CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

NEIL LINN POOLER

candidate for the Master of Science

degree.

Signed: _____ (Chairman)

_____

_____

_____

_____

Date April 26, 1979

# THE EFFECT OF NITROUS OXIDE EXPOSURE IN PREGNANT MICE ON THE CRANIOFACIAL DEVELOPMENT OF THEIR OFFSPRING


Abstract

by

NEIL LINN POOLER

Numerous teratogenic studies have shown that chemical agents administered to pregnant female laboratory animals can cause various types of craniofacial deformities in the offspring. Previous studies have also indicated that nitrous oxide exposure in pregnant rats can cause defects in osteogenesis as well as a high fetal death rate. To date, however, no specific studies have attempted to correlate nitrous oxide exposure with craniofacial defects.

This study was performed to evaluate the effect of nitrous oxide on the craniofacial growth of 158 A/J mice whose mothers had been exposed to an atmosphere containing nitrous oxide for five days during the gestation period. A control group of 71 A/J mice was also evaluated. A separate sample of 95 experimental and 115 control fetuses was used to investigate the effect of nitrous oxide on the incidence of cleft palate formation.

Three catagories of data were collected for this study:

(1) Body weight, physical condition, and general appearance

(2) Incidence of clefting

(3) Roentgenographic cephalometric measurements of the craniofacial complex.

Nitrous oxide exposure at the time periods and concentrations used produced no gross malformations in the offspring. There was no significant difference in body weight between the control and experimental offspring. The experimental offspring had a slightly higher rate of cleft palate formation than the control offspring. This difference in cleft rate between the control group and experimental group was statistically significant ($p<.05$). However, since both the control and experimental cleft rates fall within the previously reported range of spontaneous cleft palate formation in A/J mice, this difference in cleft rates was considered to be insignificant. The roentgenographic cephalometric study indicated that certain areas of the craniofacial complex were significantly altered in association with exposure to nitrous oxide, although the overall dimensions of the skull were unchanged.

Previous studies of nitrous oxide exposure in experimental animals, and surveys of pregnant women exposed to anesthetic gases including nitrous oxide, indicated that this gas might possess the potential to cause craniofacial deformities. The results of this study indicate that nitrous oxide exposure in A/J mice does not significantly increase cleft palate formation, but does cause significant dimensional changes in structural elements of the skull.

ACKNOWLEDGEMENTS

The author wishes to extend his sincere appreciation to the following individuals:

Dr. Joel Wisotzky, Professor of Oral Biology, School of Dentistry, Case Western Reserve University, who, as my advisor, provided the impetus for me to study teratology. His many hours of close attention and willingness to confront the various problems encountered in the development of this study made its successful completion possible.

Dr. Donald H. Enlow, Director of Graduate Studies and Chairman of the Orthodontic Department, School of Dentistry, Case Western Reserve University, who provided an excellent background in growth and development, and assisted in the preparation of this thesis. His genuine interest in the development of each student and skillful handling of difficult situations were greatly appreciated.

Dr. B. Holly Broadbent, Professor of Orthodontics, School of Dentistry, Case Western Reserve University, whose efforts in the field of cephalometric education provided the background necessary for this study and for future orthodontic endeavors.

Mr. William Golden, Bolton-Brush Growth Study Center, School of Dentistry, Case Western Reserve University, who prepared the illustrations for this thesis and gave many hours of assistance

during my orthodontic education.

Mr. Douglas Johnson, Photographic Department, School of Dentistry, Case Western Reserve University, who prepared the plates used in this thesis.

Mr. Robert Voight, School of Medicine, Case Western Reserve University, who helped provide the animal facilities required to maintain the breeding stock used in this study.

DEDICATED

TO

My wife, Sharon, for her
love and understanding.

My daughter, Andrea.

My parents, who made my
education possible.

My wife's parents, for
their encouragement.

TABLE OF CONTENTS


| | Page |
|---|---|
| ABSTRACT | ii |
| ACKNOWLEDGEMENTS | iv |
| DEDICATION | vi |
| LIST OF TABLES | viii |
| LIST OF PLATES | ix |
| LIST OF FIGURES | x |
| INTRODUCTION | 1 |
| REVIEW OF THE LITERATURE | 3 |
| STATEMENT OF THESIS | 14 |
| MATERIALS AND METHODS | 15 |
| RESULTS | 47 |
| DISCUSSION | 55 |
| SUMMARY AND CONCLUSIONS | 61 |
| LITERATURE CITED | 63 |

LIST OF TABLES


| Table | | Page |
|---|---|---|
| 1 | Cephalometric Analysis..... | 38 |
| 2 | Reliability Coefficients..... | 42 |
| 3 | Incidence of Clefting..... | 49 |
| 4 | Survival Rate of Offspring..... | 50 |
| 5 | Body Weight of 42-Day Postnatal Mice..... | 51 |
| 6 | Means and Standard Deviations..... | 53 |
| 7 | Statistical Analysis..... | 54 |
| 8 | Comparative Ages of Mouse and Human Embryos..... | 56 |

## LIST OF PLATES


| Plate | | Page |
|---|---|---|
| I. | Inhalation Chamber..... | 18 |
| II. | Regulator-Flowmeter and Filter..... | 20 |
| III. | Animal Cephalostat in Position on Bolton-Broadbent Cephalometer..... | 26 |
| IV. | Animal Cephalostat with A/J Mouse And Film in Position for Radiographic Exposure..... | 26 |
| V. | Print of Cephalogram..... | 31 |
| VI. | Negative Print of Cephalogram..... | 33 |
| VII. | Display of Uterus Following Hysterectomy..... | 43 |
| VIII. | Nineteen-Day Fetuses Used in Cleft Palate Study..... | 45 |

LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| 1 | Animal Groups Used in Study..... | 23 |
| 2 | Cephalometric Landmarks..... | 29 |
| 3 | Mean Tracings of Control and Experimental Animals..... | 36 |
| 4 | Cephalometric Measures..... | 39 |

# INTRODUCTION

The possible teratogenic effects of anesthetic gases, especially the effects upon the offspring of pregnant human females exposed to these gases, have been the subject of medical concern and of numerous studies in recent years. Surveys of pregnant operating room personnel exposed to anesthetic gases have shown a higher than usual incidence of miscarriage and congenital malformation. None of these studies attempted to determine which specific anesthetic gas was responsible for the effects. Experimental nitrous oxide exposure to pregnant rats is known to cause a high fetal death rate and to result in defects in skeletal growth and development.

Nitrous oxide is widespread in hospital use as one component of gas mixtures used for general anesthesia. An estimated 50,000 pregnant women receive anesthesia for medical reasons in the United States each year, although the precise percentage of this total that receive nitrous oxide in the gas mixtures in unknown. Nitrous oxide-induced "conscious sedation" has become a popular technique in recent years for sedating dental patients. Use of nitrous oxide gas has become so routine that it is administered to 98% of the patients treated in some dental offices.

The fetus is known to be particularly vulnerable to certain other agents administered to the mother during critical periods of fetal development. Because exposure of pregnant female patients and clinical personnel to nitrous oxide gas become even more widespread in recent years, the possible effects of this exposure on the human fetus call for thorough studies without delay.

The purpose of this study is to investigate the effects of maternal nitrous oxide exposure in the A/J mouse upon the craniofacial development of their offspring. This particular mouse strain was chosen because of its susceptability to clefting when exposed to teratogenic agents, as shown in previous cleft palate experiments. The incidence of clefting will be determined by examination of fetal palates. Craniofacial measurements using a roentgenographic cephalometric technique will be made to determine any alterations in the pattern and amount of growth.

## REVIEW OF THE LITERATURE

### Nitrous Oxide

Nitrous oxide was discovered by Joseph Priestly, an English chemist, in 1772. The anesthetic properties of this gas were first noticed in 1798 by Humphery Davy, but practical clinical use of nitrous oxide as an anesthetic agent did not begin until 1844, when Horace Wells publicized the use of this gas to perform painless dental extractions. In the decades that followed, interest in nitrous oxide declined as other, more potent general anesthetic agents were discovered, and as the anoxic effects of administering the 100% concentration of nitrous oxide necessary to produce general anesthesia became publicized.

In 1881, an entirely new concept, the use of an inhalation agent to produce partial analgesia and sedation while the patient remained totally conscious, was introduced to the medical profession by Klikovitsch, a Russian obstetrician. The technique consisted of administration of a mixture of 80% nitrous oxide and 20% oxygen. By 1889, nitrous oxide-oxygen analgesia was in use for dental procedures (Langa, 1976).

During the first half of the twentieth century, interest in the use of nitrous oxide analgesia was generally at a low ebb because of the high incidence of analgesic failures. Most of the failures were due to the crude gas administration machines

and the relatively impure gases available during this period. By the late 1950's, equipment and materials had improved to the point that safe, predictable nitrous oxide analgesia had become routine. Interest in the technique increased as more medical and dental practitioners became aware of its value in controlling pain and anxiety. During the 1960's, nitrous oxide analgesia became a generally accepted dental procedure, and instruction in its use became a part of the curriculum of most dental schools.

