To my Wife, Son and Parents
without whose help this work
would not have been possible.

EFFECT OF FASTING AND INSULIN ON THE SKELETAL DEVELOPMENT OF RATS

A Thesis

Presented to

The Faculty of Graduate Studies and Research

The University of Manitoba

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

by

Richard Stanley Hannah, B.Sc.

February 1970

EFFECT OF FASTING AND INSULIN ON THE SKELETAL DEVELOPMENT OF RATS

An Abstract of a Thesis

Presented to

The Faculty of Graduate Studies and Research

The University of Manitoba

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

by

Richard Stanley Hannah, B.Sc.

February 1970

The objective of this research was to study the effects, if any, of acute hypoglycemia on skeletal development in rat embryos. Hypoglycemia was produced by two methods; fasting and fasting plus protamine zinc insulin injections.

Groups of pregnant Holtzman rats were fasted for forty-eight hours at varying time intervals during early gestation. The most critical period for fasting was determined to be days nine to eleven postcoital, the major effect being retardation of ossification. However, anomalies such as supernumerary ribs and duplicated vertebral centra were also observed more often than usual.

The experiment involving fasting plus insulin injections resulted in variations in ossification rate similar to those with fasting alone, but there was an increased frequency of duplicated vertebral centra and supernumerary ribs.

ACKNOWLEDGEMENTS

I wish to thank Dr. Keith L. Moore for the opportunity to undertake this research in this department and for his generous help and guidance.

I also wish to thank my wife, Kathryn and son, Richard for their unselfish understanding during the last three years.

I greatly appreciate the capable assistance provided by the following persons: Dr. J. Moorehouse for technical assistance in blood glucose determinations, Mr. G. Reid for illustrations, Miss B. Propkop for photographic assistance and Mr. E. Clune and Mr. G. Lawson for assistance in animal care.

TABLE OF CONTENTS

CHAPTER		PAGE
I	INTRODUCTION	1
II	REVIEW OF THE LITERATURE	3
	Effect of Fasting on Embryos	4
	Effect of Fasting on Pregnancy	5
	Effect of Fasting on Metabolism	7
	Effect of Fasting on Blood Sugar in Mice	7
	Exogenous Insulin in Rats	8
	Exogenous Insulin in Mice	8
	Exogenous Insulin in Chickens	9
	Insulin and Pregnancy in Humans	9
	Placental Transmission of Insulin	10
	Intrauterine Development of the Rat	11
	Definition of Terms	13
III	MATERIALS AND METHODS	15
	Experimental Animals	16
	Environment Chamber	17
	Method of Treatments	17
	Blood Samples	19
	Caesarean Section	21
	Staining	22
	Examination of Specimens	22
	Statistical Analysis Used	22

CHAPTER		PAGE
IV	RESULTS	23
	Introduction	24
	Length of Fetuses	24
	Fetal Weight	27
	Absent Cervical Vertebral Centra	27
	Retarded Odontoid Processes	30
	Duplicated Thoracic Vertebral Centra	30
	Live Born Fetuses	35
	Resorptions	35
	Retarded Fifth Sternebral Centre	38
	Fifth Sternebra Misshapen	38
	Sternebrae Misshapen, Excluding the Fifth Sternebra	41
	Lumbar Supernumerary Ribs	44
	Asynchronous Cervical Vertebral Centra	47
	Abnormal Anterior Arch of the Atlas	50
	Effect of Acute Fasting on Maternal Blood Glucose	53
	Effect of Acute Fasting plus Protamine Zinc Insulin on Maternal Blood Glucose	53
	Effect of Protamine Zinc Insulin on Maternal Blood Glucose	58
	Retrospective Criticism of Analysis	60
	Summary of Results	61

CHAPTER		PAGE
V	DISCUSSION	63
	Environment	65
	Fasting as a Teratogen	66
	Vitamin Deficiency	68
	Effect of Acute Fasting on Abortion	69
	Fasting plus Insulin as a Teratogen	73
	Insulin and Saline as Teratogens	74
VI	SUMMARY OF RESULTS	75
BIBLIOGRAPHY		77
APPENDIX		82
FIGURES		86

I INTRODUCTION

Runner (1959) studied the effects of acute hypoglycemia on mice embryos. He concluded that hypoglycemia produced its effects on the embryo by interrupting the tricarboxylic acid cycle thereby depleting available substrate. Runner proposed that the normal functioning of the tricarboxylic acid cycle was critical for the formation of precartilaginous mesenchyme.

