethanol, as contained in alcoholic beverages, is consumed by an estimated six to nine million Americans to such an extent that they may be considered "alcohol dependent" (Committee on Alcoholism and Drug Dependence, 1971). Ethanol is so widely used that its effects should be investigated as thoroughly as possible.

Since the developing fetus may be especially vulnerable to metabolic and growth disrupting agents consumed by the mother during critical periods of fetal development, the effects on the fetus of heavy alcohol ingestion by pregnant mothers merits thorough study. Even if these effects may not be reflected by large and dramatic malformations, any effect on the regular pattern of dimensional changes due to growth should be studied. Specifically in the field of orthodontics, any factor that causes a divergence from the regular pattern of craniofacial growth is a matter of concern and must be

considered.

It will be the purpose of this study to investigate the effect of maternal ethanol inhalation on the craniofacial development of offspring in the A/J mouse. Particular attention will be paid to midfacial dimensional changes. Using radiographic cephalometrics, specific craniofacial measurements will be taken to determine any significant changes in the pattern and amount of growth. In addition, fetal palates will be examined, and the occurrence of clefting will be recorded.

REVIEW OF THE LITERATURE

Much of the early literature on the effect of long-term maternal intake of alcohol on offspring is derived from the prohibition movement in the early part of this century, and, as a result, much of it is biased, anecdotal, and without validity (Green, 1974). The "female drunkards" of a prison population were examined by Sullivan (1900), and an increased incidence of stillbirths was found. Ladrague (1901) reported on "small and sickly" children of alcoholics, and Elderton and Pearson (1910) found that children of alcoholics weighed less than children of "sober" parents. Although some of the literature may have been highly biased, there were some studies that provided data worth considering.

Early Animal Studies

The following are examples of early animal studies which were undertaken to determine the effects of alcohol intake by mothers on their offspring. Nice (1912) reported a greater number of pregnancies, but higher infant death rates, in alcoholic white mice when compared to controls. Stockard and Papanicoulaou (1916, 1918) found smaller litters, increased prenatal death rates, and decreases in the size of offspring from the descendants of alcohol-dependent guinea pigs. However, Hanson and associates (1928) found that alcohol did not lower the mean body weight of offspring of rats exposed to alcohol fumes. MacDowell and Lord (1927), Bluhm (1930), and Davenport (1932)

related decreased litter size in alcoholic pregnancies to increased prenatal mortality. Bluhm (1930) also reported decreased weight and a greater mortality rate in alcoholic offspring. Mirone (1952) reported a decrease in fertility, but no effect on litter size or newborn birth weight resultant from alcohol treatment of pregnant mice. Apparently, the use of different concentrations of alcohol, different modes of administration, and different laboratory animals has resulted in inconsistent conclusions relative to the effects of maternal ethanol intake on the developing fetus. More recent studies also reveal differences of opinion regarding the effect of ethyl alcohol on the growth and development of the fetus and newborn.

Contemporary Investigations of the Effect of Ethanol on Growth

Literature Suggesting No Adverse Effect of Ethanol on Growth

Mirone (1959) and Morgan (1960) found that 10% ethyl alcohol, as the sole drinking fluid, did not inhibit the growth of mice and rats, respectively. (However, Morgan (1960) did find that 15% ethanol markedly decreased the growth rate and fluid intake.) When exposing rats to a 12% ethanol solution, or California sherry or muscatel wines with approximately the same alcoholic content, Newell et al. (1964) found an initial reluctance of his animals to consume alcohol-containing fluids during the first six weeks of their study. This was reflected in a poorer rate of growth in treated animals than in water controls during this period. However, after this initial period of reluctance, the average weight gain of all groups

was nearly identical. No adverse effect on reproductive capacity or newborn development and behavior was reported when 10% ethanol was voluntarily consumed (Thiessen et al., 1966). Mirone (1965a,b) reported that the ingestion of 15% ethanol as the sole drinking solution had no adverse effects on the daily growth rate of weanling male mice. Using 0.5% and 5.0% ethanol solutions during all of gestation and continuing for one to three weeks postpartum, no effect of ethanol on the growth of fetuses or newborn was reported by Kahn (1968).

Literature Suggesting a Growth Retardation Due to Ethanol

Reduced growth rates have been reported in rat and mice weanlings whose dams were given ethanol solutions as their sole postpartum drinking fluid (Mirone, 1958; Pilstrom and Kiessling, 1967). There are reports, in the literature, which tend to suggest that the cause of such stunted growth could have been malnutrition, rather than a direct effect of ethanol (Collard and Chen, 1973). Specifically, ethanol may inhibit the release of oxytocin which is needed for milk ejection (Fuchs and Wagner, 1963; Fuchs, 1969). It also may cause maternal behavior that would prevent the offspring from obtaining adequate amounts of milk, or it may contaminate the milk, thereby discouraging suckling (Collard and Chen, 1973). Collard and Chen (1973), in an examination of the effects of different modes of ethanol administration on the growth of neonate mice, found that:

> . . . when ethanol was injected into the mothers of neonate mice, the pups weighed less at weaning than did

control pups. When the litters were raised in alcohol-vapor chambers, however, the pups weighed more at weaning and at seven weeks of age than did control mice.

A significant reduction in body weights, as compared to water-reared infants, was also found by Abel (1974) in infant rats of alcohol-drinking mothers. Skosyreva (1973) administered 40% alcohol intravenously (5 ml./kg.) to pregnant rats on the 8th-14th day of gestation. As a result, there was a high incidence of postimplantation death, as well as a generalized developmental retardation of the fetuses.

In summary, it is notable that some of these studies involved only the growth of weanling animals as affected indirectly by the transfer of ethanol through the mother's milk, and did not include the effect of ethanol on fetal development. Other studies, already mentioned, do seem to give evidence of a retarding effect of maternal ethanol intake on the growth of the fetus. However, there were no specific studies employing cephalometrics to determine precisely what changes in facial growth might be occurring.

Human Studies

In human subjects, it was demonstrated by Rubin and Lieber (1968) that ethanol induces an increased level of hepatic microsomal enzymes. This prompted Waltman and Iniquez (1972) to speculate that alcohol ingested by mothers may therefore affect fetal enzymes, drug metabolism, and steroid substrates. Lopez and Montoya (1971) reported vacuolization of proerythroblasts, erthyroblasts, promyelocytes and myelocytes in the bone marrow of two premature infants

whose mothers received alcohol infusions in an attempt to prevent premature labor.

Ulleland (1972) found that thirty-seven of 1,582 babies of non-alcoholic mothers, or 2.34%, were born underdeveloped in weight and length. In contrast, ten out of twelve (83%) of the offspring of alcoholic mothers were born exhibiting subnormal weight and length. In order to determine whether nutrition could have contributed to the difference between the alcoholic and non-alcoholic offspring, dietary histories were taken on seven alcoholic mothers. Five of the seven were considered to have moderate to severe deficiencies, either in protein or in calories or in both, during pregnancy. Three admitted to consuming 25% or more of their daily caloric intake in the form of alcohol. As Ulleland reported:

> Laboratory study, which included tests for hemoglobin, red cell indices, total serum protein, albumin and globulin, and the assay of various vitamins in blood and urine, did not indicate the presence of specific vitamin deficiencies, except in one mother, who had serum folate less than four micrograms per cent. One had a slightly decreased serum albumin. Five had increased globulin with normal albumin, which is more consistent with liver changes of chronic alcoholism than with malnutrition.

When evaluating the study of Ulleland (1972), it should be noted that there is the possibility that all of the alcoholic mothers were not identified. Moreover, patients may not have been completely candid in answering medical history questionnaires, and clinical tests may not have been adequate for discovering all of the chronic alcoholics or borderline chronic alcoholics. The small sample size of the alcoholic group severely limits the confidence in his conclusions that ethanol exerts an effect, exclusive of malnutrition, on newborn development.

Jones et al. (1973) presented case reports of eight unrelated offspring of three different ethnic groups, all born to mothers who were chronic alcoholics. All eight offspring demonstrated a similar pattern, characterized by craniofacial, appendicular, and cardiovascular defects, that were apparently associated with a prenatal onset of growth retardation and developmental delay. The degree of linear growth deficiency was more severe than the weight deficit at birth. This is in contrast to the study of generalized maternal malnutrition by Smith (1947), in which the newborn were found to be underweight in terms of mass per unit length. This difference suggests that a factor other than nutritional deprivation alone was adversely affecting prenatal growth in these children of chronic alcoholic mothers. Whatever the cause, the reduction in growth rate continued throughout early childhood.

In addition to the generalized growth deficiencies mentioned by Jones et al. (1973), maxillary hypoplasia, most evident in the anterior-posterior dimension, resulted in a relative prognathism. Several possible effects of maternal alcoholism on the developing fetus were discussed by Jones et al. (1973):

1. Direct ethanol toxicity (ethanol freely crosses the placental barrier as reported by Waltman and Iniquez, 1972).
2. Direct toxic effects of the breakdown products of ethanol, such as acetaldehyde or an unknown toxic agent in the alcoholic beverages which these mothers were consuming.

3. General maternal undernutrition or the deficiency of a specific nutrient or vitamin. (The degree of prenatal growth deficiency and the pattern of malformation discussed previously have not been recognized in undernourished women who were not alcoholics, as reported by Smith, 1947.)

It should be emphasized at this point that it was the report of Jones et al. (1973) of this unusual syndrome and the interesting potential it affords for deriving new knowledge in the area of prenatal and early postnatal craniofacial growth that provided the major incentive for this investigation. Thus, the present investigation was designed to analyze specifically any malformation of the maxilla in mice exposed to ethanol during fetal development.

It is difficult to isolate the effects caused by ethanol in alcoholic human females due to variables such as economic status, race, nutrition, prenatal care, maternal stress, and others (Green, 1974). Thus, it would seem that additional animal studies designed to control some of the above-mentioned variables could prove valuable in determining the effects of maternal ethanol intake on the developing fetus and subsequent postnatal development. Subtle changes in the craniofacial developmental pattern could be better observed with animal studies than with human studies, due to the difficulty in obtaining an appropriate human sample and controlled experimental conditions.

The Metabolism of Ethanol

Some of the adverse growth effects which have been reviewed in this paper could be a result of ethanol metabolism. Ethanol is converted to acetaldehyde by the transfer of hydrogen ions to a cofactor, nicotinamide adenine dinucleotide (NAD). The transfer is catalyzed by an enzyme, alcohol dehydrogenase, which serves, in general, as a remover of hydrogen ions from various compounds, including some steroids. The acetaldehyde is next oxidized to form acetic acid, which is then converted into carbon dioxide and water (Lieber, 1976).

