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

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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.

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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.

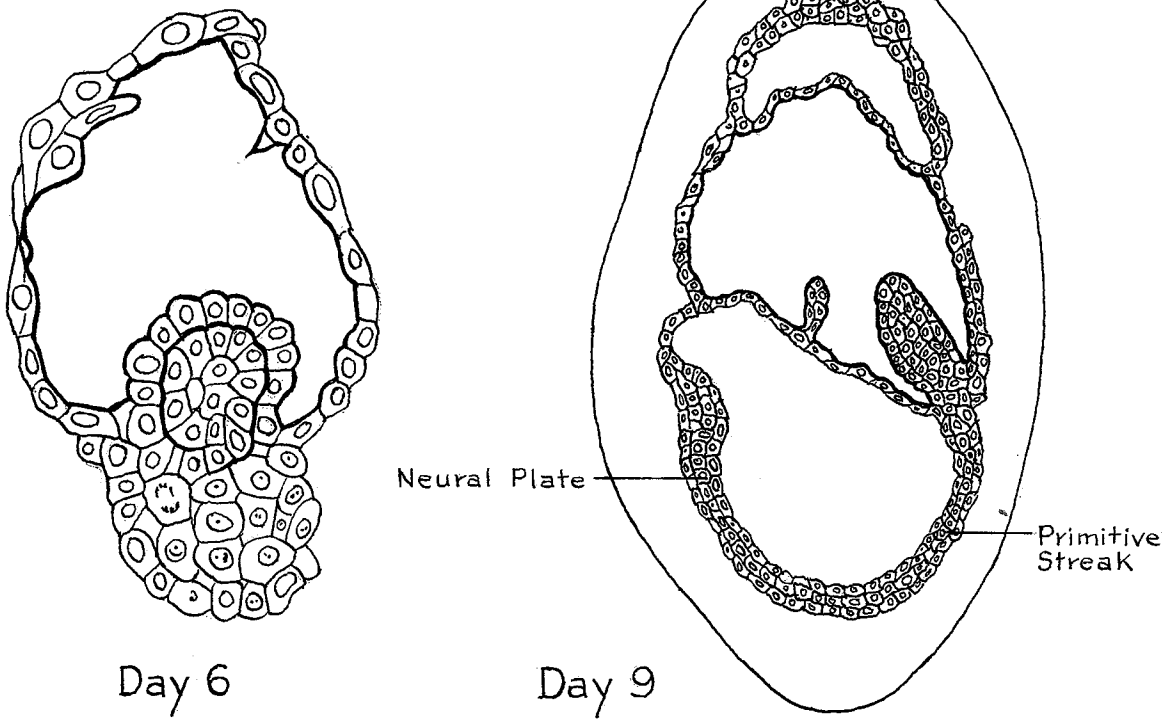
Glycogen stores in the rat embryo during early gestation are known to be limited. Therefore any decrease in maternal blood glucose would rapidly affect the fetus. Since the placental barrier prevents any direct influence of maternal insulin on the fetus, maternal hypoglycemia might produce embryonic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