Pharmacology and Physiology. Nitrous oxide is a colorless, tasteless, sweet-smelling gas. Its blood/gas solubility coefficient is 0.47, making it fifteen times more soluble in blood that nitrogen. When administered via an inhalation machine, the gas moves from the lungs into the bloodstream by displacing the less soluble nitrogen already dissolved in the blood. During the first ten to fifteen minutes of administration in human subjects, uptake of nitrous oxide is rapid as the relatively vascular organs (heart, liver, brain, kidney) reach equilibrium with the arterial nitrous oxide concentration. Approximately one hour is required for the less vascular tissues to reach equilibrium. When administration of the gas is discontinued, almost all of the nitrous oxide in the body becomes eliminated via the lungs. The elimination process requires approximately the same amount of time as was required for the body to reach equilibrium during the administration of nitrous oxide. A very small amount of the gas is excreted via the urine, skin, and sweat glands.

Nitrous oxide can produce a state of unconsciousness in some patients in only 30% concentrations, yet in other patients, unconsciousness does not occur even at the maximum safe concentration of 80%. Because of this wide variability, nitrous oxide has traditionally been classified as a weak general anesthetic agent and is typically used in combination with other agents if general anesthesia is desired.

The main site of anesthetic action of nitrous oxide is the brain. Like all other general anesthetic agents, it produces a depression of brain tissue activity, first affecting the cerebrum. It is this cerebral depression that causes sedation and partial analgesia associated with nitrous oxide administration. If a sufficiently high concentration of nitrous oxide is administered, brain tissue activity may be depressed to the point that general anesthesia is induced (Small, 1973).

The exact mechanism of physiologic action by which nitrous oxide affects the brain is unknown. Nitrous oxide has traditionally been considered to be an inert gas that does not change or combine with other substances while in the body. It produces no direct effect on the respiratory or cardiovascular systems. Its only effect on the gastrointestinal system is nausea and vomiting in a small percentage of patients. Nitrous oxide is known to cross the placental barrier (Marx, 1961).

Adverse Effects of Nitrous Oxide in Humans. Nitrous oxide was long considered to produce no harmful side effects. The first

true adverse effects were reported by Lassen, et al. (1956), who found signs of aplastic anemia in tetanus patients sedated with continuous 50% nitrous oxide, 50% oxygen gas mixture. After approximately five days of continuous sedation, leucocytopenia became apparent. In most cases, leucocyte counts fell from 12,000 per ml. prior to treatment to 2500 per ml. after ten days of nitrous oxide sedation. Bone marrow biopsies taken during this time revealed depression of hemopoiesis. Further biopsies indicated that the bone marrow began returning to normal three days after nitrous oxide sedation was discontinued. Recognizing the leucocytopenic effect of nitrous oxide, Eastwood, et al. (1963) reported the treatment of leukemia patients by adnimistration of 30% to 50% nitrous oxide. White cell counts typically declined from 170,000 per ml. to 15,000 per ml. after four to seven days of administration. These investigators were unable to determine the mechanism by which nitrous oxide causes bone marrow depression.

Numerous studies have attempted to determine the effect of prolonged exposure to low concentrations of anesthetic gases by operating room personnel. These studies generally reported rates of mortality, miscarriages, and birth defects in groups of individuals including pregnant women exposed to anesthetic gases for prolonged periods of time. None of the studies, however, attempted to determine which particular anesthetic agents were responsible for any reported effects. Personnel in all studies

had been exposed to several anesthetic gases, including nitrous oxide.

Linde and Bruce (1969), in a survey of hospital operating rooms, found an average nitrous oxide level in the room air of 130 parts per million. Cohen, et al. (1975), surveyed dental operatories in which nitrous oxide sedation was utilized and found levels of the gas in room air generally to be from 500 to 6,000 parts per million, with some levels as high as 9,700 parts per million. Whitcher, et al. (1975), noted that nitrous oxide in dental operatories could be decreased to 27 parts per million with simple, inexpensive scavenging equipment.

In the earliest study of operating room personnel, Vaisman (1967) found that of thirty-one pregnancies among 110 female subjects, eighteen ended in spontaneous abortion. He attributed this increase in abortion rate to inhalation of anesthetic gases, and to emotional strain caused by the stressful nature of operating room employment. Askrog and Harvald (1970) surveyed Danish nurses that had worked in operating rooms and other hospital areas. Their study indicated that the spontaneous abortion and premature delivery rates approximately doubled during the period of employment in operating rooms. The study also reported a relative, but not significant, decrease in the number of male children born.

Cohen, et al. (1971) surveyed female anesthetists and operating room nurses in California hospitals and found a 37% spontaneous abortion rate, compared to a 10% rate for non-operating

room nurses and female physicians. They also pointed out that about 50,000 pregnant women receive anesthetic gas for various periods of time in the United States each year. The study did not attempt to implicate any one particular anesthetic gas. Knill-Jones, et al. (1972) reported similar results in a survey of British nurses. Corbett, et al. (1974) studied the incidence of birth defects among children of nurse-anesthetists, and reported an overall birth defect rate of 16.4%, compared to 8.4% in the general population. Basically, these surveys showed a similar result, that operating room employment can result in a higher rate of spontaneous abortions and birth defects. This phenomenon may involve a composite of stress, exposure to anesthetic gases, and perhaps some other unknown factors not currently under consideration.

Recently, evidence has come to light that prolonged exposure to nitrous oxide can have deleterious effects upon the human nervous system. Layser (1978) studied twelve individuals who had been exposed to nitrous oxide in operating rooms or by self-administration. The most common sign was numbness of the extremities. Other signs included loss of dexterity, poor balance, muscular weakness, impotence, and depression. When removed from exposure to nitrous oxide, all patients improved, but the recovery rate was reported as slow. Complete recovery occurred in approximately half the cases.

Animal Studies With Nitrous Oxide. The first animal studies with nitrous oxide began after the reports of leucocytopenia in humans exposed to the gas. Kieler (1957) exposed embryonal mouse heart tissue to 50% nitrous oxide and reported the gas to be a mitotic poison, preventing interphase cells from entering mitosis and causing spindle destruction and chromosomal abnormalities in dividing cells. Green and Eastwood (1963) and Parbrook (1967) experimentally reproduced in rats the same type of leucocytopenia seen in humans after nitrous oxide exposure. Rector and Eastwood (1964) subjected incubating chicken eggs to an atmosphere of 80% nitrous oxide and 20% oxygen. They noted a hatch rate of only 20% as compared to 60% in the control group. Smith, et al. (1965) also subjected fertile chicken eggs to nitrous oxide, and noted a slight increase in the rate of congenital anomaly occurrence.

Fink, et al. (1967) showed a relationship between maternal nitrous oxide exposure and congenital anomalies in the offspring. Pregnant rats were subjected to a gas mixture of 50% nitrous oxide, 21% oxygen, and 29% nitrogen. The most common disturbances in development were death of the embryos and abnormalities in skeletal ossification. Corbett, et al. (1973) exposed pregnant rats to low levels of nitrous oxide, from 0.1% to 15%, to simulate levels that might be found in operating rooms. They found a significantly higher rate of fetal death in those animals that received nitrous oxide.

In an animal study dealing with the neurologic effects of nitrous oxide, Hayden, et al. (1974) exposed rats to a mixture of 40% nitrous oxide and 60% oxygen for periods of eight to fifteen days. Blocks of cortical tissue from the frontal, parietal, and occipital lobes of the cerebrum were examined. Observable signs of cellular damage were reported, especially in the occipital lobe. The gas mixture used in this experiment did not provide 21% oxygen, which is the normal level of oxygen found in the atmosphere. The potential effects of this hyperbaric (60%) oxygen concentration over the period of the experiment are not fully understood, although the oxygen level itself may have been responsible for some of the effects noted.

Berkowitz and Ngai (1976) used mice to show that the action of nitrous oxide resembles that of opiates, based on the observations that narcotic antagonists diminish the analgesic effects of nitrous oxide, and that a cross-tolerance could be demonstrated between morphine and nitrous oxide.

It is significant to note the similarity between the results of animal and human studies concerning nitrous oxide exposure. The animal experiments and human case reports provide reliable evidence of the ability of this gas to affect the nervous and hemopoietic systems. Animal studies have shown that nitrous oxide has definite, deleterious effects upon fetal development, while human reports tend to support, but to date have not definitely proven, this conclusion.

Cleft Palate and Craniofacial Development Studies

Most animal studies dealing with cleft palate formation and craniofacial development in the presence of potentially teratogenic agents have been carried out using inbred strains of mice or rats as the experimental model. Fraser, et al. (1951, 1954) found very high rates of cleft palate formation in mice when the pregnant females had received cortisone. Kochhar and Johnson (1965) subjected pregnant rats to high levels of vitamin A and found increased cleft palate formation in the offspring.

The mechanism of palate closure and of cleft palate formation as reported in these experiments is not well understood. Walker and Fraser (1956) suggested that an "internal force" caused the palatal shelves to close and push the tongue into a more ventral position. They defined the critical time of palate closure to be day 14½ to day 15 of gestation in the A/J mouse. Ross, et al. (1967) and Humphrey (1969) hypothesized that descent of the tongue due to fetal muscular movements is essential to normal palate closure. Jacobs (1970, 1971) paralyzed pregnant mice and separately administered muscle relaxants, both experiments being undertaken at the critical time of palate closure. The lack of cleft palate formation in these experiments suggested that muscular activity is not essential for palate closure. However, Walker and Patterson (1974), in a similar experiment, subjected pregnant mice to tranquilizers and barbiturates, some of which were the same drugs used by Jacobs (1971). They reported cleft palate rates

significantly higher than the 6% to 16.7% range for spontaneous cleft palate formation as reported by Kalter (1968) for the A/J mouse. Jelinek and Dostal (1974) studied the effect of maternal cortisone injections on the developing palatal shelves in mice. They reported that cortisone-induced clefting was due to a slower rate of proliferation of palatal tissues, caused by decreased mitoses. A/J mice were used in all of these cleft palate experiments because of the suceptability of this strain to cleft palate formation when exposed to teratogenic agents.