The excessive accumulation of hydrogen ions from ethanol causes an added oxidative load. This unbalances the liver cell chemistry by causing an abnormal shunting of hydrogen into one or more pathways, creating several adverse effects. One such effect is the blocking of gluconeogenesis by the diversion of pyruvate to lactate instead of to glucose (Lieber, 1976). This will lead to hypoglycemia; especially, but not solely, when there is no dietary source of carbohydrates and when glycogen stores have been depleted (Madison et al., 1967; Lieber, 1976). Another adverse effect is the increased production of lipids, specifically triglycerides. Besides forming lipids, the excessive hydrogen ions also prevent the normal oxidation of lipids in the liver cell. Instead of oxidizing lipids, the mitochondria of the liver cell utilize the hydrogen ions from the ethanol to produce energy. This causes an abnormal accumulation of lipids in the liver cells (Lieber, 1976).

Lipids either can be converted to water soluble ketone

bodies which are secreted into the bloodstream, or the lipids can be secreted directly. Before lipids can directly enter the bloodstream, however, they must be made water soluble by being wrapped in a thin coat of protein to form lipoproteins (Leiber, 1976).

This diversion of proteins could be one of several specific adverse effects that might cause a growth inhibition. Ethanol and its primary conversion product, acetaldehyde, could also affect growth (adversely) by interfering with the activation of vitamins by the liver cells, and also by impairing the absorption of vitamins in the small intestine (Tomasulo et al., 1968; Lieber, 1976).

Although most ethanol metabolism takes place in the liver, some acetaldehyde-oxidizing capacity is present in other tissues, such as: kidney, heart, small intestine, adipose, lung, brain and adrenal (Maxwell and Topper, 1961). The existence of a multi-tissue reactivity with ethanol opens the possibility that similar chemical reactions could conceivably take place in young tissues during their rapid growth phase and when their forming cells are undergoing active metabolism.

Placental Transfer of Ethanol

It has been established that ethanol crosses the placenta from the time of placental development and throughout gestation (Waltman and Iniquez, 1972; Ho et al., 1972). Ethanol metabolism does occur in the fetus in the same manner as in the adult, but at a slower rate due to low fetal levels of alcohol dehydrogenase (Pikkarainen, 1967). This slower rate of fetal ethanol metabolism

causes ethanol and its degradation products, including acetaldehyde, to remain in fetal tissues longer than in the mother's, possibly resulting in fetal effects that are of longer duration than the maternal effects (Ho et al., 1972).

The actual mechanism of placental transfer is explained by Waltman and Iniquez (1972) as follows:

> The fundamental mechanism of placental transfer involves not only diffusion (simple and facilitated) but probably active transport, Starling-Landis filtration, filtration through pores, pinocytosis, phagocytosis and vesiculation as well. In terms of rate of diffusion, molecules such as methanol and ethanol with one or two hydroxyl and hydrocarbon groups will predictably permeate rather rapidly.

The rapid permeation of ethanol could explain the findings by Ho et al. (1972) that both the placenta and amniotic fluid contain relatively high concentrations of ethanol, especially during late preganacy (Table I).

In reference to Table I, the amount of ethanol in the amniotic fluid (after an intraperitoneal injection in the pregnant hamster) is reasonably similar to that in the maternal blood, at least during the first ninety minutes after injection. It can also be seen that, during this time interval, the maternal blood and amniotic fluid ethanol levels are usually higher than those of the fetus.

From the data in Table I, and references previously reviewed, it can be concluded that ethanol passes from the mother to the fetus and the amniotic fluid via the placenta, and as a result, could conceivably create adverse effects upon the developing fetus.

TABLE I

LEVELS OF UNCHANGED ETHANOL IN HAMSTER TISSUES FOLLOWING INTRAPERITONEAL INJECTION OF 1.5 g./kg. (mg./g. of tissue)

Time after Injection	Days of Gestation	Maternal Blood	Fetus	Placenta	Amniotic Fluid
5 min.	6	1.79	0.32		
	15	1.47	0.68	0.32	1.40
30 min.	6	0.14	0.78		
	15	0.58	0.80	0.40	0.55
90 min.	6	0.62	0.20		
	15	0.47	0.21	0.21	0.39
4 hrs.	6	0.06	0.03		
	15	0.16	0.16	0.11	0.06

After Ho et al., 1972, Table 2

Midfacial Growth

A major portion of the present investigation will be to determine whether maternal ethanol inhalation in the A/J mouse can duplicate the reduced maxillary advancement that Jones et al. (1973) described as one of the characteristics of children of chronic alcoholic mothers. The possible etiology of a retruded maxilla in fetuses of alcoholic mothers will be examined as it relates to two contemporary concepts of midfacial growth (the nasal septum and functional matrix hypotheses).

Nasal Septum Theory

Scott (1953, 1954, 1956) argued that growth of the nasal septal cartilage separates the facial bones from each other and from the cranial portion of the skull, allowing growth to take place at the sutures by a process of secondary deposition. He further stated that the cartilage of the nasal septum is an extension of the cartilage of the cranial base and when the nasal septal cartilage was replaced by bone (usually around age six in humans), the septum no longer contributed to midfacial growth.

Histological examination (Latham, 1971) of two cyclopic specimens revealed that the maxilla, in the late fetal period, is capable of growing downward and forward in the absence of the nasal septum, although a degree of anterior-posterior deficiency was noted. This additional non-septal source of growth was said to be due to surface deposition of bone on the posterosuperior aspect of the maxillary bones. Latham and Scott (1970) felt that a mass of

fibro-fatty tissue (as seen in the examination of fifty-five human specimens from six weeks prenatal to six months postnatal) was acting as a cushion against which the pressure from this surface deposition could react, and as a result, push the nasomaxillary complex downward and forward.

In addition to the mechanism of nasal septal growth and surface deposition, the presence of a fibrous connection between septum and premaxilla was suggested by Young (1960) and Latham (1970) to be transmitting forces from the septum to the maxillae as tension rather than pressure. Latham (1970) concluded that the septum pulled the developing maxillae and premaxillae forward via this connection, termed the septopremaxillary ligament. His findings were consistent with Gange (1973) and Gange and Johnston (1974), who severed the septopremaxillary ligament of newborn rats and demonstrated a resulting anteroposterior midfacial deficit.

Additional evidence for the nasal septum theory comes from studies involving septal ablation. Sarnat and Wexler (1966) demonstrated a lack of forward growth of the rabbit upper face after surgical resection of the nasal septum. An anteroposterior midfacial deficit was also created in septal resection experiments by Ohyama (1969) and Kvinnsland (1974a). From these investigations, it seems apparent that resection or detachment of the septum results in loss of some anterior-posterior growth.

In view of the preceeding nasal septum investigations, it might be hypothesized that the nasal septum has its own growth potential. Support for such a hypothesis was presented by Kvinnsland

(1973). He observed that autotransplanted nasal septal cartilage grows at about the same rate as septal cartilage in situ. Thus, nasal septal cartilage does seem to have an "intrinsic" ability to grow.

The previously cited investigations have been conducted to test the nasal septum hypothesis, and their results support the contention that the cartilaginous nasal septum has its own growth potential and serves, at least during fetal development, to pull the nasomaxillary complex forward via the septopremaxillary ligament.

However, some investigators have taken an opposing position, namely, that the growth of the midface is completely independent of nasal septum growth. Controversy exists as to whether the nasal septal cartilage influences translation and transformation of bones, or whether the translation and transformation of bones is due to function occurring within functional matrices.

Functional Matrix Theory

Moss (1960, 1962) described a "Method of Functional Cranial Analysis" which, according to him, would replace "many classic theories of cranial structure, as well as derivative conjectures about facial growth processes." He explained (Moss, 1968) this new approach to craniology as follows (p. 65, italics his):

> The head is a region of the body within which a number of relatively independent *functions* are carried out; respiration, olfaction, vision, hearing, balance, chewing, digestion, swallowing, speech, and neural integration. Each of these functions is carried out by a separate *functional cranial component*. Each component consists of all of the

> tissues, organs, spaces, and skeletal parts necessary to carry out a given function completely. The tissues, organs, and functioning spaces, taken as a whole, comprise the *functional matrix*; while the skeletal tissues (osseous, as well as cartilaginous) related to this specific functional matrix are the *skeletal unit*.

The soft tissues of a functional cranial component are the functional matrices, either periosteal or capsular. Periosteal matrices are soft tissues (muscles, teeth, glands, etc.) that act to alter (transform) the size and shape of the various skeletal tissues. Capsular matrices are those soft tissues (brain, orbit, spaces, etc.) that are responsible, by way of their expansion, for translative growth or changes in spatial position of bones imbedded within the capsular matrix itself (Moss, 1969b).

Moss (1969a) stated that the growth of the nasal cartilages, and also the condylar and cranial base cartilages, are "secondary, compensatory, and mechanically obligatory events, in all ways homologous with the compensatory growth observed at the sutural margins of all intramembranous cranial bones."

Moss et al. (1968) interpreted deformities resulting from the removal of nasal septum in twenty-day-old rats as being due to a collapse of the roof of the nasal cavity. Stenström and Thilander (1970) concluded similarly that the nasal septum in guinea pigs is not a primary growth center, but rather a mechanical support for the nasal bones. After "very comprehensive early extirpations of the septal cartilage" in young guinea pigs, they found deformities which "were only moderate and most often were a downward tipping of the nasal bones with a slight ventral tipping of the whole snout,

together with a posterior relation of the mandible."

However, as Gange and Johnston (1974) pointed out, collapse implies a vertical deficit, and the radiographs of Moss et al. (1968) revealed more than "collapse;" namely, that an anteroposterior deficit of the maxillae was also evident in their experimental animals. Gange and Johnston also noted that Stenström and Thilander (1970) and Moss et al. (1968) did not comment on the fact that their results demonstrated an anteroposterior deficit, as well as vertical collapse. This additional effect is illustrated by Johnston (1976, Fig. 4).

Based largely on the previously cited references, the ideas presented by Moss seem to be marred by a lack of either proof or a testable physical basis. Indeed, Daniels and Kremenak (1971) described Moss' "Method of Functional Cranial Analysis" as a formulation containing a set of ingenious but hard to test hypotheses, and suggested that "it not be regarded as a theory now." Latham's most recent views (Latham, 1970, 1971), as presented previously in the present review, seem more acceptable as a testable provisional hypothesis.