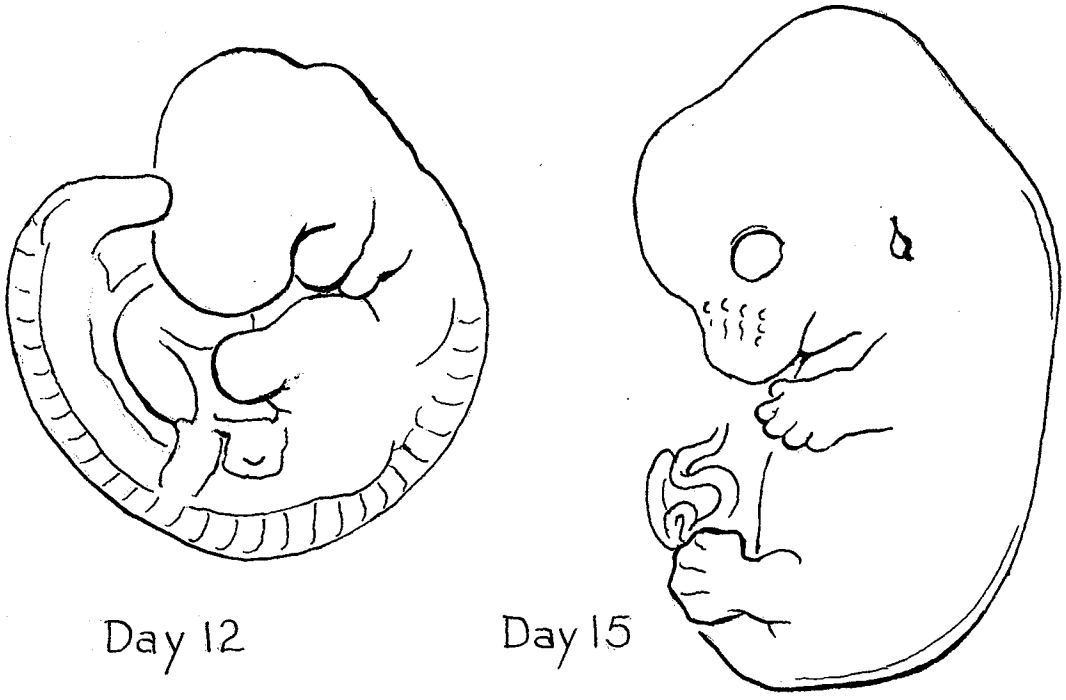
Intrauterine Development of the Rat

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

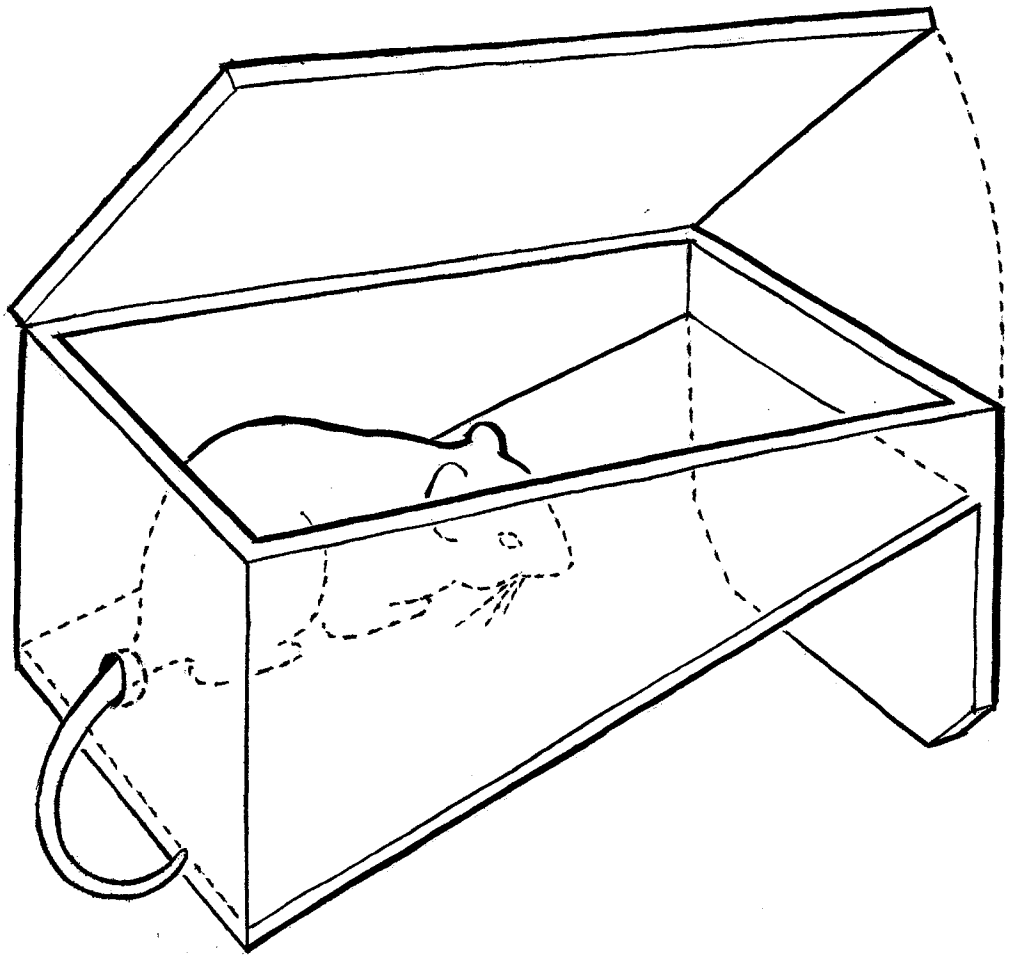


Diagram of Rat Restraining Cage

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

TABLE 2

Mean Length of Fetuses Per Group (F-1)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	42.80	1.62
II	Fast	9-11	41.80	1.99
III	Fast	11-13	42.30	1.06
IV	Control	-	43.50	0.97
V	Control	-	43.50	0.97