The objective of this research was to study the effects of acute hypoglycemia on the skeletal development of rat embryos. Hypoglycemia was produced by two methods. A single 48 hour period of fasting was imposed on pregnant rats, for varying times during early gestation. To produce more severe hypoglycemia, protamine zinc insulin was administered to fasting pregnant animals.

Runner (1959) observed that some drugs which stressed carbohydrate metabolism, produced an additive effect when administered during a fasting state in mice. In this research, insulin was administered during fasting to determine if an additive effect occurs in rats.

Although it is unlikely that pregnant women would fast themselves for approximately three weeks, it is possible that nausea occurring during the first two months of pregnancy may result in a depletion of carbohydrate stores. This effect may accentuate the teratogenicity of a drug taken during this time period while the woman is unaware of her pregnancy.

II REVIEW OF THE LITERATURE

Effect of Fasting on Embryos

Runner and Miller (1956) studied the effects of fasting on embryonic mice. They observed that during days eight and nine post coitum the embryos were most vulnerable to the effects of fasting. Anomalies such as malformed vertebral centra, supernumerary ribs and exencephaly were observed. Almost complete protection was afforded to the embryos by administering small quantities of carbohydrate or amino acid during the fasting period.

In 1959, Runner studied the types of nutrients that would protect the embryonic mice during a twenty-four hour fast on days eight and nine. The nutrients were glucose, ketone bodies and amino acids such as methionine and glutamic acid, all of which provide substrates for the tricarboxylic acid cycle (TCA). The effect of fasting would be to slow down the TCA cycle by depleting the amount of available substrate. Runner also administered TCA cycle blocking agents such as insulin, x-methyl folic acid and iodoacetate, all of which produced anomalies similar to those produced by fasting. Runner suggested that normal carbohydrate metabolism is critical for morphogenesis of the neural tube and for differentiation of precartilaginous mesenchyme.

Runner (1964) postulated that a decrease in energy production by the TCA cycle, due to lack of available substrate, affects either the rate of protein synthesis or cell membrane

permeability. Such action on the cell could alter the affect of an organizer or inductor, thus producing an abnormal condition.

Effect of Fasting on Pregnancy

McClure (1958) fasted pregnant mice for a forty-eight hour period, from the fourth to the sixth day post coitum, during which time implantation occurs. As a result of this treatment McClure observed complete embryonic mortality. Starvation for a forty-eight hour period later in gestation caused fewer deaths. By feeding only starch and sucrose in place of the regular food during the forty-eight hour periods, he was able to maintain a larger number of pregnancies. This effect was not obtained when egg white was used in lieu of starch and sucrose. This suggested that the carbohydrate fraction of food may be the critical element during this period of pregnancy. A histological study of mice uteri, fasted from two to four days by McClure (1961 a), showed an initial haemorrhage and necrosis of the decidua resulting in the secondary death of the embryos. This was followed by a leukocytic invasion with liquifaction and absorption of the debris.

McClure found that periods of at least seventy-two hours were necessary to produce comparable results in rats but shorter periods of fasting did reduce the littering rate (McClure 1961 b).

There are two major hypotheses concerning the etiology of haemorrhaging of the decidua. One is that the stress of fasting stimulates hypersecretion of corticosterone. Robson and

Sharaf (1952) and Macfarlane et al (1957) produced embryonic mortality in rats by administering large doses of ACTH and cortisone. However, McClure (1961 b) using adrenalectomized rats showed that the adrenal cortex was not involved to any great extent with embryonic mortality. Normal rats and adrenalectomized rats fasted for the same time period showed comparable degrees of embryonic mortality.