Alcohol and Palate Formation

No report exists in the literature relating heavy alcohol consumption to cleft palate, but several investigators share a common theory that growth inhibition may be responsible for preventing palatal fusion (Kochhar, 1968; Zimmerman et al., 1970; Andrew and Zimmerman, 1971; Andrew et al., 1972; Jelinek and Dostal,

1974; Greene and Kochhar, 1975). Due possibly to decreased fetal movement from sedation with barbiturates and tranquilizers, Walker and Patterson (1974) claimed a significant increase in the incidence of clefting. Since ethanol has been related to growth inhibition and to decreased motor activity, it would seem appropriate to determine if ethanol would cause any significant increase in the incidence of cleft palate.

STATEMENT OF THESIS

It will be the purpose of this investigation to analyze the craniofacial dimensions of forty-two-day-old A/J mice that were exposed to ethanol during gestation via maternal ethanol inhalation. Specifically, it will be determined whether there was any change in size, shape, or position of the nasomaxillary complex in the experimental animals. The effect of ethanol on the incidence of clefting will also be determined.

METHODS AND MATERIALS

Sample

One hundred sixty-nine A/J mice from thirty-one litters were employed to discern any craniofacial dimensional effects from exposure to ethanol. It was possible to obtain 363 fetuses from forty-six litters for determination of clefting. The breeding stock was purchased from Jackson Laboratories, Bar Harbor, Maine.

Pregnant mice were obtained by housing two to three females with one male in a 20 x 25 x 46 cm. metal box with a twelve-hour light and dark cycle. Daily observations for vaginal plugs were made within two hours after the dark cycle. The presence of a plug was taken as the midpoint of the first twenty-four hours of gestation (Kalter, 1968b). At that time, the female was placed in a separate 12 x 12 x 28 cm. plastic cage, identified according to the estimated time of possible conception, and provided with water and Purina rat-mouse chow ad libitum.

Experimental Design

A total of thirty-one litters were used in this investigation to analyze craniofacial growth and were divided into the following three groups:

1. Twelve litters (sixty-nine mice) were exposed to ethanol via maternal ethanol inhalation from the eleventh through the fifteenth day of gestation.

2. Nine litters (forty-eight mice) were exposed to ethanol via maternal ethanol inhalation from the fourteenth through the eighteenth day of gestation.
3. Ten litters (fifty-two mice) served as controls. Their mothers were not placed in the chamber and consequently inhaled no ethanol vapor.

Twenty-one litters (164 fetuses) were used to determine the incidence of clefting after exposure to ethanol vapor from 11-14 days of gestation. Twenty-five litters (199 fetuses) served as controls. Palates for the cleft investigation were examined at eighteen days of gestation after terminating the pregnancy with the removal of the uterus. This was done to avoid the problem of maternal cannibalism after a normal term delivery.

Ethanol Exposure Times

The exposure times (11-15 days and 14-18 days) were chosen to determine any differences in effects from exposure at different times in fetal development. The palatal shelves are actively growing during the former period, and the nasal septum is active throughout both periods (Rugh, 1968; Walker, 1969; Latham, 1969b, 1970). During their time in the chamber, mice were only removed for thirty minutes each day for weighing. All dams were provided with water and Purina rat-mouse chow ad libitum before, during, and after exposure to ethanol.

Care of Litters for Growth Analysis

For the growth analysis, all newborn were left in isolated cages with their mothers until forty-two days postnatal. In this way, a natural weaning could occur at approximately twenty-one days with minimal disturbance to both mother and offspring. The litters were handled as little as possible to minimize maternal cannibalism which occurred in twelve litters, mostly during the first five postnatal days. However, all cages were changed every two weeks to avoid unsanitary conditions (Green, 1966). After forty-two days, the mice were labeled, weighed, killed with an overdose of ether, photographed and radiographed.

Exposure to Ethanol

Inhalation Chamber

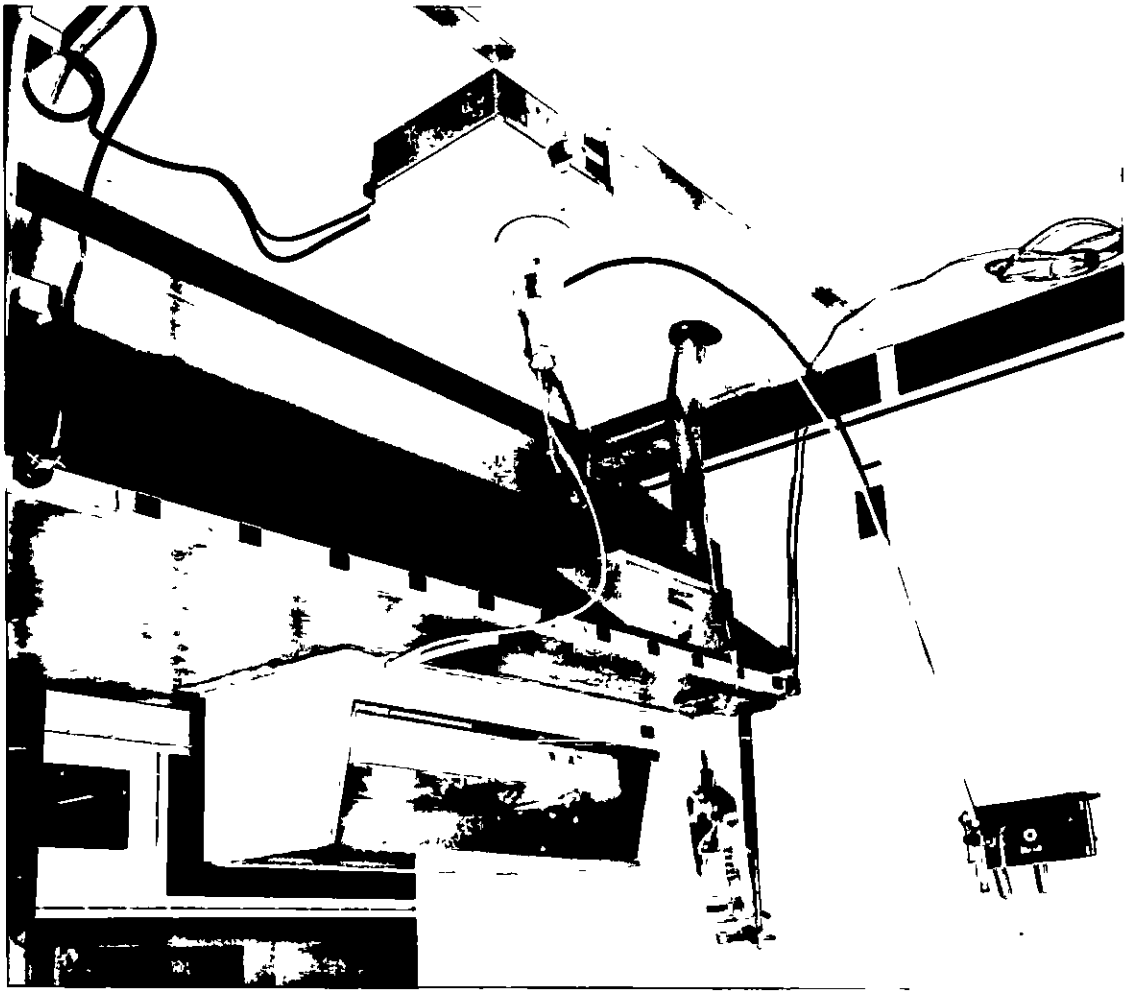
In order to obtain a constant ethanol exposure that would result in blood levels established as biologically significant, an inhalation chamber was devised. The choice of an inhalation approach was predicated upon dissatisfaction with the constancy of blood-ethanol levels that could be obtained with injections and voluntary or forced liquid consumption. Mice, due to their high metabolic rate, eliminate ethanol so rapidly that it becomes difficult to maintain constant high levels with injections (Goldstein and Pal, 1971). Moreover, due to an initial reluctance to drink ethanol in water, and since they drink only limited daily amounts, mice do not consume enough ethanol in the drinking water to achieve significant ethanol blood levels (Newell et al., 1964; Goldstein and Pal, 1971).

The inhalation chamber utilized in this study was approximately 90 liters in volume, with a transparent plastic top and front. A constant 5 l./min. flow of compressed air was regulated by a flowmeter (Burdox, Inc., Gateway Division, Cleveland, Ohio). The air was delivered into a 1000 ml. Erlenmeyer flask, in which mixture with vaporizing drops of ethanol occurred. Two hundred proof ethanol (Rossville Gold Shield Alcohol, Commercial Solvents Corporation, Terre Haute, Indiana) was dripped into the same Erlenmeyer flask by gravity using a 1000 ml. intraveneous bottle and tubing (Baxter Laboratories, Division of Travenol Laboratories, Inc., Morton Grove, Illinois) to achieve a constant drop rate from a 0.2 ml. pipette which formed drops measured to be 0.02 ml. To avoid deaths, the drop rate was monitored daily and regulated to an average of four drops per minute (range: 3-5 drops per minute). To obtain immediate vaporization and avoid accumulation of ethanol in the bottom of the flask, the Erlenmeyer flask was heated to a constant temperature of 56.5°C with a hot plate (Thermolyne Corporation, Dubuque, Iowa). The mixture of air and ethanol was then fed into the chamber at the same airflow rate of 5 l./min. An exit hose was provided at a corner of the chamber opposite from the entrance of the ethanol-air mixture. Up to twenty mice at one time could be accomodated in the chamber with Purina rat-mouse chow and water provided ad libitum.

The airflow rate of 5 l./min. was determined adequate for the respiratory needs of the mice, since Goldstein and Pal (1971) reported flow rates of 2-3 l./min. being more than adequate for twenty mice.

PLATE I

INHALATION CHAMBER



In order to prove, further, adequacy of the ventilation, twenty test mice were placed in the chamber for five days with the same 5 l./min. airflow, but no ethanol, and exhibited no adverse effects from being in the chamber.

The amount of food and water consumed and the changes in weight differed only slightly between control and chamber-enclosed mice. This appears to confirm the observations of Griffiths, Littleton and Ortiz (1973) that mice inhaling ethanol ate and drank only slightly less than controls, and showed little change in weight as compared to controls.

Determination of Blood-Ethanol Levels

Collection of Blood Samples

Blood levels were established for different ethanol drop rates by the use of twenty-four test mice. After a period of exposure in the chamber, a test animal was immediately decapitated and bled into a wax-lined 50 ml. plastic tube containing sodium oxalate, an anti-coagulant. About 1 to 1.5 cc. of blood was accumulated in this manner (a mouse's total blood volume is approximately 2 cc.). Once mixed with the anti-coagulant, the blood was quickly transferred to a 10 x 75 mm. culture tube and sealed with a paraffin sheet (Parafilm "M", American Can Company, Neenah, Wisconsin). Each such sample was stored in a refrigerator until a group was collected for ethanol analysis. No samples were allowed to be stored for more than one week before testing.