GRAPH 1

Experiment Number I

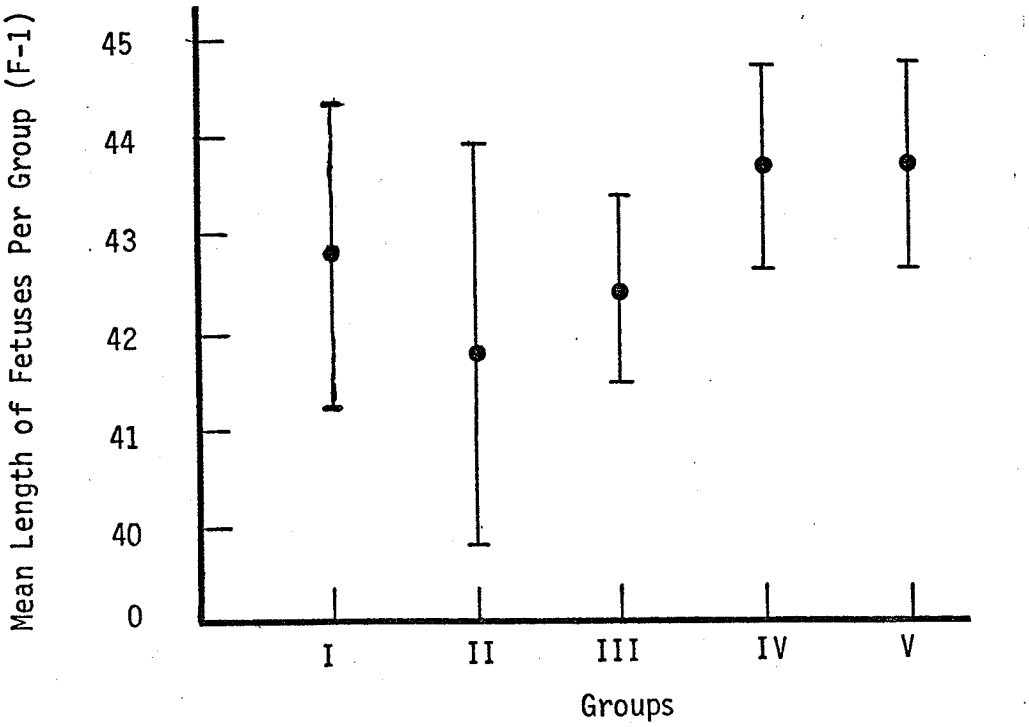


TABLE 3

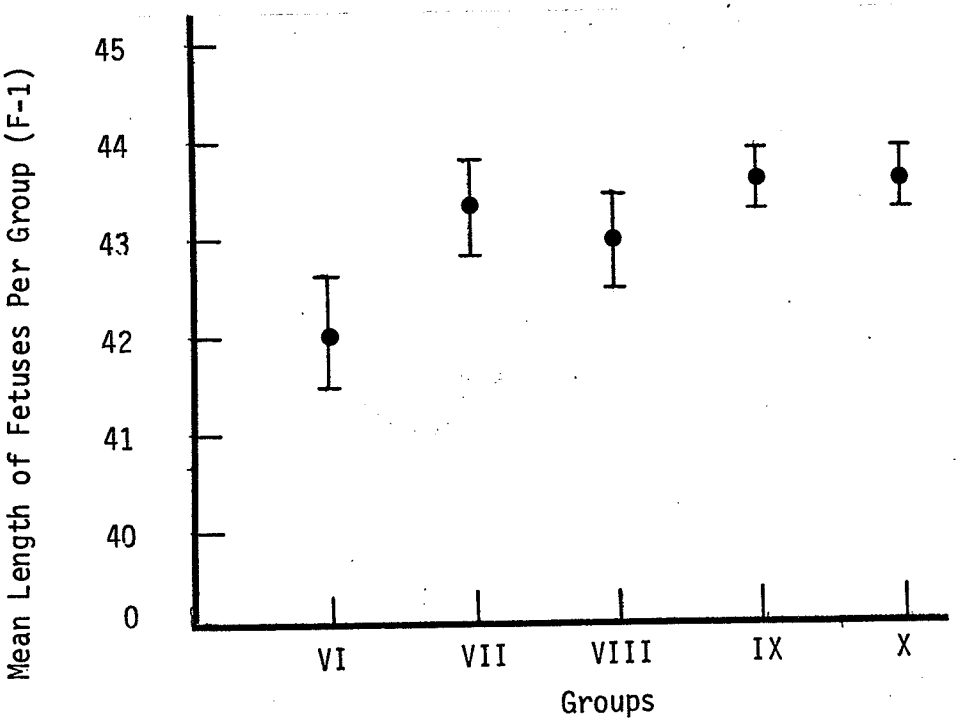
Mean Length of Fetuses Per Group (F-1)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	42.00	0.58
VII	Fast	9-11	43.20	0.47
VIII	PZI	9-11	43.00	0.42
IX	Saline	9-11	43.60	0.27
X	Control	-	43.60	0.27

GRAPH 2

Experiment Number II



ossification in this group, it should not be surprising that there is also a decrease in length.

The results from Experiment II (table 3, graph 2) show that length of fetuses is not affected in any group, including those whose mothers were fasted and yet there were about as many skeletal variations as in Experiment I. Therefore, despite the statistical findings, little emphasis may be placed on the affect of fasting on fetal length.

Factor - 2. Fetal Weight (Table A-1, See Appendix)

Fetal weight was not found to be statistically different in any of the groups studied.

Factor - 3. Absent Cervical Vertebral Centra (Tables 4 and 5, Graphs 3 and 4, Figure 5)

In Experiment I (table 4, graph 3) there was a statistically significant decrease in the number of cervical vertebral centra present in fetuses in Group II (fast days 9-11) and Group III (fast days 11-13). These findings indicate a delay in the rate of ossification. In Experiment II (table 5, graph 4) none of the fetuses in any group showed a significant decrease in the number of cervical vertebral centra present. All centra were present in the thoracic, lumbar or sacral regions.

TABLE 4

Mean Number of Absent Cervical Vertebral Centra Per Group (F-3)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	18.10	3.29
II	Fast	9-11	26.90	3.27
III	Fast	11-13	26.40	3.63
IV	Control	-	14.90	1.79
V	Control	-	13.60	2.19