Paulus (1976) investigated the effects of maternal ethanol inhalation on both cleft palate formation and overall craniofacial development in A/J mice. The ethanol had no effect on cleft palate formation, but affected craniofacial development, as revealed by comparative dimensions taken from cephalometric radiographs.

Nitrous Oxide and Craniofacial Development

There have been no studies to date attempting to relate nitrous oxide exposure to craniofacial development or to cleft palate formation. Previously cited studies have shown that nitrous oxide can cause various fetal abnormalities in experimental animals, and thus may have the potential to disrupt fetal development during human pregnancy. This gas has been shown to be pharmacologically sinilar to opiates, a class of drugs similar to the barbiturates and tranquilizers used experimentally to produce a high cleft rate in several strains of mice, including the A/J strain. Published reports indicate that a decrease in

mitoses can prevent normal palatal closure, a fact that is significant in light of the report that nitrous oxide is a "mitotic poison".

Considering these findings and the fact that nitrous oxide is presently in routine use in medicine and dentistry, it is appropriate to determine if this drug can affect the normal progress of prenatal development.

## STATEMENT OF THESIS

This investigation is intended to determine the effect of nitrous oxide on cleft palate formation in A/J mice. It will also determine the effect of nitrous oxide on overall craniofacial development by analyzing the craniofacial dimensions of 42 day old A/J mice.

## MATERIALS AND METHODS

### Sample

A breeding stock of A/J mice (Jackson Laboratories, Bar Harbor, Maine) was six weeks of age when received. The animals were housed in the Case Western Reserve University animal facility for two weeks with a twelve hour light and dark cycle so that they could adjust to the new environment. During this two week period, the mice were housed in 45 X 25 X 20 cm. cages, separated by sex, with ten males per cage and twenty females per cage. The females were intentionally crowded in the cages in order to produce a more regular estrous cycle, thus making for easier breeding when the animals are subsequently placed in an uncrowded environment, as reported by Whitten (1959).

At eight weeks of age, the mice were housed with two females and one male per 25 X 15 X 13 cm. cage, with a twelve hour light and dark cycle. The females were inspected each morning during this breeding period for presence of vaginal plugs. The first day of pregnancy was considered to have started twelve hours prior to finding the vaginal plug, as reported by Kalter (1968). Pregnant females were identified and returned to the breeding cages, where they were housed until the start of the experiment. All animals were provided with Purina rat-mouse chow and water ad libitum throughout the breeding and experimental

periods.

A total of 301 A/J mice from 52 litters were obtained in this manner to determine any craniofacial effects of nitrous oxide exposure. Thirty litters with a total of 207 prenatal fetuses were also obtained to discern any effect upon the rate of cleft palate formation.

## Experimental Design

This experiment consisted of exposing pregnant mice to nitrous oxide in a specially constructed inhalation chamber from day eleven through day fifteen of pregnancy. Fifty-two litters were used to determine any effects on craniofacial growth, and were divided into the following groups:

1. Twenty seven litters (158 newborn mice) produced by mothers that had been exposed to 50% nitrous oxide.
2. Twelve litters (72 newborn mice) produced by mothers exposed to 10% nitrous oxide.
3. Thirteen litters (71 newborn mice) were used as controls. Their mothers were placed in the same inhalation chamber but breathed filtered, compressed air passed through the chamber in place of the nitrous oxide gas mixture.

After removal from the chamber, the pregnant mice were placed in isolated cages. All newborn mice remained in the cages with their mothers until forty-two days after birth. These cages were disturbed as little as possible and the infants not handled in order to minimize maternal cannibalism. At age forty-two days,

the mice were weighed, examined, killed, and radiographed.

The incidence of cleft palate formation was determined by inspecting fourteen prenatal litters (95 fetuses) whose mothers had been exposed to 50% nitrous oxide in the experimental chamber. Sixteen litters (112 fetuses) served as controls. As in the craniofacial growth portion of this experiment, mothers of the control group were housed in the inhalation chamber from day eleven through day fifteen, and breathed filtered air from a compressed source at essentially atmospheric pressure. In order to eliminate the problem of maternal cannibalism of the offspring, the litters used in the cleft palate portion of this experiment were obtained by harvesting the fetuses at the ninteenth day of gestation through removal of the uterus.

## Nitrous Oxide Exposure

Of two levels of nitrous oxide exposure, the 50% level was chosen as a maximal dose because this is the highest level typically used in nitrous oxide sedation in human subjects. The 10% level was chosen because it approximates the highest reported level of nitrous oxide waste gas in the atmosphere of a dental or medical operatory. Thus, 10% represents the highest level of chronic, unintentional human exposure to this drug. Custom gas mixtures of U.S.P. grade nitrous oxide, oxygen, and nitrogen were obtained (Linde Div., Union Carbide Corp.) in "T" size tanks containing 3500 liters. The actual gas mixtures used were 50% nitrous oxide, 21% oxygen, 29% nitrogen for one mixture, and 10%

PLATE I

INHALATION CHAMBER

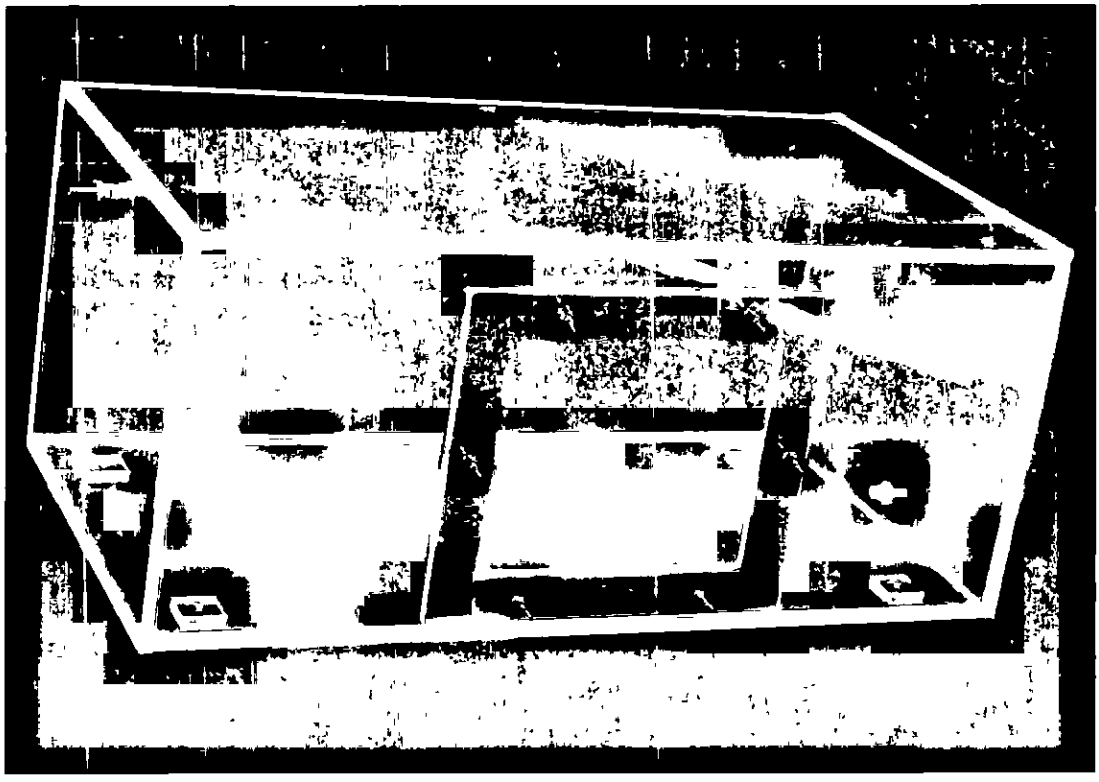
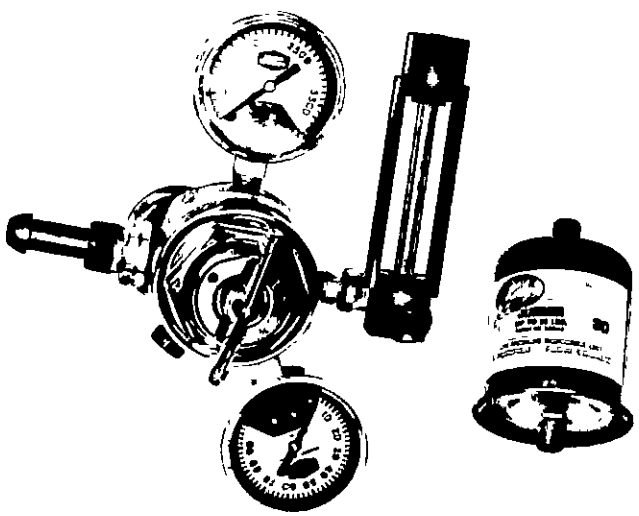