The alternate hypothesis concerns a failure in gonadotrophic function of the adenohypophysis. Pomerantz and Mullinos (1939) and Werner (1939) produced pseudohypophysectomy by starvation. In 1949, Rinaldini observed a decrease of gonadotrophin content in the pituitary upon chronic inanition. McClure (1961 b) fasted pregnant mice for a forty-eight hour period from the third to the fifth day. By administering small amounts of either progesterone or chorionic gonadotrophin, he was able to maintain the pregnancies for two days longer (before the animals aborted). McClure suggests that by using the right method and dose of these hormones, pregnancy could be maintained to full term. One possible explanation of this phenomenon is the effect of hypoglycemia on the nervous system. McIlwain (1959) states that hypoglycemia 30 percent to 37.5 percent below normal levels is sufficient to cause changes in electrical conductivity in the central nervous system. Puskarev (1964) noted that in cats hypoglycemia disturbed the function of ganglionic structures and presynaptic endings. It is possible that this effect on

the central nervous system may inhibit the gonadotrophin releasing factors from the adenohypophysis.

McClure concludes, for mice at least, that embryonic mortality produced by short term fasting is caused by a depressed hypophyseal function.

The Effect of Fasting on Metabolism

Wood et al (1960) showed that gluconeogenesis is accelerated during fasting and endogenous lipid stores replace exogenous carbohydrate as the major source of fuel for TCA cycle.

In a study by Buchanan and co-workers (1969) insulin levels were shown to decrease during starvation, thus removing part of the block on gluconeogenesis provided by high insulin levels and allowing the animal to maintain a relatively constant blood glucose level.

Effect of Fasting on Blood Sugar in Mice

McClure (1967) reported that, by removing the food from pregnant mice for a forty-eight hour period, the blood glucose level dropped to one-half of the "normal" value within four hours and remained at that level until the food was returned to the animals. With feeding resumed, the blood sugar level rapidly rose to normal values for that time of pregnancy.

Exogenous Insulin in Rats

Lichtenstein et al (1951) administered subcutaneously, seven to eight units of protamine zinc insulin daily to pregnant albino rats throughout their pregnancies. This treatment produced very large mortality rates among the mothers, varying from 15 percent to 75 percent at different times during the experiment. Observations made on the fetuses of surviving mothers, showed significant differences from the non-treated control group in the following parameters: lower weight, more resorptions, decrease or absence of ossification centres of the sternum, and irregularities in shape of ribs and long bones.

Exogenous Insulin in Mice

McClure (1967) injected pregnant albino mice with varying doses of protamine zinc insulin from the first to the fifth day of pregnancy and reported a significant decrease in the littering rate.

Smithberg et al (1956) injected pregnant albino mice intraperitoneally with protamine zinc insulin (0.1 unit) at eight and one-half days post coitum. They noted abnormalities such as exencephaly, umbilical hernia, and fusion of ribs in the treated animals. Sixty-three percent of the treated animals had one or more of these conditions while only five percent of the control animals were affected.

Exogenous Insulin in Chickens

Landaurer (1948) injected insulin into the yolk sac of chick embryos and produced rumplessness and abnormalities of the beak, eyes and extremities.

Duraiswami (1950) produced skeletal defects in chickens by injecting crystalline insulin into fertilized eggs from the first to the sixth days. He observed that the growth rate of the embryo was retarded in direct proportion to the dose of insulin administered.