GRAPH 3

Experiment Number I

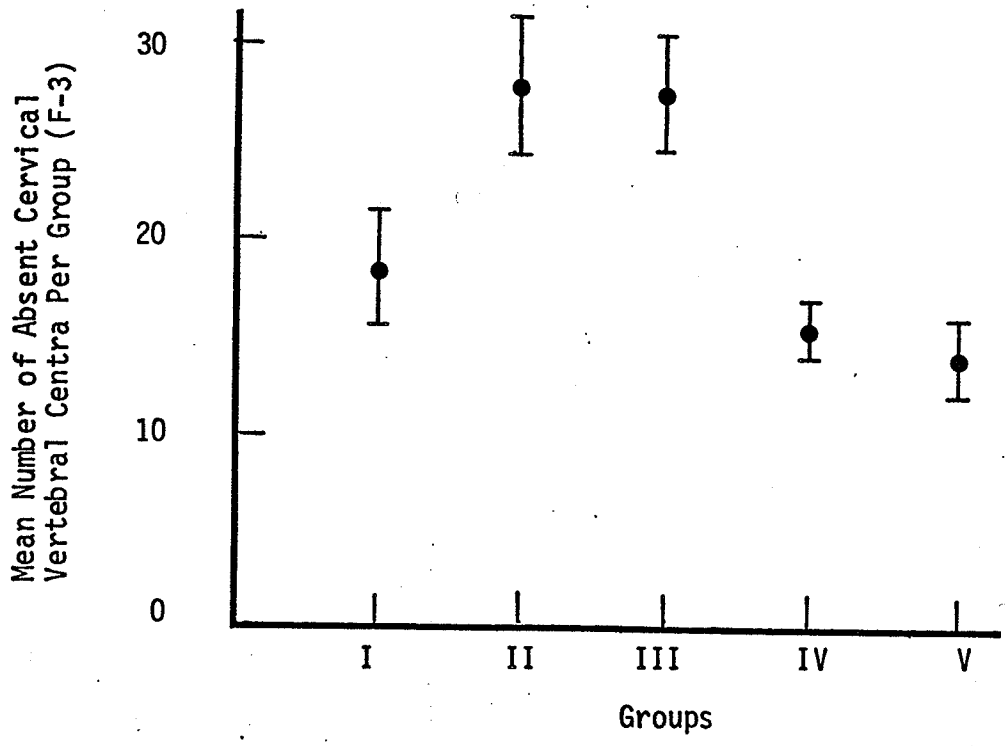


TABLE 5

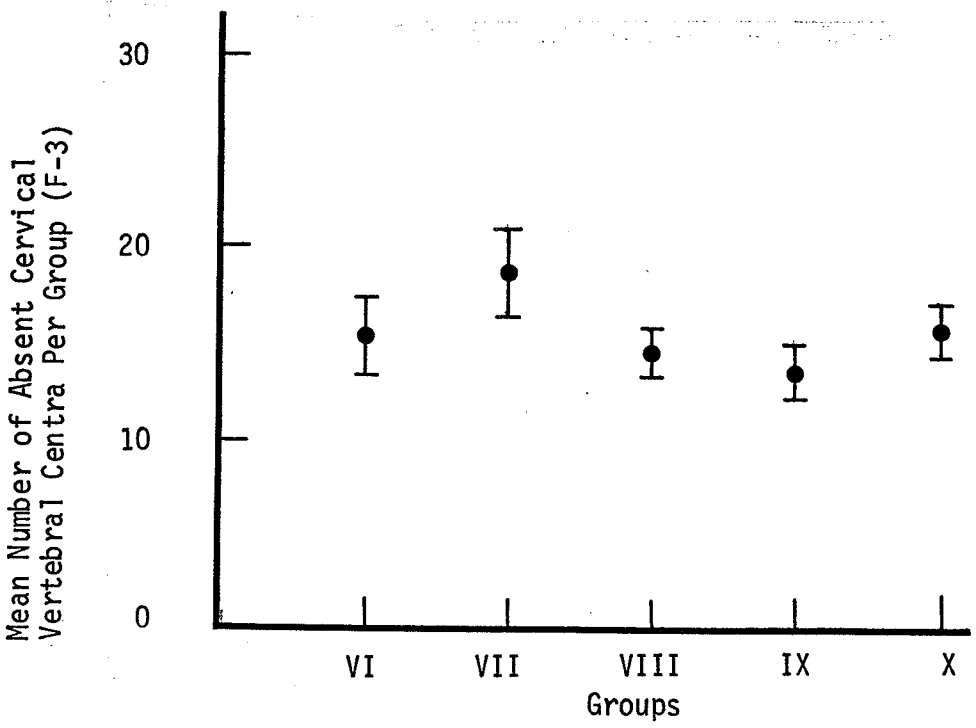
Mean Number of Absent Cervical Vertebral Centra Per Group (F-3)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	14.60	2.58
VII	Fast	9-11	18.40	2.29
VIII	PZI	9-11	13.10	1.10
IX	Saline	9-11	12.30	1.27
X	Control	-	14.10	1.30

GRAPH 4

Experiment Number II



Factor - 4. Retarded Odontoid Processes (Tables 6 and 7, Graphs 5 and 6, Figure 6)

In Experiment I (table 6, graph 5) the odontoid process of the second cervical vertebra showed a significant decrease in ossification in Group I (fast days 7-9) and Group III (fast days 11-13). There was no significant effect on ossification of the odontoid process in Group II (fast days 9-11), or in Experiment II (table 7, graph 6) which was conducted exclusively on days 9-11. However, as may be seen on Graph 6, there was a slight although insignificant decrease for those treatments (with the exception of Group IX, saline), occurring on days 9-11. Over the six day period of treatment the odontoid process was less susceptible to the effects of hypoglycemia on days 9-11 than on the other four days. It is possible that this effect was due to some other mechanism occurring over this time period, in this area, which provides some protection to the odontoid process.