PLATE II

REGULATOR-FLOWMETER AND FILTER



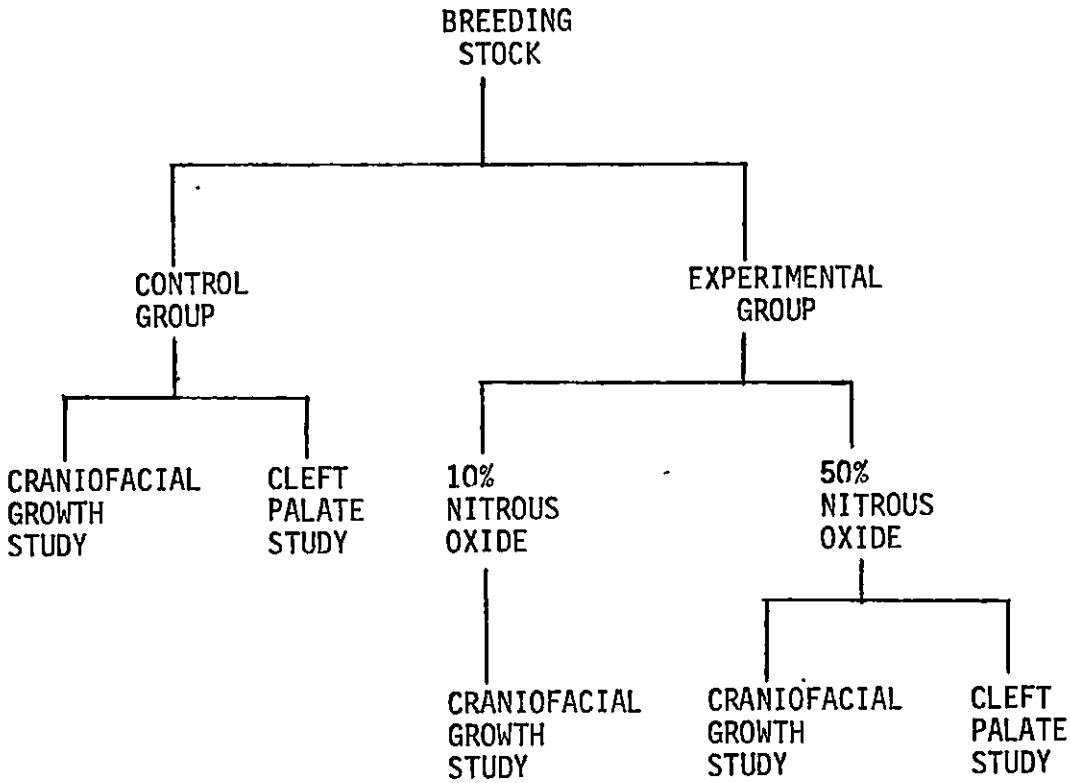
nitrous oxide, 21% oxygen, 69% nitrogen for the other. The gas mixture in each tank was tested by the supplier and reported to be accurate to within 0.1%. Both gas mixtures were designed to provide the same level of oxygen that is in ordinary room air, so as to prevent any effect of hyperbaric oxygen concentration.

The inhalation chamber was a clear plastic box, 60 X 25 X 32 cm., containing approximately 48 liters of gas. A flow rate of two liters of gas per minute through the chamber was maintained for both experimental and control groups. This flow rate was considered to be adequate for the maximum of fifteen animals that were housed in the chamber, based on the work of Goldstein and Pal (1971), and Fink, et al. (1967). Regulation of the flow rate for both experimental and control groups was accomplished with a commercial flowmeter-regulator (Burdox Co., Gateway Div., Cleveland, Ohio). A beaker containing 100 grams of soda lime, a mixture of sodium hydroxide and calcium oxide (Fisher Scientific, Fair Lawn, N.J.), was placed in the chamber and changed daily to act as a carbon dioxide absorbent, as described by Fink, et al. (1967).

The female mice were placed in the chamber on the eleventh day of pregnancy and removed on the fifteenth day. All mice survived while in the chamber. Upon removal, the mice were grouped into two divisions for the craniofacial growth investigation or for the cleft palate study. A chart indicating the various groups of animals used in the entire experiment is shown in Figure 1. The order in which the groups were placed in the chamber was:

FIGURE 1

ANIMAL GROUPS USED IN STUDY


BREEDING STOCK
CONTROL GROUP
EXPERIMENTAL GROUP
CRANIOFACIAL GROWTH STUDY
CLEFT PALATE STUDY
10% NITROUS OXIDE
50% NITROUS OXIDE
CRANIOFACIAL GROWTH STUDY
CRANIOFACIAL GROWTH STUDY
CLEFT PALATE STUDY

1. Control group.
2. 50% exposure group.
3. 10% exposure group.

## Examination of Fetuses for Cleft Palate Study

The female mice to be used in the cleft palate study were killed after nineteen days of pregnancy. The fetuses were removed by hysterectomy, and the sex of each fetus was determined by measuring the distance from the anus to the genital tubercle. This distance is approximately one millimeter greater in males. The fetuses were then examined under a dissecting microscope for presence of cleft palate. Results of the cleft palate study at 50% nitrous oxide exposure were such that it was decided not to repeat this portion of the experiment at 10% nitrous oxide, as explained later.

## Cephalometric Technique

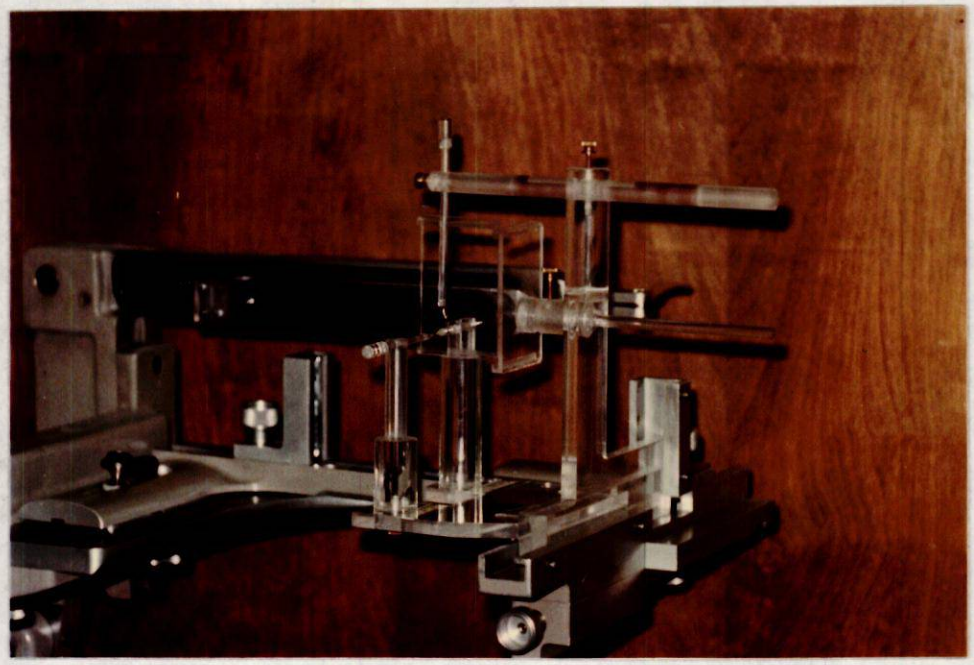
A cephalostat especially constructed for use with small experimental animals was utilized to obtain cephalometric radiographs. This cephalostat is basically a scaled down version of the Bolton-Broadbent cephalometer (Series 1K550, Bolton Fund, Cleveland, Ohio). It was designed to fit into the Bolton-Broadbent cephalometer and maintain the same sixty inch distance from radiation source to midsaggital plane. All planes of reference remained the same as in human cephalometric radiology, except that the ear rods were inserted into the external auditory

PLATE III

ANIMAL CEPHALOSTAT IN POSITION ON
BOLTON-BROADBENT CEPHALOMETER

PLATE IV

ANIMAL CEPHALOSTAT WITH A/J MOUSE AND FILM
IN POSITION FOR RADIOGRAPHIC EXPOSURE





meatus and then positioned at the base of the occipital bone. This change was necessary in order to visualize all cranial structures on the radiographs. The central ray still passed through the ear rods, but not through the transporionic axis.

All radiographs were taken on dental occlusal film (DF46, Kodak Co., Rochester, N.Y.) at 80 KV and 10 mA for five seconds. Processing was accomplished in an automatic developer to insure consistency of technique.

## Cephalometric Landmarks

In order to more accurately measure the small dimensions involved, each cephalogram was first enlarged five times with a photographic enlarger and printed on photographic paper (Kodak Ecktomatic SC-F, Kodak Co., Rochester, N.Y.). The following points were then identified and marked with a small pin hole (Figure 2);

1. the tip of the nasal bone (N),
2. the most superior point 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 surface of the maxillary first molar (Mx),
6. the intersection of the mandibular alveolar process

FIGURE 2

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 posterosuperior point on the condylar curvature, averaged right and left; (SOS) the most superior (dorsal) point of the spheno-occipital 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.