Gas Chromatographic Analysis

Blood samples were centrifuged to separate red blood cells, and the plasma was injected into a gas chromatograph (7620A Hewlett-Packard Research Chromatograph, Hewlett-Packard, Loveland, Colorado). The column temperature was 200°C, and gas flow rates were as follows:

carrier nitrogen--50 ml./min.

hydrogen--40 ml./min.

air--300 ml./min.

A read-out was given on a pen recorder, but calculations of ethanol content were based on arbitrary units of area given from an integrator (3373B, Hewlett-Packard, Loveland, Colorado). Sample readings were all related to either a .16 gm.% or .32 gm.% standard ethanol reading.

Relating Drop Rate to Ethanol Blood Levels

Blood-ethanol levels of twenty-four representative test mice were found to be related to the chamber drop rate in the following manner:

6 drops/min.--.47 gm.%--death (100%)

5 drops/min.--.33±.04 gm.%--unconsciousness and some death (10%)

4 drops/min.--.12±.03 gm.%--decreased motor activity

3 drops/min.--.06±.01 gm.%--decreased coordination

2 drops/min.--.03±.01 gm.%--minimum exposure level

The drop rate of the chamber was monitored daily and, when necessary, adjusted to minimize deaths. An attempt was made to achieve the

maximum blood-ethanol levels consistent with survival over the five-day period in the chamber.

For approximately 75% of their time in the chamber, the animals were exposed to a rate of four drops per minute. As a result, it was determined that the surviving mice experienced (during approximately 75% of their stay in the chamber) a blood-ethanol level of .13 gm.%, as calculated from the best-fit exponential equation, that was derived using the blood-ethanol levels and the corresponding drop rates previously noted. The equation generated, which best describes the relationship between the blood-ethanol levels and the drop rates ($r = .992$), is as follows:

$$y = .008e^{.689x}$$

from the general form, $y = ae^{bx}$, where

x = the dropping rate (drops/min.)

y = blood-ethanol level (gm.%)

Solving the equation for the four drop per minute rate yielded the .13 gm.% level seen in the surviving experimental mice. This equation was generated to permit extrapolation to drop values not measured experimentally (i.e. 3.5, 4.5, etc.), and to validate the experimentally obtained values. As noted in Table II, the experimentally derived value of a four drop per minute rate, using seven test animals, was .12 gm.%±.03 gm.%. The equation generated from an analysis of animals placed in a chamber with a dropping rate of two, three, four, five and six drops per minute would seem to be quite accurate, inasmuch as the mathematically obtained value, .13 gm.%, is very close to the experimentally obtained value.

TABLE II

CHAMBER CONCENTRATIONS AND RESULTANT BLOOD-ETHANOL LEVELS

Drops/Min.	Calculated Estimate of Chamber Concentration at Equilibrium (mg./l.)	Time to Equilibrium (min.)	Mouse Blood-Ethanol Levels (gm.%)	Number of Determinations (N)	Physical Condition
3	9.48	150	.06±.01	8	Decreased Coordination
4	12.64	150	.12±.03	7	Decreased Motor Activity
5	15.80	150	.33±.04	4	Unconsciousness and 10% Death
6	18.95	150	.472	1	100% Death

TABLE III

THE AVERAGE EFFECTS OF ETHANOL IN HUMANS

Bottles of Beer or Cocktails	Physical Condition	Blood-Ethanol Level (gm.%)
1-2	Flushing of skin Inhibitions begin to recede Heart speeds up Gaiety	.04
3-4	Judgement is slower Giddiness Coordination decreased	.06
5-6	Vision blurred Speech slightly impaired Reaction time increased	.10*
6-8	Staggering Diplopia Loss of Balance	.16
15-20	Skin is clammy Dilated pupils Unconsciousness	.40
20-25	Death	.50

*Legal intoxication in many states

After Burgess, 1973, p. 41

However, it must be remembered that blood-ethanol levels may flucuate in the same animal and among animals due to variability in respiration and a changing capacity of the liver to metabolize ethanol after increasing time spent in the chamber. Regardless, the surviving animals of this investigation experienced a constant presence of ethanol in their blood ranging from .06 gm.% to .25 gm.%, with a median level of .13 gm.%. It is interesting (and very supportive) to note that the experimentally determined blood levels and the value generated by the equation were associated with biological manifestations of ethanol intoxication that agree very closely to corresponding data reported for the human (Table III).

Relating Drop Rate to Concentration of Chamber

For this investigation, one drop of ethanol was found to have a volume of .02 ml. and, as calculated from its density, a mass of 15.8 mg. The concentration of the vapor chamber at any time approaching and once at equilibrium can be determined by using the following formula (Golumb and Shanks, 1965):

$$S_{(t)} = \frac{VX}{A} - S_c e^{-t/V/A}$$

or

$$C_{(t)} = \frac{X}{A}\left(1 - \frac{1}{e^{t/V/A}}\right)$$

where $S_{(t)}$ = the amount of ethanol in the chamber at a given time (mg.),

V = the volume of the chamber (92.31 liters),

X = the amount of ethanol vaporized per minute (mg./min.),

S_c = the amount of ethanol in the chamber at equilibrium (mg.),

A = airflow (l./min.),

t = a given time,

$C = \frac{S(t)}{V}$, concentration of ethanol in the chamber (mg./l.).

Once equilibrium has been reached, the formula becomes:

$$C = \frac{X}{A}$$

The time to reach equilibrium for any constant drop rate was calculated to be approximately 150 minutes. The relationship between drop rates, chamber concentration, time to equilibrium, and mouse blood-ethanol levels is shown in Table II.

Data Collection

Forty-two days after birth, the mice were labeled and then weighed to the nearest tenth of a gram on an analytical balance (P5N, Mettler Instrument Corporation, Hightstown, New Jersey). Next, the mice were killed with an overdose of ether, and then photographed and radiographed.

Cephalometric Technique

Cephalograms

A specially constructed cephalostat, similar to one used by Spence (1940), and modified after Gange (1973), Fuller (1974) and Bernabei (1975), was used to immobilize the animal (Plates II and III).

The radiographs were taken on standard dental occlusal film (Kodak DF-46): one at 0.8 seconds at 10 milliamperes and 65 kilovolts, and the second at 0.2 seconds at 15 milliamperes and 90 kilovolts. The central ray was perpendicular to the film and passed through the trans-porionic axis, and the source-to-film distance was held constant at 50 cm. All films were developed in an automatic processor to insure consistency of technique.

Cephalometric Landmarks

With the aid of a dissecting microscope and magnifying glass, the following points were punched directly on the radiograph (Figure 1):

1. the tip of the nasal bone (N),
2. the most superior portion of the frontonasal suture (Na),
3. the most inferior (ventral) point of the jugum limitans fossa olfactoria (JL)
4. the intersection of the lingual periodontal ligament of the maxillary incisor and the plane of the bony palate (I),
5. the intersection of the maxillary bone and the anterior

PLATE II

CEPHALOSTAT WITH EAR ROD IN POSITION

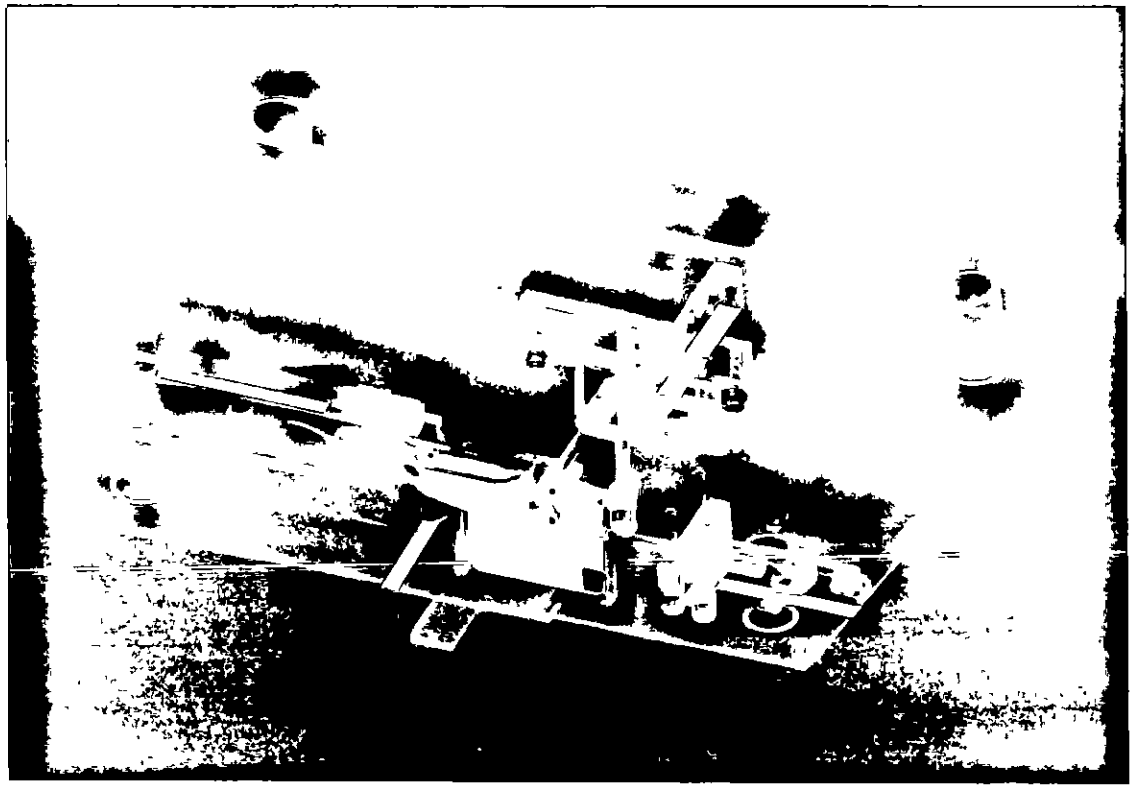


PLATE III

CEPHALOSTAT WITH EAR ROD SWUNG
OUT OF POSITION
(to permit radiographic
exposure)

surface of the maxillary first molar (Mx),

6. the intersection of the mandibular alveolar process and the anterior surface of the lower first molar (Mn),
7. the most superior (dorsal) point of the intersphenoidal synchondrosis (ISS),
8. the most dorsal (superior), posterior point on the curvature of what would represent the average position of the left and right condyles (C),
9. the most superior (dorsal) point of the sphenoccipital synchondrosis (SOS),
10. opisthion, the most dorsal (superior) point of the foramen magnum located in the mid-sagittal plane on the occipital bone (O),
11. the most superior (dorsal) point on the external surface of the parietal bone (P), and
12. the most anterior point on the margin of the foramen magnum (Ba).