Insulin and Pregnancy in Humans

Insulin coma therapy is used to treat human emotional disturbances such as schizophrenia. Wickes (1954) reported several cases of pregnant women who received insulin coma therapy during the first ten weeks of pregnancy, resulting in many severe fetal malformations, for example, skull deformities, mental defects and optic atrophy. Sobel (1960) reported that out of a total of 17 insulin coma therapy treated mothers, there resulted a 35.3 percent frequency of fetal damage similar to that observed by Wickes (1954). This compares unfavorably to the control group of 202 women, in which the frequency of fetal damage was only seven percent. Sobel also reported that fetal malformations were observed when the mothers were treated up to the fourteenth week of pregnancy, which is a longer period of susceptibility than reported by Wickes (1954).

It is not known whether the malformations caused by insulin coma therapy were due to hypoglycemia, anoxia, or insulin itself. At the present time, there is no definite evidence in the literature that a pharmacological dose of insulin, used for example in the treatment of diabetes mellitus, is teratogenic in humans.

Placental Transmission of Insulin

In 1932, E.L. Corey examined placental permeability of insulin in albino rats, using methods crude by today's standards. He proposed that in the latter third of gestation, the placenta is highly permeable to insulin from mother to fetus. However, recent work, described in the next two paragraphs, has proven Corey's early observations to be in error.

Goodner and Freinkel (1961) injected I^{131} labelled bovine insulin into pregnant albino rats, by using single injections and constant infusion techniques. They found that little or none of the labelled insulin crossed the placenta into the fetal circulation. They also noted intraplacental sequestration of insulin.

Clark et al (1968) working with albino rats, measured immunoreactive insulin levels in mother and fetus while injecting varying doses of insulin into the maternal circulation. The corresponding rise in maternal insulin was not observable in the fetus. This would seem to be substantial proof that exogenous

insulin does not cross the placenta from mother to fetus.

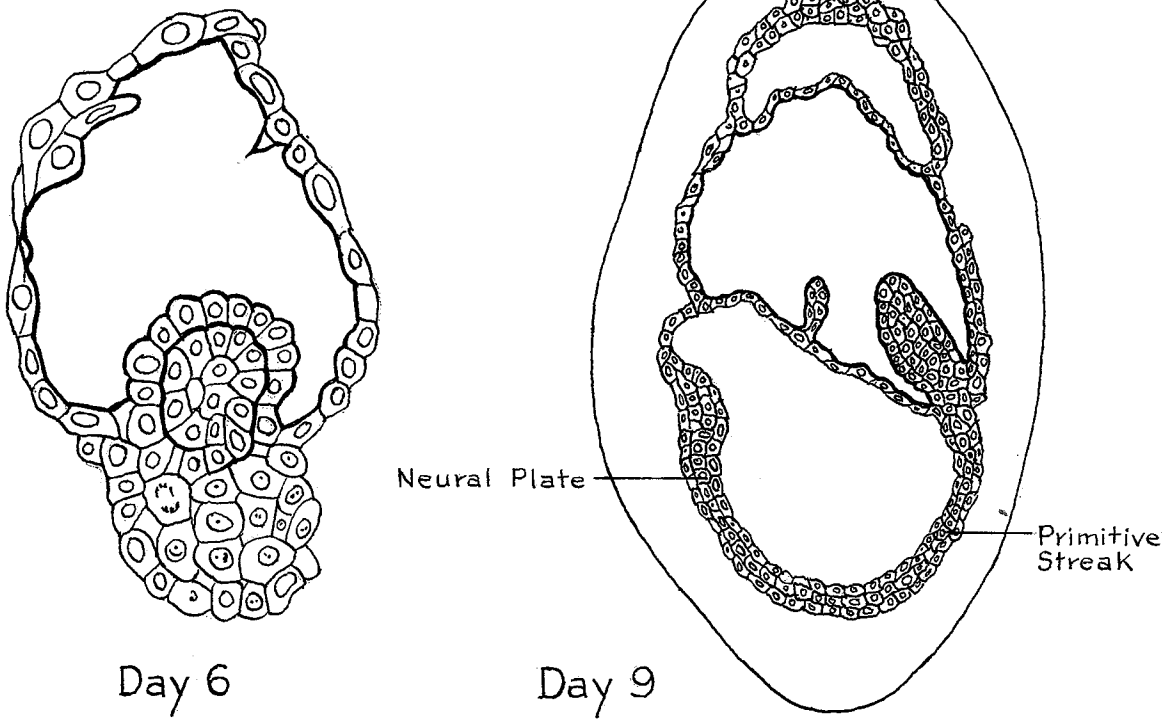
Glycogen stores in the rat embryo during early gestation are known to be limited. Therefore any decrease in maternal blood glucose would rapidly affect the fetus. Since the placental barrier prevents any direct influence of maternal insulin on the fetus, maternal hypoglycemia might produce embryonic defects by this indirect method, Goodner et al (1969).