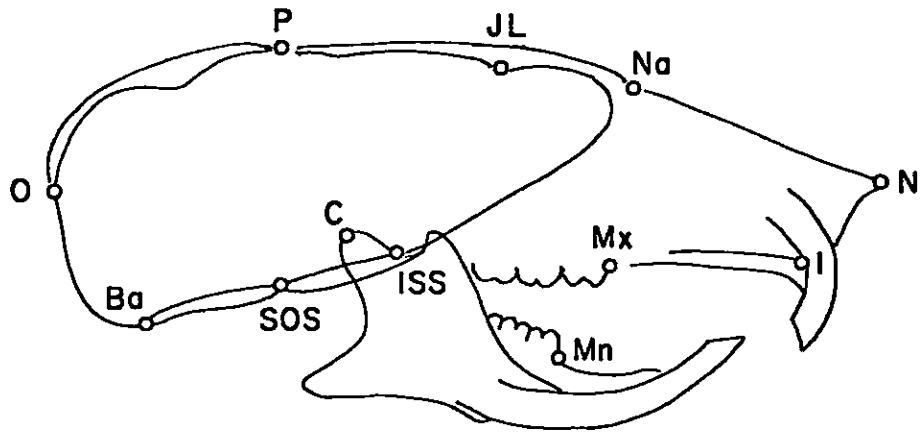

P
JL
Na
O
N
C
Mx
I
ISS
Ba
SOS
Mn

PLATE V

PRINT OF CEPHALOGRAM
(5X)

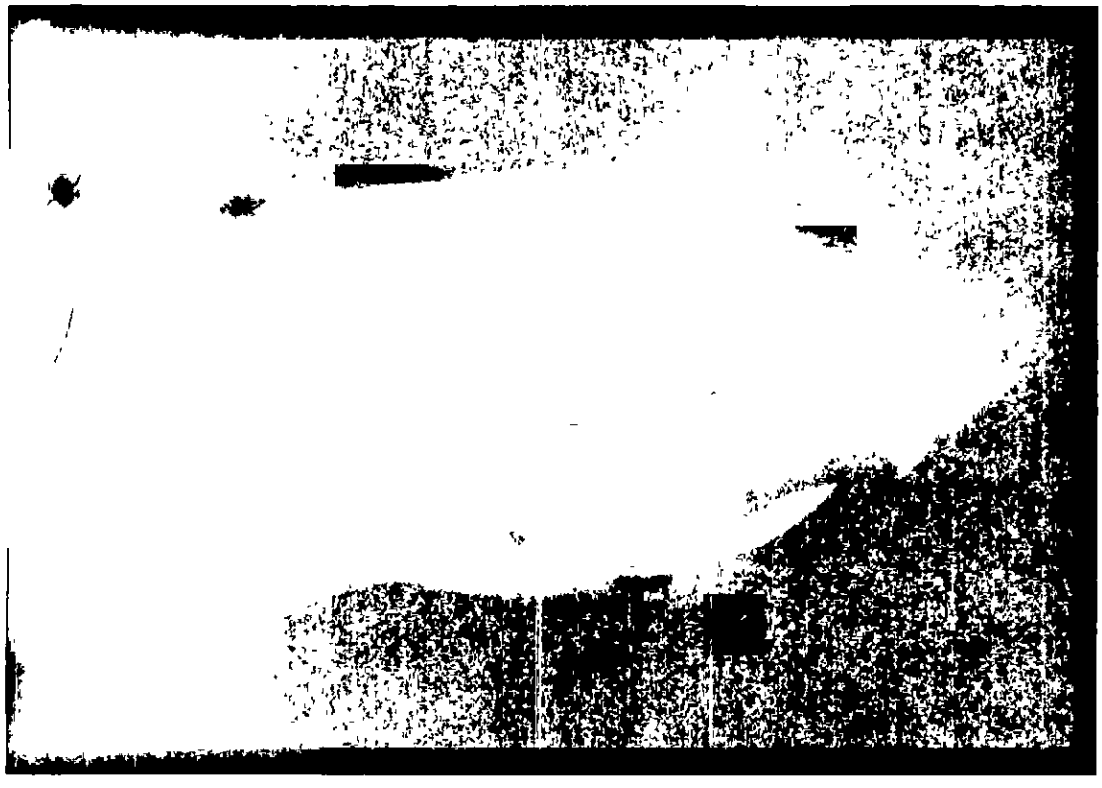


PLATE VI

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

and the anterior surface of the lower first molar (Mn),

7. the most superior (dorsal) point of the inter-sphenoidal synchrondrosis (ISS),
8. the most posterosuperior point on the condylar curvature, averaged right and left (C),
9. the most superior (dorsal) piont of the spheno-occipital synchondrosis (SOS),
10. opisthion, the most dorsal (superior) point of the foramen magnum located in the midsagittal 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).

Cephalometric tracings of all negative prints were made for the control and experimental groups. Tracings were paired and averaged to obtain a mean tracing for each group, using the same method as was used to obtain the Bolton standards of human craniofacial growth and development (Broadbent, et al., 1975). These mean tracings are shown in Figure 3 and illustrate the dimensional differences between the two groups. The measures used are listed in Table 1.

## Error Study

To estimate the reliability of the cephalometric technique, twenty animals were chosen at random and the cephalograms were

FIGURE 3

MEAN TRACINGS OF CONTROL AND
EXPERIMENTAL ANIMALS

——————— CONTROL

- - - - - - - - - - EXPERIMENTAL

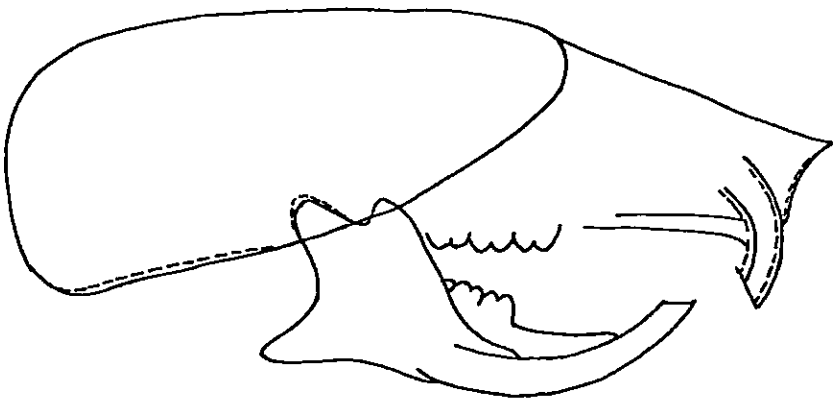


TABLE 1

CEPHALOMETRIC ANALYSIS

| Anatomical Dimensions-Direct Measurement | | |
|---|---|---|
| Measure | | Landmarks |
| A | Snout Length | JL-N |
| B | Neurocranium Length | JL-O |
| C | Overall Skull 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 4

CEPHALOMETRIC MEASURES

Direct Measurements

(A) Snout Length (JL-N)

(B) Neurocranium Length (JL-O)

(C) Overall Skull 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)

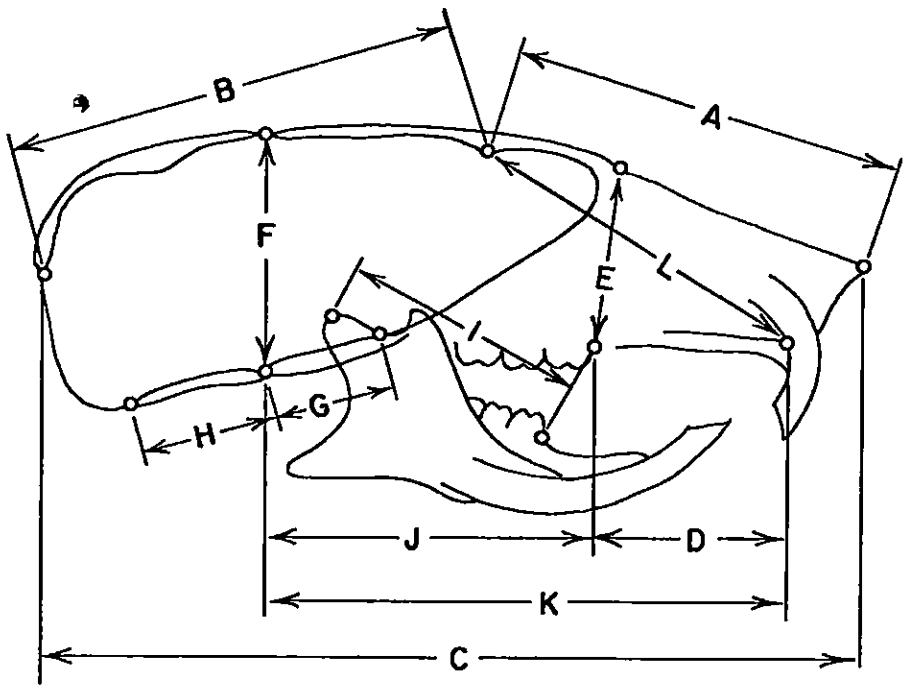
(H) Basioccipital Length (SOS-Ba)

(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
E
L
I
G
H
J
D
K
C

re-enlarged. Measurements were taken and a coefficient of correlation (Snedecor and Cochran, 1967, pg. 172) was calculated between the first and second measurements for each dimension. The square of this coefficient, $r^2$, constitutes a measure of the 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).