Analysis

Each cephalogram was enlarged five times with a photographic enlarger and printed on photographic paper (Kodak Ecktomatic SC-F, Eastman Kodak Company, Rochester, New York).

Mean tracings of the photographs were produced to illustrate any possible differences among control and ethanol-treated animals employing the method used to obtain the Bolton Standards (Broadbent et al., 1975). Cephalometric tracings of negative prints of the enlarged cephalograms for each group were paired and averaged until

FIGURE 1

CEPHALOMETRIC LANDMARKS

CEPHALOMETRIC LANDMARKS: (N) the tip of the nasal bone, (Na) the most superior portion of the frontonasal suture, (JL) the most inferior (ventral) point of the jugum limitans fossa olfactoria, (I) the intersection of the lingual periodontal ligament of the maxillary incisor and the plane of the bony palate, (Mx) the intersection of the maxillary bone and the anterior surface of the maxillary first molar, (Mn) the intersection of the mandibular alveolar process and the anterior surface of the lower first molar, (ISS) the most superior (dorsal) point of the intersphenoidal synchondrosis, (C) the most dorsal (superior), posterior point on the curvature of what would represent the average position of the left and right condyles, (SOS) the most superior (dorsal) point of the sphenoccipital synchondrosis, (O) opisthion, the most dorsal (superior) point of the foramen magnum located in the midsagittal plane on the occipital bone, (P) the most superior (dorsal) point on the external surface of the parietal bone, (Ba) the most anterior point on the margin of the foramen magnum.

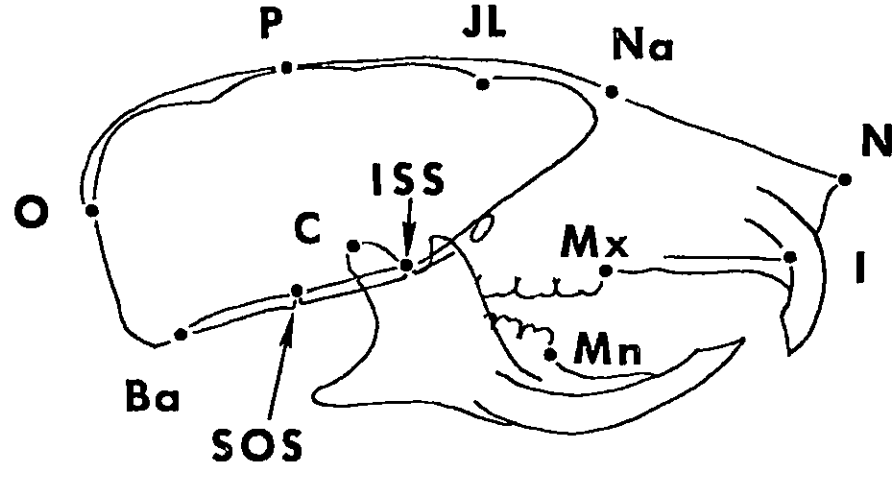
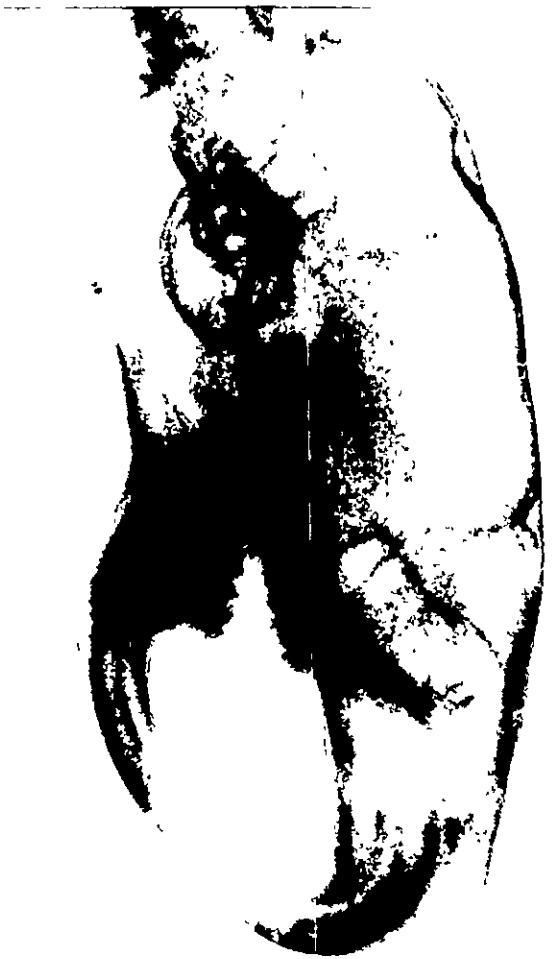

P
JL
Na
N
O
ISS
C
Mx
I
Mn
Ba
SOS

PLATE IV

PRINT OF CEPHALOGRAM

PLATE V

NEGATIVE PRINT OF CEPHALOGRAM
(at 5X, used for taking
measurements)



a group representation was achieved.

Twelve direct linear measurements were performed on the photographs to characterize any dimensional differences among treatment groups. The measures used are listed in Table IV.

Measurements were taken to the nearest .05 millimeter with dial vernier calipers (Helios Calipers, DCA Corporation, Washington, D.C.), and calculations were performed on a programmable electronic calculator (Model 9100A, Hewlett-Packard, Loveland, Colorado).

Data Reduction

Treatment effects were examined by way of an analysis of covariance where the weight of each animal at the time of killing served as the covariate. Inasmuch as an analysis of variance for the weights of the various treatment groups was non-significant ($p>.05$), it is assumed that the weights were not influenced by the treatments.

A linear model was assumed and may be expressed:

$$Y_{ij} = \mu + a_i + \beta X_{ij} + \varepsilon_{ij} \text{ , where}$$

Y_{ij} = a measurement for the ith treatment in the jth mouse ($i = 1, 2, 3$ and $j = 1, 2, \ldots J_i$, where J_i is the number of mice receiving the ith treatment),

μ = the mean population,

a_i = the difference in the y-intercepts of the regression lines.

β = the slope of the common regression line

TABLE IV

CEPHALOMETRIC ANALYSIS

Anatomical Dimension--Direct Measurements	
Measure	Landmarks
A Snout Length	JL-N
B Neurocranium Length	JL-O
C Overall Length	N-O
D Premaxillary, Maxillary Length	I-Mx
E Posterior Height of Snout	Mx-Na
F Posterior Height of Neurocranium	SOS-P
G Basisphenoid Length	SOS-ISS
H Basioccipital Length	SOS-Ba
I Mandibular Length	Mn-C
J Maxillary Advancement	Mx-SOS
K Premaxillary, Maxillary Advancement	I-SOS
L Premaxillary, Maxillary Advancement	I-JL

FIGURE 2

CEPHALOMETRIC MEASURES

Direct Measurements

(A) Snout Length (JL-N)

(B) Neurocranium Length (JL-O)

(C) Overall Length (N-O)

(D) Premaxillary, Maxillary Length (I-Mx)

(E) Posterior Height of Snout (Mx-Na)

(F) Posterior Height of Neurocranium (SOS-P)

(G) Basisphenoid Length (SOS-ISS)

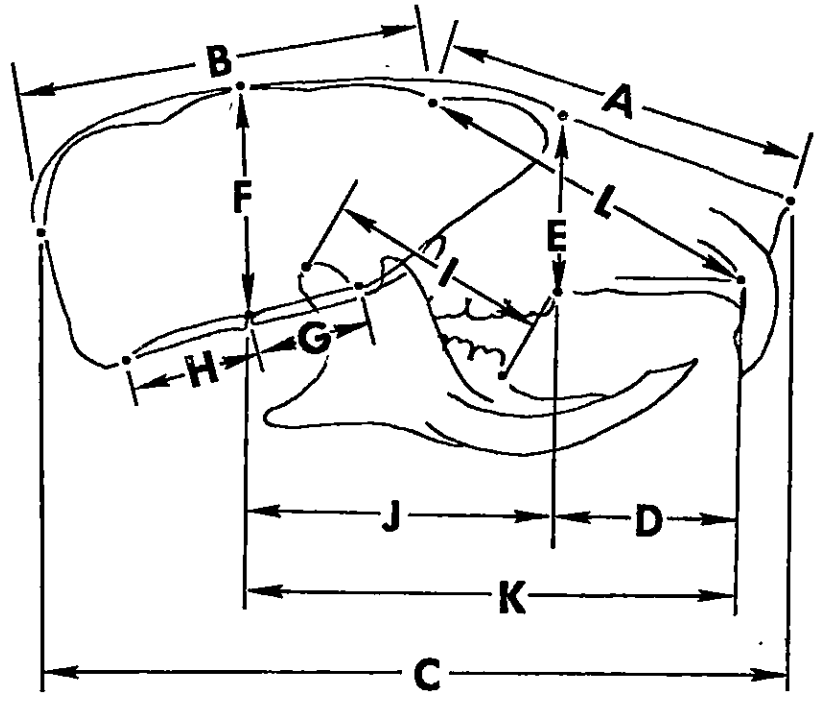
(H) Basioccipital Length (SOS-Ba)

(I) Mandibular Length (Mn-C)

(J) Maxillary Advancement (Mx-SOS)

(K) Premaxillary, Maxillary Advancement (I-SOS)

(L) Premaxillary, Maxillary Advancement (I-JL)


B
A
F
L
E
I
G
H
J
D
K
C

in the population,

X_{ij} = the deviation of any weight, associated with the ith treatment in the jth mouse, from the total mean,

ε_{ij} = the experimental error associated with the ith treatment in the jth mouse.

Error Study

To determine the reliability of the measurements, twenty animals were chosen at random, and measurements were retaken from a separate set of photographs derived from an independent set of cephalograms. A coefficient of linear correlation was calculated between the first and second determination of each dimension. The square of this coefficient, r_{xx}^2, constitutes a measure of reliability associated with each dimension, and is an estimate of ". . . the proportion of their variance that is true variance" (Guilford, 1965; Gange and Johnston, 1974).

Procedure for Examination of 18-Day Fetuses

After eighteen days of pregnancy, female mice designated for palatal investigation were killed by cervical dislocation. A hysterectomy was performed, and fetuses counted and labeled. Under a dissecting microscope, palates were examined and designated as being normal or cleft.