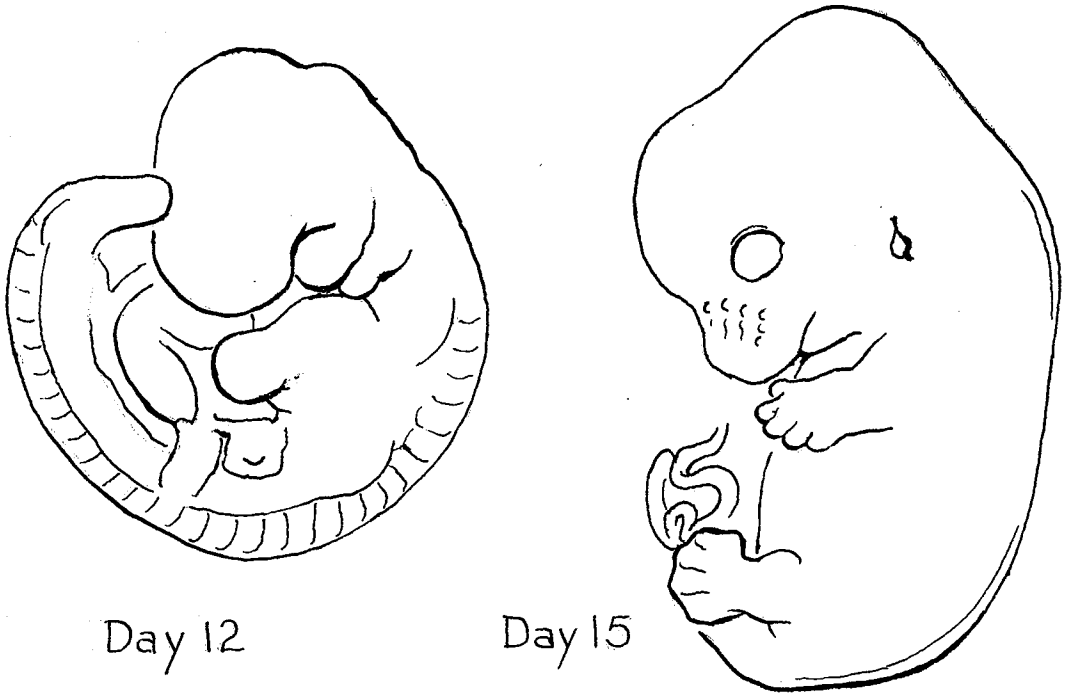
Intrauterine Development of the Rat

Early development of the rat (days 7-13) will be outlined, according to Witschi (1962), (see figure 1), to show stages of gestation when treatments were performed in this study. During days 7 to 9 implantation occurs and the primitive streak becomes prominent. From days 9 to 11, the neural tube begins rapid development. By day 11 somites 1-25 (lower thoracic) have appeared and the arm and leg buds are recognizable. During days 11 to 13 somites 26-48 (caudal) develop and by day 13 the embryo is well formed.