TABLE 2

RELIABILITY COEFFICIENTS

| Measure | r | $r^2$ |
|---|---|---|
| A | .952 | .905 |
| B | .942 | .887 |
| C | .972 | .945 |
| D | .870 | .776 |
| E | .726 | .527 |
| F | .855 | .731 |
| G | .826 | .682 |
| H | .897 | .795 |
| I | .886 | .785 |
| J | .917 | .841 |
| K | .931 | .866 |
| L | .941 | .886 |

PLATE VII

DISPLAY OF UTERUS FOLLOWING HYSTERECTOMY

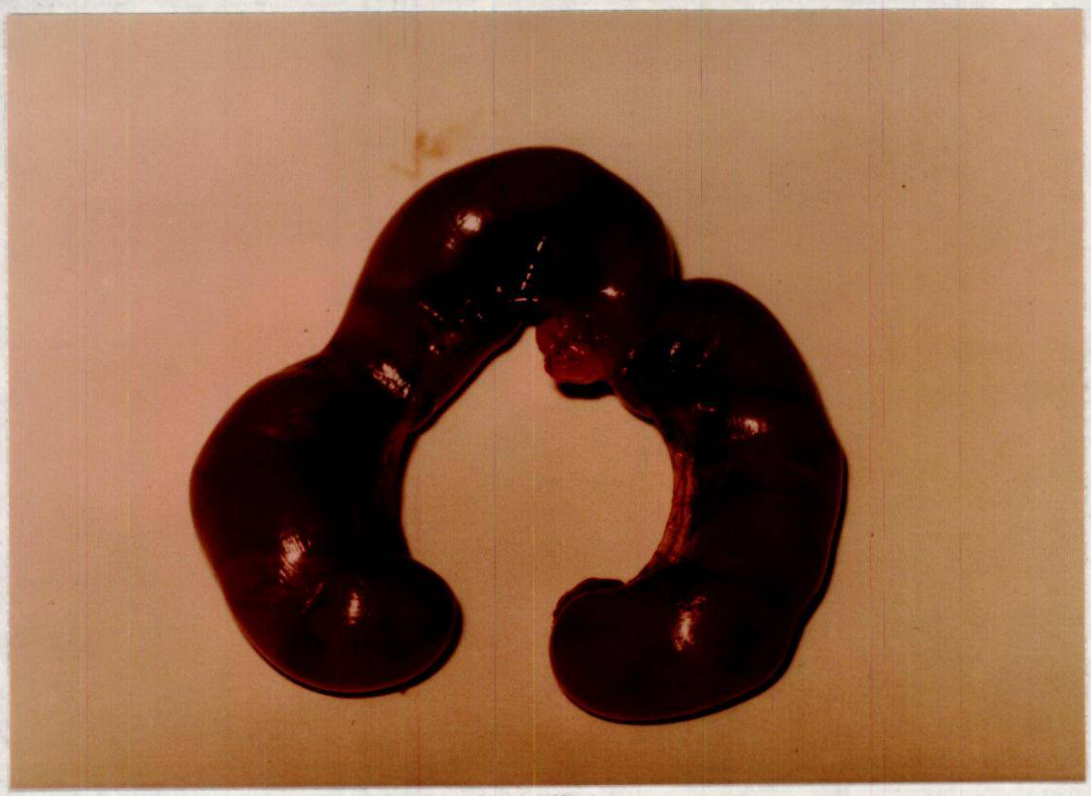
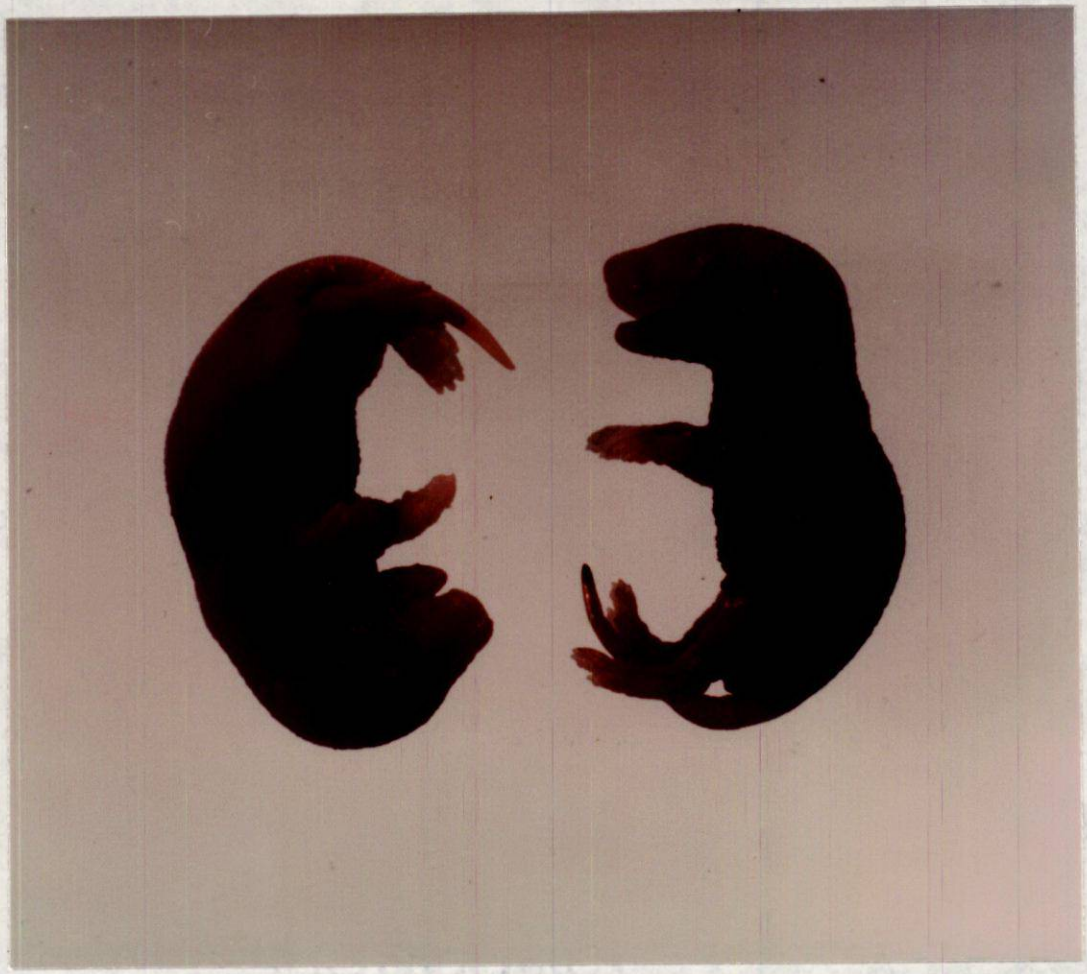


PLATE VIII

NINETEEN-DAY FETUSES USED IN CLEFT PALATE STUDY



## RESULTS

### Incidence of Clefting

Among the 112 control fetuses, 8 (7.1%) had clefting. In the experimental group exposed to 50% nitrous oxide, 13 of 95 (13.7%) had clefting. A breakdown of the clefting by sex for both the control and experimental groups is shown in Table 3.

Kalter (1968) reported a spontaneous clefting rate of 6% to 16.7% for the A/J mouse. The experimental cleft rate at 50% exposure differs significantly from the control rate, using the Chi-square test ($p < .05$). However, the experimental rate is within the reported range of spontaneous cleft rates for this animal, and as such, the effect of 50% nitrous oxide on clefting, although statistically significant in this study, reflects a minimal increase that may at times be seen in this strain when not exposed to any agent. There was no significant difference between the male cleft rate and the female cleft rate. Since this maximal nitrous oxide exposure did not produce an abnormally high cleft palate rate, it was decided not to repeat this portion of the experiment at the lower (10%) exposure.

### Survival Rate of Offspring

The survival rate of experimental animals (offspring of pregnant mothers) exposed during the fetal period to 10% or 50%

nitrous oxide, and of the control animals is shown in Table 4. The 50% exposure level was extremely lethal, with only 4 of the 158 offspring (2.5%) surviving. Exposure to 10% nitrous oxide caused moderate lethality, as 44 of the 72 offspring (61.1%) survived. The experimental animals in both groups were all born alive, and any deaths occurred within two days after birth. Survival rate within the control group was 100%.

General Appearance and Body Weight

The experimental animals were examined grossly and found to be apparently normal with respect to color, coat, eyes, dentition, and overall appearance.

The mean body weights of the 42 day old control and 10% exposure level experimental groups are shown in Table 5. A statistical analysis utilizing the two tailed t-test ($p > .05$) revealed no significant difference between the control group and the 10% exposure group. The 50% exposure group was not used for statistical analysis because of the small number of survivors.

TABLE 3

INCIDENCE OF CLEFTING

| | Control Group | 50% Exposure Group |
|---|---|---|
| Total Animals | 112 | 95 |
| Male | 57 | 47 |
| Female | 55 | 48 |
| Cleft Animals | 8 (7.1)* | 13 (13.1) |
| Male | 4 | 6 |
| Female | 4 | 7 |

* Numbers in parentheses are percentages.

For control vs. experimental cleft rate, $X^2=6.06$, df = 1, p = .015.