TABLE V

SUMMARY TABLE FOR ANALYSIS OF COVARIANCE

Source	Degrees of Freedom (df)	Sum of Squares (SSQX) (ΣX^2)	Sum of Cross Products (SCP) (ΣXY)	Sum of Squares (SSQY) (ΣY^2)	Degrees of Freedom (df)	Sum of Squares (SSQY′)	Mean Squares (MSQY′)[a]
Treatment (Tr)	2	$SSQX_{Tr}$	SCP_{Tr}	$SSQY_{Tr}$	2	$SSQY'_{T} - SSQY'_{er} = SSQY'_{Tr}$	$\frac{SSQY'_{Tr}}{2}$
Error (er)	166	$SSQX_{T} - SSQX_{Tr} = SSQX_{er}$	$SCP_{T} - SCP_{Tr} = SCP_{er}$	$SSQY_{T} - SSQY_{Tr} = SSQY_{er}$	165	$SSQY_{er} - \frac{(SCP_{er})^2}{SSQX_{er}} = SSQY'_{er}$	$\frac{SSQY'_{er}}{165}$
Total (T)	168	$SSQX_{T}$	SCP_{T}	$SSQY_{T}$	167	$SSQY_{T} - \frac{(SCP_{T})^2}{SSQX_{T}} = SSQY'_{T}$	

[a] $F = MSQY'_{Tr} / MSQY'_{er}$; $F_{(2,165).95} = 3.07$; $F_{(2,165).99} = 4.79$

TABLE V--Continued

$$SSQX_T = \Sigma\Sigma X_{ij}^2 - \frac{T_{X_{++}}^2}{N} \qquad SSQX_{Tr} = \frac{\Sigma T_{X_{i+}}^2}{n_i} - \frac{T_{X_{++}}^2}{N}$$

$$SCP_T = \Sigma\Sigma X_{ij}\, Y_{ij} - \frac{(T_{X_{++}})(T_{Y_{++}})}{N} \qquad SCP_{Tr} = \frac{\Sigma T_{X_{i+}} T_{Y_{i+}}}{n_i} - \frac{(T_{X_{++}})(T_{Y_{++}})}{N}$$

$$SSQY_T = \Sigma\Sigma Y_{ij}^2 - \frac{T_{Y_{++}}^2}{N} \qquad SSQY_{Tr} = \frac{\Sigma T_{Y_{i+}}^2}{n_i} - \frac{T_{Y_{++}}^2}{N}$$

TABLE VI

RELIABILITY COEFFICIENTS

Measure	r	r^2
A	.997	.994
B	.982	.964
C	.989	.978
D	.964	.929
E	.989	.978
F	.953	.908
G	.941	.885
H	.943	.889
I	.957	.916
J	.993	.986
K	.980	.960
L	.993	.986

PLATE VI

DISPLAY OF UTERUS FOLLOWING HYSTERECTOMY



RESULTS

The results of the present study will be presented under the following headings: (1) General Appearance, Physical Condition, and Body Weight, (2) Cephalometric Measurements, and (3) Incidence of Clefting.

General Appearance, Physical Condition And Body Weight

Examination of experimental animals revealed no gross deformities in the shape of the head and snout. The incisors were in proper occlusion, and coat, eyes, and color appeared normal.

The mean body weights of the three groups are shown in Table VII. An analysis of variance revealed no significant among-treatment differences ($p>.05$).

Cephalometric Measurements

Descriptive Statistics

Means and standard deviations for the twelve direct craniofacial dimensions are presented in Table IX. For all measurements, except H, the control dimensions were larger than the two groups exposed to ethanol. Mean tracings of fifty-two control, sixty-nine experimental (11-15 day exposure), and forty-eight experimental (14-18 day exposure) animals are presented in Figure 3.

TABLE VII

BODY WEIGHT OF 42-DAY POSTNATAL MICE

	Control	11-15 day ethanol exposure	14-18 day ethanol exposure
$\bar{X}$ (gms.)	15.03	14.82	14.26
S.D.	2.42	3.80	4.35

TABLE VIII

ANALYSIS OF VARIANCE: BODY WEIGHT OF 42-DAY POSTNATAL MICE

Source	df	SSQ	MSQ
Treatment	2	16.03	8.02
Error	166	2170.25	13.07
Total	168	2186.29	

$F_{(2,166)} = 0.61$; $p > .05$

$F_{(2,166)}$ .95 = 3.07

FIGURE 3

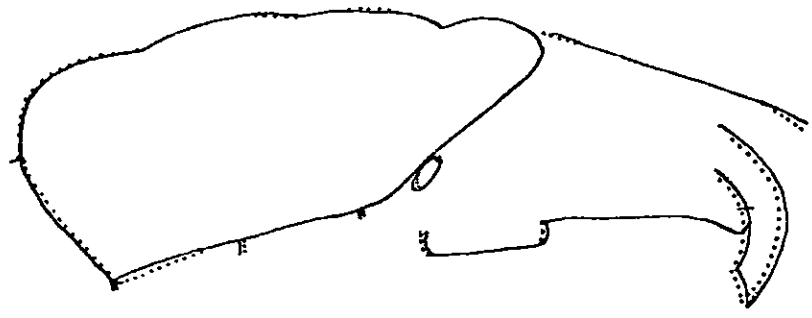
MEAN TRACINGS (5X)

A. Average of 52 controls and 69 experimental (11-15 day exposure to ethanol)

B. Average of 52 controls (same as above) and 48 experimental (14-18 day exposure to ethanol)

Control ———— ; Experimental ·····

A.



B.

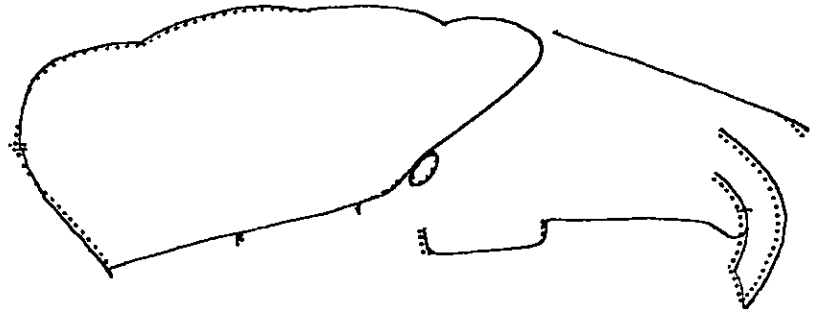


TABLE IX

MEANS AND STANDARD DEVIATIONS
(in millimeters X 5)

Measure	Animal Group		
	Control	11-15 day ethanol exposure	14-18 day ethanol exposure
A	49.76 (2.04)*	49.21 (3.07)	49.00 (3.68)
B	57.40 (1.49)	56.98 (2.17)	56.91 (2.93)
C	103.01 (3.35)	101.97 (5.11)	101.59 (6.50)
D	26.27 (.96)	25.79 (1.61)	25.55 (2.07)
E	24.25 (.70)	23.98 (1.10)	24.03 (1.34)
F	29.77 (.71)	29.52 (.98)	29.43 (1.24)
G	15.93 (1.17)	15.78 (1.60)	15.78 (1.36)
H	17.63 (.88)	17.74 (1.25)	17.35 (1.73)
I	33.66 (1.82)	33.52 (2.18)	33.65 (2.33)
J	40.23 (1.49)	39.34 (2.34)	39.40 (2.69)
K	66.71 (2.46)	65.04 (3.91)	64.97 (4.60)
L	46.48 (1.72)	45.52 (2.65)	45.27 (3.19)

*Numbers in parentheses are standard deviations.

Inferential Statistics

The results of the analysis of covariance are presented in Table X. Measurements C, D, J, K, and L showed differences that were statistically significant ($p<.05$). The significantly shorter measurements in the ethanol groups showed that the maxillary incisors and maxillary molars were positioned more posteriorly in both ethanol groups than in the control group (measures J, K, and L). It is also evident that the distance between the maxillary incisors and the maxillary molars was decreased in the ethanol groups as compared to the controls (measure D).

The length of the cranium (measure B), the length of the cranial base (measures G and H), the length of the upper snout (measure A), and the length of the mandible (measure I) could not be shown to be significantly different ($p>.05$). Non-significant among-treatment differences were also shown for the vertical height of the posterior snout (measure E) and the vertical height of the cranium (measure F).

The means of the significant measures were adjusted for body weight according to the following formula (Snedecor, 1966):

$$\bar{Y}_A = \bar{Y}_M - b(\bar{X}_M - \bar{X}_T), \text{ where}$$

$\bar{Y}_A$ = the adjusted mean value,

$\bar{Y}_M$ = the mean measurement before adjustment,

b = the regression coefficient,

$\bar{X}_M$ = the mean body weight,

$\bar{X}_T$ = the total mean of the body weights.

TABLE X

STATISTICAL ANALYSIS: CEPHALOMETRIC MEASUREMENTS

Measure	Analysis of Variance				Analysis of Covariance			
	Sum of Squares (Y)			$F_{(2,166)}$	Sum of Squares′ (Y′)			$F'_{(2,165)}$
	Treatment	Error	Total		Treatment′	Error′	Total′	
A	15.76	1490.81	1506.57	0.88	4.73	156.83	161.56	2.49
B	7.38	837.01	844.39	0.73	3.20	136.72	139.92	1.93
C	55.47	4336.65	4392.12	1.06	17.69	476.09	493.79	3.07*
D	13.33	424.75	438.08	2.61	5.59	73.94	79.53	6.24**
E	2.38	191.47	193.84	0.21	1.80	52.68	54.48	2.82
F	3.18	162.23	165.41	1.63	1.25	39.01	40.25	2.64

TABLE X--Continued

Measure	Analysis of Variance				Analysis of Covariance			
	Sum of Squares (Y)			$F_{(2,166)}$	Sum of Squares′ (Y′)			$F'_{(2,165)}$
	Treatment	Error	Total		Treatment′	Error′	Total′	
G	0.79	286.31	287.10	0.23	0.46	218.87	219.32	0.17
H	4.31	287.13	291.45	1.25	1.86	154.17	156.02	0.99
I	0.70	746.64	747.34	0.08	3.51	362.82	366.98	0.80
J	26.97	827.12	854.09	2.71	17.65	112.46	130.11	12.95**
K	103.96	2345.57	2449.53	3.68*	55.70	216.13	270.65	21.26**
L	41.95	1107.15	1149.10	3.15*	21.29	104.75	126.05	16.77**

*Significant at 95% level, $F_{(2,165)}$ .95 = 3.07, $F_{(2,166)}$ .95 = 3.07

**Significant at 99% level, $F_{(2,165)}$ .99 = 4.79

The adjusted values are shown in Table XI.