Wright et al (1958) studied rat prenatal skeletal development in the Wistar rat. Fetuses were stained with toluidine blue for cartilage and alizarin red for bone. The first appearance of cartilage was at 15 days in the third to the ninth ribs. Bone first appeared in the embryo at 15 days in the body of the mandible. The majority of bones appeared during days 15½ to 16. The first sign of ossification of vertebral arches appeared in the first

FIGURE 1





Four Stages in the Development of the Rat Embryo.

cervical arch and proceeded caudully. By day 21 all arches except the more distal caudal ones showed some signs of ossification.

The ossification of the vertebral bodies first appeared in the mid-thoracic region (thoracic 4-13) on day 18, and proceeded in both directions but more rapidly in the rostral direction. By day 21 all the bodies were ossified except the first and second cervical vertebral bodies and the most distal caudal bodies.

Complete cartilage models of all ribs were observed to be present by day 17½.

The first two sternabrae were the first to ossify (day 19). Sternabrae three and four and the xiphoid process ossified one-half day later. The fifth sternabrae was not ossified until day 20.

Definition of Terms

There is great variability in the literature, in defining the severity of teratological defects. For the purpose of this work the following definitions were used:

1. Variations

Variations are slight deviations from "normal" development, of a structure or in the timetable of development, which may not persist to the time of maturity of the animal. As an example, in this research, a decrease in the rate of fetal ossification has

been considered as a variation or deviation of development.

2. Anomalies

Anomalies are considered as abnormalities, which may usually persist until maturity and represent a marked deviation of development. Anomalies usually do not handicap or threaten the life of the animal. As an example, in this research, supernumerary ribs were defined as anomalies.

3. Congenital Malformations

Congenital malformations are serious developmental abnormalities and persist throughout the lifetime of the animal and usually severely handicap or threaten its life, e.g. cardiac abnormalities. No congenital malformations were observed in this research.

III MATERIALS AND METHODS

Experimental Animals

Female albino rats, of the Holtzman strain, Madison, Wisconsin were used. This strain is descended from the Sprague-Dawley strain.

Newly acquired animals were placed four to a cage and appropriate markings were made on their ears for identification. Using the random numbers table, animals were randomized into groups of ten.

Albino Holtzman males were placed with females at 1700 hours and separated at 0900 hours, the next morning. The estimated time of coitus was 0100 hours ± 8 hours. Therefore gestational age of the fetuses could be placed within this 16 hour period, (Everett and Sawyer, 1950).

At 0900 hours vaginal smears were obtained from each female, using the following method. The animal was secured by holding it in a vertical position with the left hand just under the forelimbs. A cotton swab, saturated with physiological saline (to reduce trauma to the vagina) was inserted into the vagina and rotated 360°.

The swab was rolled on a labelled glass microscope slide. The slide was then stained for one minute with Methylene Blue, dipped into water to remove excess stain and allowed to air dry. The stained specimen was examined under a microscope for the presence of spermatozoa. When spermatozoa were discovered, the female was considered pregnant, and in day one post-coitus which

corresponds to day one of gestation for the embryos, (see figure 4).

All animals were fed ad libitum a diet of "Victor Fox Food Cubes" supplemented with "RexAnd Feeding Oil". In accordance with experimental design, water was available at all times.

Environment Chamber

Pregnant animals were placed in separate cages, after their weight was recorded. The animals and cages were then placed in an environmental chamber where the temperature was maintained at 75°F ± 1° and relative humidity at 50 percent ± 20 percent.

The light cycle in the chamber consisted of 12 hours light from 0800 hours to 2000 hours and 12 hours darkness from 2000 hours to 0800 hours.

Method of Treatments

Fasting. Three groups of ten animals each were fasted for 48 hour periods at the following times; days 7-9 (Group I), days 9-11 (Group II) and days 11-13 (Group III) post coitum (see table 1).