TABLE 4

SURVIVAL RATE OF OFFSPRING

| | Control Group | 10% Exposure Group | 50% Exposure Group |
|---|---|---|---|
| Total Animals | 71 | 72 | 158 |
| Surviving Animals | 71 | 44 | 4 |
| Survival Percent | 100 | 61.1 | 2.5 |

TABLE 5

BODY WEIGHT OF 42-DAY POSTNATAL MICE

| | Control Group | 10% Exposure Group |
|---|---|---|
| Number of Animals | 71 | 44 |
| Mean (gms.) | 17.30 | 16.95 |
| Standard Deviation | 2.32 | 1.88 |

Calcualted t = .843.

Table t (p=.05) = 1.9813

## Cephalometric Analysis

Twelve direct craniofacial measurements as listed in Table 1 were taken from enlarged cephalometric radiographs of the control and 10% experimental groups. No measurements or statistics will be presented for the 50% group, due to the small sample size resulting from the high neonatal mortality rate. Measurements were made to the mearest 0.1 millimeter with vernier calipers, and calculations were performed on a programmable electronic calculator. Means and standard deviations for these measurements are shown in Table 6. Results of a statistical analysis of these measurements in the two groups utilizing the two tailed t-test ($p<.05$) are shown in Table 7.

Seven measurements showed statistically significant differences ($p<.05$). Two of these measurements, JL-O, neurocranium length and Mn-C, mandibular length, were larger in the experimental group. Five measurements, SOS-P, height of posterior neurocranium, I-Mx, premaxillary length, SOS-Ba, basioccipital length, Mx-SOS, maxillary advancement, and I-SOS, premaxillary advancement, were larger in the control group. Mean tracings of the control and experimental groups are shown in Figure 3.

TABLE 6

MEANS AND STANDARD DEVIATIONS
(in millimeters X 5)

| Measure | Control Group | 10% Exposure Group |
|---|---|---|
| A | 52.19 (1.86)* | 51.95 (1.66) |
| B | 59.28 (1.63) | 60.03 (1.02) |
| C | 107.63 (3.02) | 107.56 (2.51) |
| D | 27.34 (1.10) | 26.87 (0.90) |
| E | 25.52 (1.18) | 25.31 (1.09) |
| F | 30.89 (1.07) | 30.51 (0.56) |
| G | 15.96 (2.11) | 16.39 (0.69) |
| H | 18.17 (1.02) | 17.78 (0.68) |
| I | 32.33 (1.46) | 32.89 (1.08) |
| J | 42.17 (1.92) | 41.30 (1.37) |
| K | 69.35 (2.75) | 68.08 (2.22) |
| L | 47.20 (1.79) | 47.40 (1.48) |

* Numbers in parentheses are standard deviations.

TABLE 7

STATISTICAL ANALYSIS

| Measure | t-calculated |
|---|---|
| A | .392 |
| B | 2.736* |
| C | .129 |
| D | 2.382* |
| E | .955 |
| F | 2.176* |
| G | 1.307 |
| H | 2.244* |
| I | 2.197* |
| J | 2.619* |
| K | 2.584* |
| L | .931 |

* Significant at 95% level.

Table t = 1.981 for df=113, p=.05.

$$\text{Calculated } t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1 + n_2 - 1}} \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

## DISCUSSION

The objective of the investigation was to determine the effects of maternal nitrous oxide exposure on craniofacial growth and cleft palate formation in A/J mice. Two experimental groups were treated with 10% or 50% nitrous oxide from day 11 through day 15 of pregnancy. It was planned to study craniofacial development and cleft palate incidence for both experimental groups, but the high mortality rate among the offspring of those animals subjected to 50% nitrous oxide resulted in an insufficient sample to investigate craniofacial development. Therefore, the craniofacial development portion of this investigation is limited to animals exposed to 10% nitrous oxide.

Pregnant mice in this experiment received nitrous oxide for five days, or approximately one-fourth of the gestation period. This is the equivalent of exposure from day 30 to day 75 of the human gestation period (Table 8), according to Rugh (1968). Therefore, it would be necessary for a pregnant human to breathe 10% or 50% nitrous oxide for 24 hours per day from day 30 to day 75 of gestation for the fetus to receive the same proportionate extent of exposure as the animals in this study. This prenatal period covers the interval of time when the palatal shelves and nasomaxillary complex are actively growing (Atherton, 1967; Rugh, 1968; Latham, 1969), and the period during which palatal contact and fusion occurs (Walker and Fraser, 1956).

TABLE 8

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 |
| 8 | 20.5 |
| 9 | 25.5 |
| 10 | 27 |
| 11 | 30.25 |
| 12 | 36 |
| 13 | 38 |
| 14.5 | 47 |
| 15.5 | 65 |
| 16.5 | 84.5 |

*After Rugh, 1968.

The 10% exposure level was chosen because it approximates the highest level of nitrous oxide to which operatory personnel would be exposed on a chronic basis (Cohen, et al., 1975), with the exception of self-administration. It was decided to use a 24 hour per day exposure, rather than the eight hours per day that operatory personnel might receive, in order to maximize the exposure time during the period of palatal and nasomaxillary proliferation. This increased exposure time was intended to heighten any potential effects on craniofacial development, thereby making those effects more readily apparent so that they could be detected utilizing the methods employed in this investigation.

The cephalometric study revealed several significant differences in the skull dimensions of animals exposed to nitrous oxide. Specifically, the exposed animals showed an increased neurocranial length and mandibular length, while the premaxilla was shorter and the entire maxillary complex positioned more posteriorly. It is significant to note that the overall length of the skull did not differ between the two groups. One possible explanation for the manner by which this group of disharmonious elements combined to form an overall structure, the skull, of the same size as in the control group lies in the process of compensation. Enlow (1975) defined compensation as "a feature of the developmental process; it provides for a certain latitude of imbalance in some areas to offset the effects of disproportions in other areas." In this experiment, the nitrous oxide exposure

might have caused a disproportion in one area of the skull, while the process of compensation provided a biologic mechanism by which another area was changed to offset the original imbalance.

The cleft palate portion of this experiment was also planned to be undertaken at both 10% and 50% nitrous oxide, but the essentially negative effect of exposure at the maximal level made it unnecessary to repeat this portion of the experiment at the lower dose. It should be noted that this negative result is, nonetheless, significant. The exposure level of nitrous oxide was so great that 94% of the offspring died, yet the cleft palate rate was within the reported range of spontaneous cleft formation. It can therefore be stated that nitrous oxide does not increase the incidence of cleft palate formation in the A/J mouse. Of course, extrapolation of the results of this study to possible human cleft formation should be done with caution.

For many years, nitrous oxide was considered to be an "inert" gas, with respect to its long term effects upon the human body. Numerous reports of animal and human studies in recent years have proven this supposition to be false. Nitrous oxide has the ability to affect rapidly proliferating cells, as shown by its depressive effect on hemopoiesis in both animals and humans, and by the congenital malformations seen in the offspring of pregnant animals exposed to nitrous oxide. This gas can also affect some mature cells, or their actions, in the adult

individual, as shown by its pathological effects upon the nervous systems of humans and animals.

The exact mode of action by which nitrous oxide produces these deleterious effects is unknown, but one possible explanation was revealed in the work of Ebert and Hornsey (1958), who produced evidence that so-called "inert" gases, such as nitrous oxide, compete with oxygen for access to specific but unidentified binding sites within the cell. By causing a decreased level of oxygen available to certain areas of the cell, any element of the entire range of cellular activities could be altered or destroyed. The specific binding sites that could be affected by the presence of nitrous oxide may be different in various types of cells, thereby causing the numerous effects reported in different systems of the body. The reported increase in the percentage of female offspring born to mothers exposed to nitrous oxide during gestation could also be due to the ability of this "inert" gas to selectively affect only male cells, although the results of this study did not show a male-female difference in cleft rate.

There is no immediate explanation for the high mortality rate seen in animals exposed to 50% nitrous oxide. The drug may have affected the nervous or hemopoietic systems, as reported in other experiments, or it might have caused some previously unreported pathology. It is also possible that the offspring were normal, but that the drug had affected the mothers.

Neonatal mice will die if not suckled shortly after birth. If nitrous oxide caused a change in the lactation process of the mothers, the result would be death of the offspring. Therefore, it should be noted that high mortality among the offspring does not necessicarily indicate that the drug had a direct effect upon the offspring.

Future studies of maternal nitrous oxide exposure should attempt to determine whether the lethality is due to the effect of the drug upon the offspring or upon the mothers. An examination of the offsprign of those animals previously treated during the fetal period, the F2 generation, should also be included in future studies, in order to determine the tendency for this drug to affect subsequent generations.

## SUMMARY AND CONCLUSIONS

Nitrous oxide has been implicated as a possible teratogenic agent in studies of pregnant women exposed to this gas. Experiments utilizing laboratory animals have shown a definite relationship between maternal nitrous oxide exposure and congenital defects in the offspring. No studies have attempted to determine the effect of nitrous oxide on cleft palate formation or to characterize any specific craniofacial changes caused by exposure to this gas.

This study was performed to evaluate the effect of nitrous oxide exposure of the craniofacial growth of 158 A/J mice whose mothers lived in an atmosphere containing this gas for five days during the gestation period. A control group of 71 mice was also evaluated. A separate sample of 95 experimental and 115 control fetuses was used to investigate the effect of nitrous oxide of the incidence of cleft palate formation.