Incidence of Clefting

Of 199 control fetuses, twenty-four, or 12.06%, had clefting. For the group exposed to ethanol from the eleventh to the fourteenth day of gestation, twenty-one out of 164, or 12.81%, had cleft palates (Table XII). A spontaneous rate of from 6% to 16.7% is reported by Kalter (1968, 1969) for the A/J mouse. As judged by a zI test "for comparing two samples of isolated occurrences with each other" (Langley, 1970), the observed incidence of clefting in the ethanol group does not differ significantly from that of the control group ($p>.05$).

TABLE XI

ADJUSTED MEANS
(in millimeters X 5)

Measure	Animal Group		
	Control	11-15 day ethanol exposure	14-18 day ethanol exposure
C	102.60 (103.01)*	101.83 (101.97)	102.22 (101.59)
D	26.14 (26.14)	25.75 (25.79)	25.74 (25.74)
J	40.05 (40.23)	39.28 (39.34)	39.67 (39.40)
K	66.41 (66.71)	64.94 (65.04)	65.44 (64.97)
L	46.27 (46.48)	45.45 (45.52)	45.59 (45.27)

*Numbers in parentheses are means before adjustment to different body sizes.

TABLE XII

CLEFT INCIDENCE

Group	Number of Normal Fetuses	Number of Fetuses with Cleft	Total Number of Fetuses	Percentage of Fetuses with Cleft
Control	175	24	199	12.060
Ethanol	143	21	164	12.805

$Z = -.05$

$p > .05$

PLATE VII

NON-CLEFT--DORSAL VIEW

PLATE VIII

NON-CLEFT--PALATAL VIEW



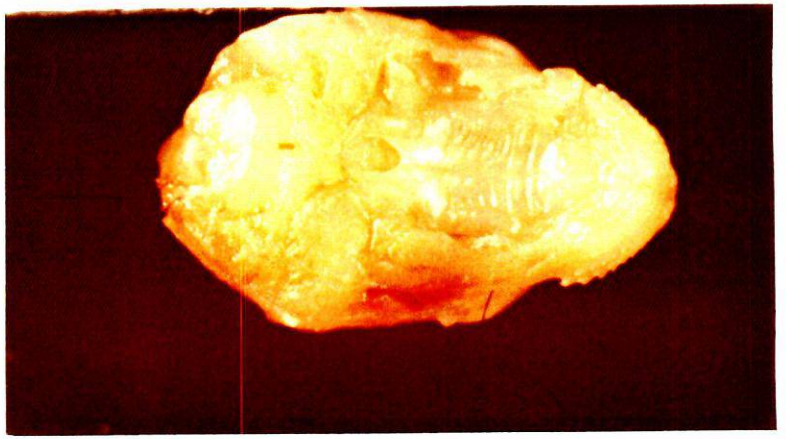
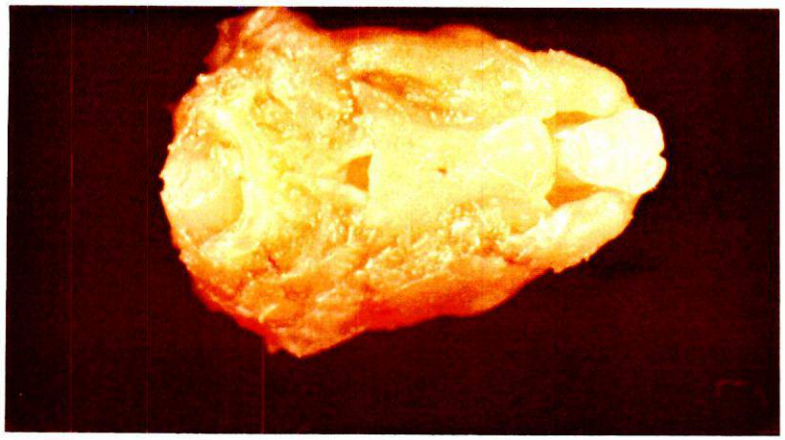


PLATE IX

ISOLATED CLEFT OF THE SOFT PALATE

PLATE X

MIDLINE CLEFT OF THE SOFT PALATE
AND POSTERIOR HARD PALATE



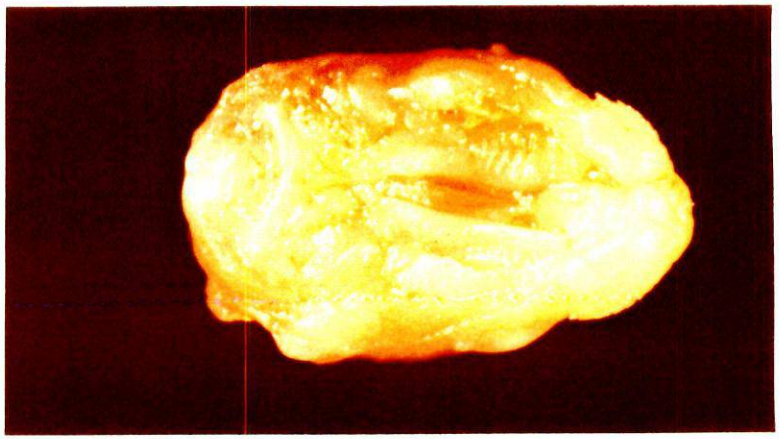


PLATE XI

UNILATERAL CLEFT LIP AND CLEFT PALATE

PLATE XII

BILATERAL CLEFT LIP AND CLEFT PALATE
(with tongue between
palatal shelves)

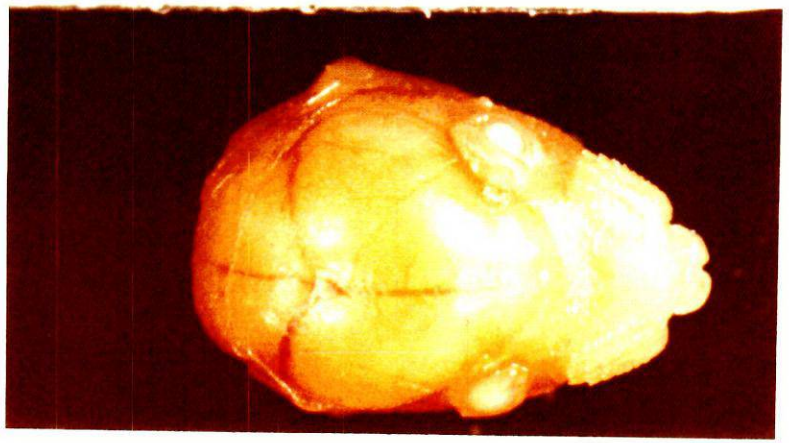


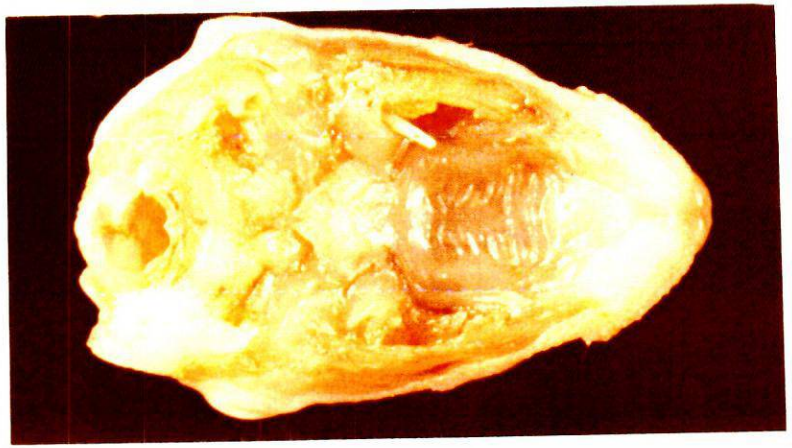


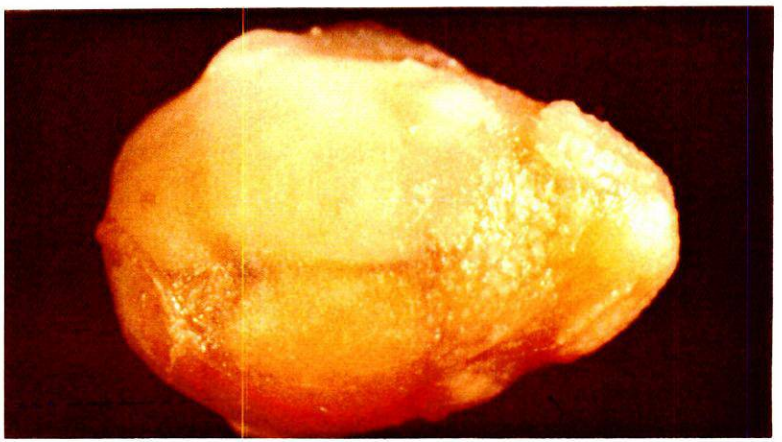
PLATE XIII

BILATERAL CLEFT LIP AND CLEFT PALATE
(with tongue removed)

PLATE XIV

BILATERAL CLEFT LIP AND CLEFT
PALATE--DORSAL VIEW





DISCUSSION

The purpose of this investigation was to determine the effect of ethanol on facial growth and on the incidence of clefting. To determine the significance of treatment effects among control and experimental groups, an analysis of covariance was used with body weight as the covariate.

A high correlation between body weight and the different measures of facial size indicated that a large portion of the variability of each measurement may have been a reflection of differences in body size (Table XIII). An analysis of variance, however, showed no significant differences among the body weights of the control and ethanol-treated groups ($p>.05$); therefore, it was concluded that body weight was not affected by the treatments and could be used as a covariate to eliminate variability due to differences in body size.

In order to test the appropriateness of the assumptions underlying the analysis of covariance (equality of variances and regression), Bartlett's test for homogeneity of variance and a test of the hypothesis, $\beta_1 = \beta_2 = \beta_3$, were performed. The null hypothesis of unequal variances was rejected ($p>.05$) using the following chi-square test (Snedecor, 1966, p. 287):

$$\chi^2_{k-1} = 2.3026[(\log \bar{s}^2)\Sigma(n-1) - \Sigma(n-1)(\log s^2)]$$

where: $\bar{s}^2 = \frac{\Sigma x^2}{(n-1)}$, $\Sigma x^2 = s^2(n-1)$,

s^2 = the mean square,

n-1 = the degrees of freedom.

The test of the hypothesis of equal regressions was made by the following F test (Snedecor, 1966, p. 378):

$$F_{\nu_1,\nu_2} = \frac{\text{Mean Square for Regression Coefficients}}{\text{Mean Square Within Samples}}$$

The sample regression coefficients were not found to differ significantly ($p>.05$).