The food was removed from the cage at 0900 hours on the appropriate day and returned 48 hours later at 0900 hours. Water was supplied continuously ad libitum. Weights were recorded for each animal at the beginning of the fast, after 24 hours and at the end of the fast. Blood sugar levels were recorded on control

TABLE 1

METHOD of TREATMENTS

Group	Days of Gestation	Treatment			
		Fast	Fast+Insulin	Insulin	Saline
I	7-9	+	-	-	-
II	9-11	+	-	-	-
III	11-13	+	-	-	-
IV	-	-	-	-	-
V	-	-	-	-	-
VI	9-11	-	+	-	-
VII	9-11	+	-	-	-
VIII	9-11	-	-	+	-
IX	9-11	-	-	-	+
X	-	-	-	-	-

Day 1 = Day sperm found

animals every 12 hours throughout the period of the fast.

Controls. Twenty control animals (Groups IV and V) were supplied with food and water ad libitum throughout pregnancy.

Protamine Zinc Insulin and Fasting. One group of ten animals (Group VI) was given a total of 0.4 units of protamine zinc insulin during a fasting period from days 7-9. 0.2 units of protamine zinc insulin was given to each animal at 0900 hours on day 7 and 0.2 units at 0900 hours on day 8 (table 1).

The protamine zinc insulin was diluted from stock solution of 40 units per cc to 1 unit per cc using sterile physiological saline. The injection was given subcutaneously in the dorsal region of the neck and shoulder.

Controls. Control animals consisted of three groups of ten animals each, in which one group (Group VIII) was given a total of 0.4 units of protamine zinc insulin, but not fasted, one group (Group IX) received 0.4 cc of saline and one group (Group X) fasted from days 7-9 (table 1).

Blood Samples

Blood samples of 0.2 cc each were obtained in the following manner. The animals were handled for approximately 2 minutes to make sure they were calm, and to prevent them from becoming excited later. They were then placed in a restraining cage.

The restraining cage (figure 2) was designed to ensure that the animals would neither become excited, nor feel con-