Three catagories of data were collected for this study:

(1) Body weight, physical condition, and general appearance

(2) Incidence of clefting

(3) Roentgenographic cephalometric measurements of the craniofacial complex.

Nitrous oxide exposure at the time periods and concentrations used produced no gross malformations in the offspring. There was no significant difference in body weight between the control and experimental offspring. The experimental offspring had a slightly higher rate of cleft palate formation than the control offspring. This difference on cleft rate between the control group and the experimental group was statistically significant at the .05 level. However, since both the control and experimental cleft rates fall within the previously reported range of spontaneous cleft palate formation in A/J mice, this difference in cleft rates was considered to be insignificant. The roentgenographic cephalometric study indicated that certain areas of the craniofacial complex were significantly altered in association with exposure to nitrous oxide, although the overall dimensions of the skull were unchanged.

Previous studies of nitrous oxide exposure in experimental animals, and surveys of pregnant women exposed to anesthetic gases including nitrous oxide, indicated that this gas might possess the potential to cause craniofacial deformities. The results of this study indicate that nitrous oxide exposure in A/J mice does not significantly increase cleft palate formation, but does cause significant dimensional changes in structural elements of the skull.

## LITERATURE CITED


Andrew, F.D., Bawen, D., and Zimmerman, E.F.: Glucocorticoid inhibition af RNA synthesis and the critical period for cleft palate induction in inbred mice, Teratology 7: 167-176, 1972.

Askrog, V., and Harvald, B.: Teratogen effects of inhalation anesthesia, Anestetika Saertryk Nord. Med. 83: 49, April, 1970.

Atherton, J.D.: A descriptive study of the face in human fetus with unilateral cleft lip and palate, Cleft Palate J. 4: 104-114, 1967

Berkowitz, B.A., and Ngai, S.H.: Nitrous oxide "analgesia": resemblance to opiate action, Science 194: 967-968, 1976.

Broadbent, B.H., Sr., Broadbent, B.H., Jr., and Golden, W.H.: Bolton Standards of Dentofacial Developmental Growth, St. Louis: C.V. Mosby Co., 1975.

Cohen, E.N., Bellville, J.W., and Brown, B.W.: Anesthesia, pregnancy, and miscarriage: a study of operating room nurses and anesthetists, Anesthesiology 35: 343-347, 1971.

Cohen, E.N., Brown, B.W., and Bruce, D.L.: A survey of anesthetic health hazards among dentists: report of an ad hoc committee of the effects of trace anesthetic agents on the health of operating room personnel, J. Am. Dent. Assoc. 90: 1291-1296, 1975.

Corbett, T.H., Cornell, R.G., and Endres, J.L.: Effects of low concentrations of nitrous oxide on rat pregnancy, Anesthesiology 39: 299-301, 1973.

Corbett, T.H., Cornell, R.G., and Endres, J.L.: Birth defects among the children of nurse-anesthetists, Anesthesiology 41: 341-344, 1974.

Eastwood, D.W., Green, C.D., and Lambdin, M.A.: Effect of nitrous oxide on the white-cell count in leukemia, New England J. of Med. 268: 297-299, 1963.

Ebert, M., and Hornsey, S.: Effect of inert gases on oxygen-dependent radiosensitivity, Nature 181: 613-616, 1958.

Enlow, D.H.: Handbook of Facial Growth, Philadelphia: W.B. Saunders Co., 1975.

Fink, B.R., Shepard, T.H., and Blandau, R.J.: Teratogenic activity of nitrous oxide, Nature 214: 146-148, 1967.

Fraser, F.C., and Fainstat, T.D.: Production of congenital defects in the offspring of pregnant mice treated with cortisone, Pediatrics 8: 527-533, 1951.

Fraser, F.C., Kalter, H., Walker, B.E., and Fainstat, T.D.: The experimental production of cleft palate with cortisone and other hormones, J. Cell. and Comp. Physiol. 43: Suppl 1: 237-259, 1954.

Gange, R.J., and Johnston, L.E.: The septopremaxillary attachment and midfacial growth, Am. J. Orthod. 66: 71-81, 1974.

Goldstein, D.B., and Pal, N.: Alcohol dependence produced in mice by inhalation of ethanol: grading the withdrawl reaction, Science 172: 288-290, 1971.

Green, C.D., and Eastwood, D.W.: Effects of nitrous oxide inhalation on hemopoiesis in rats, Anesthesiology 24: 341-345, 1963.

Greene, R.M., and Kochhar, D.M.: Some aspects of cortocosteroid-induced cleft palate: a review, Teratology 11: 47-56, 1975.

Guilford, J.P.: Fundamental Statistics in Psychology and Education, New York: McGraw-Hill, Inc., 1965.

Hayden, J., Allen, G.D., Butler, L.A., Lewis, G.B., and Schultz, R.: An evaluation of prolonged nitrous oxide-oxygen sedation in rats, J. Am. Dent. Assoc. 89: 1374-1380, 1974.

Humphrey, T.: The relation between human fetal mouth opening reflexes and closure of the palate, Am. J. Anat. 125: 317-344, 1969.

Jacobs, R.M.: Normal closure of secondary palate in paralyzed mouse embryos, J. Dent. Res. 49: 1495-1497, 1970.

Jacobs, R.M.: Failure of muscle relaxants to produce cleft palate in mice, Teratology 4: 25-30, 1971.

Jelinek, R., and Dostal, M.: Inhibitory effect of corticoids on the proliferative pattern in mouse palatal processes, Teratology 11: 193-198, 1974.

Kalter, H.: Sporadic congenital malformations of newborn inbred mice, Teratology 1: 193-200, 1968.

Kalter, H.: How should times during preganacy be called in teratology, Teratology 1: 231-234, 1968.

Kieler, J.: Cytotoxic effect of nitrous oxide at different oxygen tensions, Acta Pharmacol. et Toxicol. 13: 301-308, 1957.

Knill-Jones, R.P., Moir, D.D., Rodrigues, L.V., Spence, A.A.: Anesthetic practice and pregnancy: controlled survey of women anesthetists in the United Kingdom, Lancet 1: 1326-1328, 1972.

Kochhar, D.M., and Johnson, E.M.: Morphological and autoradiographic studies of cleft palate induced in rat embryos by maternal hypervitaminosis A, J.Embryol. Exp. Morph. 14: 223-238, 1965.

Langa, H.: Relative Analgesia in Dental Practice, Philadelphia: W.B. Saunders Co., 1976.

Lassen, H.C., Henriksen, E., Kristensen, H.S., and Neukirch, F.: Treatment of tetanus: severe bone marrow depression after prolonged nitrous oxide anesthesia, Lancet 1: 527-530, 1956.

Latham, R.A.: The pathogenesis of the skeletal deformity associated with unilateral cleft lip and palate, Cleft Palate J. 6: 404-414, 1969.

Layzer, R.B.: Myeloneuropathy after prolonged exposure to nitrous oxide, Lancet 2: 1227-1230, 1978.

Linde, H.W., and Bruce, D.L.: Occupational exposure of anesthetists to halothane, nitrous oxide, and radiation, Anesthesiology 30: 363-368, 1969.

Marx, G.F.: Placental transfer and drugs used in anesthesia, Anesthesiology 22: 294-313, 1961.

Parbrook, G.D.: Leucopenic effects of prolonged nitrous oxide treatment, Brit. J. Anes. 39: 119-127, 1967.

Paulus, W.D.: The effect of maternal ethanol inhalation during pregnancy on the craniofacial development of the A/J mouse, Thesis, Department of Orthodontics, Case Western Reserve University, Cleveland, 1976.

Rector, G.H., and Eastwood, D.W.: The effects of an atmosphere of nitrous oxide and oxygen on the incubating chick, Anesthesiology 25: 109, 1964.

Ross, L.M., and Walker, B.E.: Movement of palatine shelves in untreated and teratogen treated mouse embryos, Am. J. Anat. 121: 509-522, 1967.

Rugh, R.: The Mouse - Its Reproduction and Development, Minneapolis: Burgess Co., 1968.

Small, S.C.: The status of nitrous oxide in dentistry, Oral Health 63: 6-14, 1973.

Smith, B.E., Gaub, M.L., and Moya, F.: Teratogenic effects of anesthetic agents: nitrous oxide, Anes. and Analg. 44: 726-734, 1965.

Snedecor, G.W., and Cochran, W.G.: Statistical Methods, Sixth Edition, Ames, Iowa: Iowa State University Press, 1967.

Vaisman, A.: Working conditions in surgery and their effect on the health of anesthesiologists, Eksp. Khir. Anesteziol. 3: 44-49, 1967.

Walker, B.E., and Fraser, F.C.: Closure of the secondary palate in three strains of mice, J. Embryol. Exp. Morph. 4: 176-189, 1956.

Walker, B.E., and Patterson, A.: Induction of cleft palate in mice by tranquilizers and barbiturates, Teratology 10: 159-164, 1974.

Whitcher, C., Zimmerman, C., and Tonn, E.: Control of occupational exposure to nitrous oxide in the operatory, NIOSH Contract No. CDC-210-75-0007, Dec., 1975.

Whitten, W.K.: Occurence of anoestrus in mice caged in groups, J. Endochrinology 18: 294-313, 1961.