Comparison of Blood-Ethanol Concentrations in Mice and Humans

Significant levels of blood-ethanol seem to result in biological responses in mice which are similar to those observed in humans at equivalent concentrations. As shown in Table III, a human blood-ethanol concentration of .10 gm.% affects motor activity to a degree comparable to a .12 gm.% concentration in the mouse. A concentration of .40 gm.% causes unconsciousness in both mice and humans, and a .50 gm.% level results in death for both species.

The pregnant mice of this investigation experienced a blood-ethanol level of .13 gm.% for five days, or one-fourth of the gestation period. To achieve comparable levels, a human would need to consume from five to seven bottles of beer or its equilavent within two to three hours (Burgess, 1973). In order to maintain such levels for a comparable period of time, the same drinking rate would have to continue for nine weeks during pregnancy. The equivalent periods of fetal development in the human would be from

TABLE XIII

CORRELATION BETWEEN BODY WEIGHT AND MEASURES

Measure	Treatment		
	Control	11-15 day Ethanol Exposure	14-18 day Ethanol Exposure
A	.856	.954	.968
B	.846	.897	.953
C	.891	.948	.958
D	.699	.929	.952
E	.734	.818	.924
F	.789	.884	.891
G	.603	.587	.504
H	.574	.732	.672
I	.561	.731	.787
J	.866	.942	.937
K	.896	.952	.972
L	.867	.960	.970

1 to 3.25 months and from 1.5 to 3.75 months *in utero*. This interval of time would include the period of development when the palatal shelves and nasal septum are actively growing (Atherton, 1967; Rugh, 1968; Latham, 1969b).

The Incidence of Clefting

The exposure to ethanol, although it resulted in an average blood level of ethanol that has been reported to be associated with significant biological manifestations, did not increase the incidence of cleft palate in the A/J mouse. In support of the validity of this observation, it should be noted that there have been no previous reports in the literature that have linked ethanol to an increase in the incidence of clefting.

Walker and Patterson (1974), however, have reported that barbiturates and tranquilizers cause a slight but significant ($p<.05$) increase in the incidence of cleft palate in three strains of mouse. Based on the observation (Walker, 1969) that fetal movements were critical in closure of the secondary palate, it was their contention that agents capable of reducing movements of the fetus could have the potential of inducing cleft palate.

Since ethanol is reported to decrease motor activity (Burgess, 1973; Holtzman, 1974), it was suggested that ethanol might have the potential to decrease fetal movement and thus cause clefting. This was part of the rationale for testing ethanol in the present investigation. The findings, however, proved negative; this suggests that the proposed relationship between fetal movement

TABLE XIV

COMPARATIVE AGES OF MOUSE AND HUMAN EMBRYOS (in days)

Mouse	Human
0	1
1	2
2	3
4	4
5	5-6
5.5	7-8
6	9-10
6.5	11-13
7	14-17
7.5	18-20
8	20.5
8.33	21.0
8.5	22.0
8.66-8.75	23.0
8.80	24.33
9	25.5
9.5-9.66	26
10	27
10.5	28.5
11	30.25
11.5	33.5
12	36
12.33	36.5
13-13.5	38
14.5	47
15.5	65
16.5	84.5

After Rugh, 1968.

and incidence of clefting, as proposed by Walker and Patterson (1974), merits further evaluation.

Ethanol Effects

A significant reduction in measures C, D, J, K, and L signifies a smaller and more retruded midface. Such a reduction in midface advancement duplicates one of the findings of Jones et al. (1973) who described a "hypoplastic" maxilla as being characteristic of the children of chronic alcoholic mothers.

The results of the present investigation are, in several respects, similar to those obtained by Gange and Johnston (1974) as a result of electrolytic lesions placed in the region of the fibrous attachment of septum to premaxilla. Johnston (1976) presented a graphic representation of the mean craniofacial configuration for forty-three controls and forty-four experimental animals from an earlier paper by Gange (1973). This figure reveals a reduction in the advancement and length of the nasomaxillary complex and a ventral flexure of the anterior tip of the nasal bone.

The mean drawings of the present investigation (Figure 3) reveal not only a retruded and smaller nasomaxillary complex, but also a ventral flexure of the anterior tip of the nasal bone. This result can, perhaps, be explained by a consideration of the following factors and sequences of biological events. Long, Greulich, and Sarnat (1968) reported active proliferation in the cartilaginous nasal septum of growing rabbits, the anteroinferior and posterior areas being the most active. If this cellular proliferation of the

cartilage is retarded by a direct cytotoxic effect of ethanol, then the forward growth of the septum would be diminished. Retarded septal advancement could inhibit the forward bony depositional movement of the tip of the nasal bone via a fibrous connection between the nasal bone and the septum, as is described by Young (1960). This interplay of altered growth rates and fibrous tensions thus causes a ventral flexure of the tip of the nasal bone.

In septal ablation experiments (Sarnat and Wexler, 1966; Ohyama, 1969; Kvinnsland, 1974a), septal cartilage is removed and therefore presumably not available to contribute to the forward translation of the midface; no other areas of the cranium are affected. However, there have been questions raised as to the direct effect of surgery and resultant post-operative scarring accompanying these procedures. It should be noted that the effect of ethanol was similar to that of the surgical procedures, although of lesser magnitude; it occurred in the absence of scarring.

The studies of Rönning (1968) and Kvinnsland (1974b, 1974c) involved the parenteral administration of papain, an enzyme that has been shown to affect the growth of cartilage (Hulth, 1958; Spicer and Bryant, 1958). The enzyme reaches the cartilage via the bloodstream and then acts to split the chondroitin sulfate bonds of the cartilage matrix (Spicer and Bryant, 1958), and thus interferes with cartilage growth. However, its action is non-specific, and the growth of a variety of cartilages is affected.

By way of contrast, the results of the present investigation suggest that ethanol is acting preferentially on the nasal septal

cartilage. A preferential action of ethanol on the septum can, perhaps, be explained by the morphology of the nasal septum, itself: a thin, relatively avascular sheet of cartilage presenting a large surface area, surrounded in the nasal cavity by ethanol-containing amniotic fluid. Indeed, the cartilage is separated from the ethanol molecules by only one cell layer of pseudostratified ciliated columnar epithelium, a thin layer of submucosal glands, and a thin perichondrium (Tandler, 1976).

The permeability of the septal mucoperichondrium to the ethanol molecule (Waltman and Iniquez, 1972) would permit easy access to the cartilage and, subsequently, to the matrix of the cartilage, itself. Once within that matrix, the ethanol molecules could not be carried away readily by the bloodstream, due to the avascularity of the cartilage. Thus, the elevated concentration of ethanol molecules and the unique morphology of the nasal septal cartilage might provide the basis for a direct (and preferential) cytotoxic effect on septal chondrocytes above and beyond that which other craniofacial tissues would have to endure: ethanol would not have direct access to other "growth centers;" they would be only exposed to the somewhat lower level of ethanol seen in the circulating blood. However, the nature of the proposed direct cytotoxic effect requires some discussion.

Although it has been reported that liver mitochondrial function is adversely affected by ethanol (Rubin et al., 1970; Rubin et al., 1972; Gordon, 1973), no studies have been done to characterize the effects of ethanol on the biology of the chondrocyte;

however, it would seem possible that if ethanol molecules were permitted to accumulate in the cartilage, that the chondrocytic mitochondrial function would be affected in a manner similar to that reported for liver cells.

Reduced mitochondrial activity could result in a decrease of cartilage growth by decreasing the available energy needed for the growth process (Wisotzky, 1976). Some ethanol oxidation has been suggested by Pikkarainen and Räihä (1967) as occurring in other tissue cells besides those of the liver and, hence, could conceivably be oxidized in nasal cartilage chondrocytes. This would tend to overload the oxidative capacity of these cells and result in shunting of some energy needed for cartilage synthesis.

Moreover, Oudea et al. (1973) have reported that ethanol causes a 34% decrease in the surface density of the rough endoplasmic reticulum of the hepatic parenchymal cell. It is possible that an equivalent effect in exposed cartilage would result in decreased protein synthesis and concomitant inhibition of the growth of cartilage. For example, Gregory (1974) points out the need for at least two types of link proteins in the attachment of side chains to the disaccharide chain of the cartilage matrix, and ethanol might well cause a decrease in the availability of these proteins.

Results in View of Contemporary Hypotheses

The results of this investigation indicate that the experimental creation of an amniotic ethanol bath for the nasal septum produces an isolated midfacial deficit similar to that produced by

a variety of techniques that produce specific septal damage. Given an isolated effect of ethanol on septal cartilage, the results would seem to argue that the nasal septum plays an important role in naso-maxillary growth, as hypothesized by Scott (1953, 1954, 1956) and numerous other workers.

It is possible, of course, that ethanol prevents the septal cartilage from growing secondarily in response to the expansion of the nasopharyngeal space, as has been suggested by Moss (1968a, 1968b, 1969) for postnatal experimental intervention; however, in the present study it is difficult to determine--or even to imagine--the morphogenetic properties of a "functioning" airspace that does not yet exist. In the absence of clarification of the role of the "functional matrix" in early prenatal development, the present results would seem to support the concept of a primary morphogenetic role for the growing nasal septum.

SUMMARY AND CONCLUSIONS

Jones et al. (1973) have described a syndrome affecting children born to chronically alcoholic mothers. In addition to a variety of general signs, one of the most specific features was a retruded, underdeveloped maxilla. To date, however, no specific studies have been conducted to characterize precisely the nature of the attendant facial signs.

A study was performed to evaluate the effect of ethanol on the midfacial growth of 117 mice whose dams had been exposed to an ethanol vapor during five days of gestation; fifty-two mice served as controls. The effect of ethanol on the incidence of clefting was also investigated in a separate sample of 164 experimental and 199 control fetuses.

Data were collected in three categories:

(1) General appearance, physical condition, and body weight

(2) Roentgenographic cephalometric measurements of the craniofacial complex

(3) Incidence of clefting

In general, the experimental animals did not reveal any gross distortion or malformation due to ethanol. No significant effects of ethanol on body weights were seen. As demonstrated by analysis of covariance, an isolated reduction in maxillary advancement was seen in mice exposed to ethanol prenatally--the maxillary

incisors and molars were retruded and the length of the snout was decreased. No change in overall cranial height or cranial base length was noted. There was no increase in the incidence of clefting.

Anecdotal reports of the symptoms of children born to alcoholic mothers indicate a retruded and hypoplastic maxilla. The results of the present investigation support those findings. In light of contemporarily received opinion regarding the unique anatomical configuration of the septum, it is postulated that ethanol may have caused a selective growth retardation of the cartilaginous nasal septum.

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