FIGURE 2

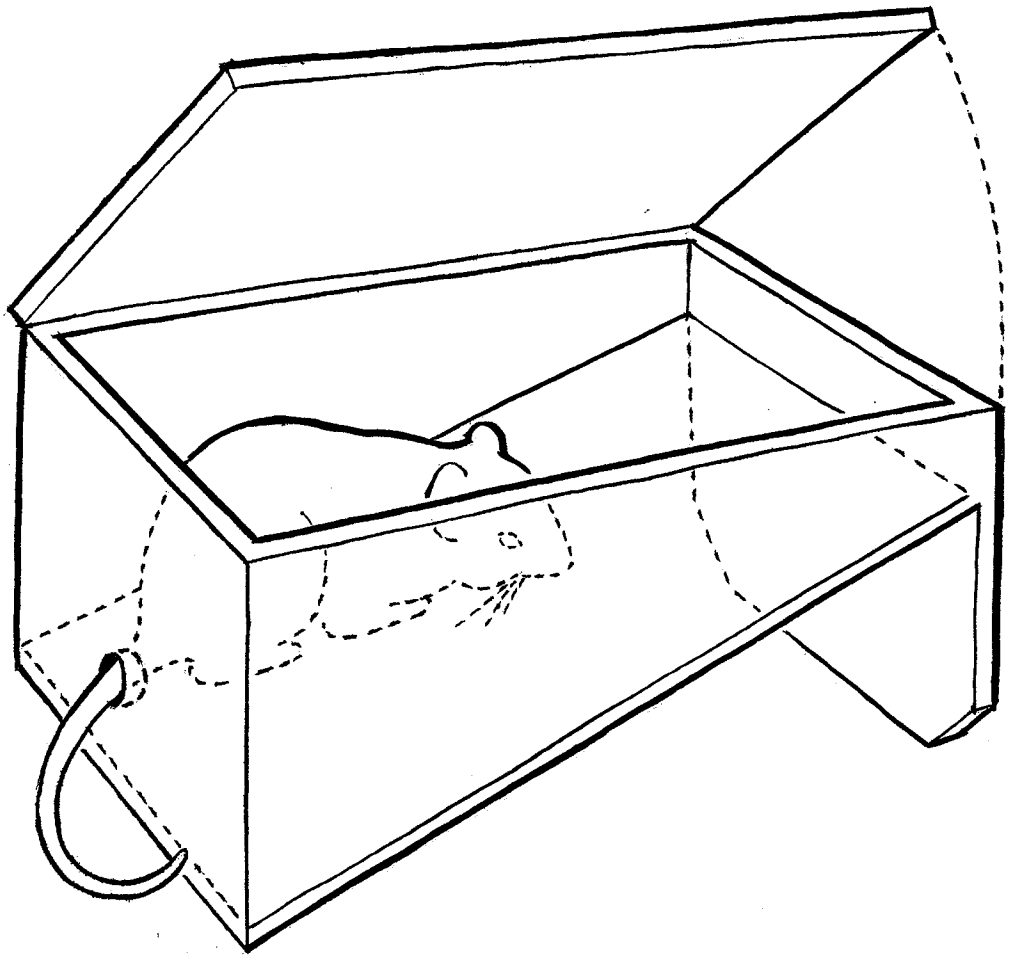


Diagram of Rat Restraining Cage

Used in Obtaining Blood Samples

strained during the procedure. This was accomplished by maintaining darkness in the cage by the use of the lid and by allowing room for lateral movement. As may be seen from the diagram, there was a hole in the end of the cage, 3/8" in diameter, through which the rat's tail was extended. The floor of the cage was on an incline of 40° and slippery to prevent the animal from forcibly pulling its tail out of the hole.

After the animal's tail was extended through the aperture, it was swabbed with ethanol for sterilization. Approximately 1 mm of the tip of the tail was removed with a razor blade and 0.2 cc of blood removed using a Unopette pipette. The blood sample was then analyzed using the method of Grant and Moorhouse (1966) in an autoanalyzer, (Techicon Instruments Corporation).

Caesarean Section

Caesarean sections were performed on pregnant females twenty-one days post coitus. A mid-line abdominal incision was made from the base of the sternum to the proximal edge of the vagina and the bi-cornuate uterus was disected from the abdominal cavity. The uterus was positioned on the table in a relatively straight line. On the side of the uterus opposite the placentae, a lengthwise incision from one terminal end to the other exposed the fetuses. Progressing from left to right, the fetuses were separated from the placentae by severing the umbilical cords. Each fetus was weighed, measured and evicerated and placed in

individual labelled glass jars containing 95 percent ethanol.

Staining

The fetuses were processed using Dawson's Alizarin Staining Technique. From the ethanol the fetuses were placed in acetone to remove the fat. This was followed by immersion in potassium hydroxide to clear the specimens. They were then stained with Alizarin stain which is specific for osseous formations and finally stored in glycerin.

Examination of Specimens

Examination of specimens took place under a disecting microscope.

Statistical Analysis Used

The data on bone development, was analyzed using an analysis of variance with a Duncan's Multiple-Range Test.

The blood glucose data was anlyzed using the Student "T" Test.

IV RESULTS

Introduction

The statistical analysis performed has been described in the Materials and Methods.

The statistically significant effects on developing rat embryos of maternal fasting and insulin injections have been summarized in Table 23.

Tables with the means and standard error of the means are included for all factors studied (tables 2 to 21), while graphs are included here only for those factors found to be statistically significant (graphs 1 to 20). Tables for those factors not found to be statistically significant may be found in the Appendix.

The points on graphs have not been joined because they are not linear components and joining them would be misrepresentative.

Factor - 1. Length of Fetuses (Tables 2 and 3, Graphs 1 and 2)

In Experiment I (table 2, graph 1), Group II (fast days 9-11) fetuses showed a statistically significant decrease in length from the control values. The general impression of the table and graph is that none of the groups differ significantly, however, a Student "T" Test was done which confirmed the finding shown by the analysis of variance.

As may be seen from Table 13, Group II fetuses were the most severely affected group in the fasting study. With such widespread variations and definite decreases in the rate of