Factor - 5. Duplicated Thoracic Vertebral Centra (Tables 8 and 9, Graphs 7 and 8, Figure 7)

In Experiment I (table 8, graph 7) the number of duplicated thoracic vertebral centra present were observed to be statistically different from normal (groups IV and V) for Group I (fast days 7-9) and Group II (fast days 9-11). Group III (fast days 11-13) did not have a significantly different number of duplicated centra from normal present.

In Experiment II (table 9, graph 8) duplicated vertebral

TABLE 6

Mean Number of Retarded Odontoid Processes Per Group (F-4)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	1.30	0.42
II	Fast	9-11	2.50	0.75
III	Fast	11-13	1.30	0.50
IV	Control	-	3.90	1.04
V	Control	-	4.10	0.81

GRAPH 5

Experiment Number I

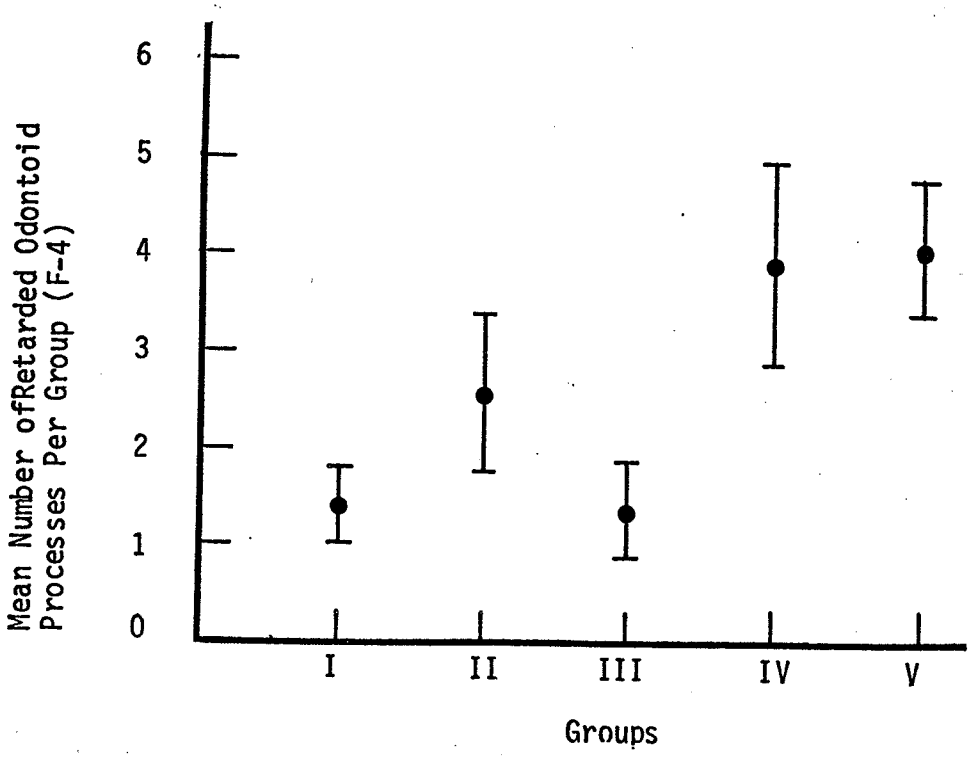


TABLE 7

Mean Number of Retarded Odontoid Processes Per Group (F-4)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	2.60	0.81
VII	Fast	9-11	2.70	0.67
VIII	PZI	9-11	2.20	0.20
IX	Saline	9-11	3.60	0.69
X	Control	-	3.20	0.61

GRAPH 6

Experiment Number II

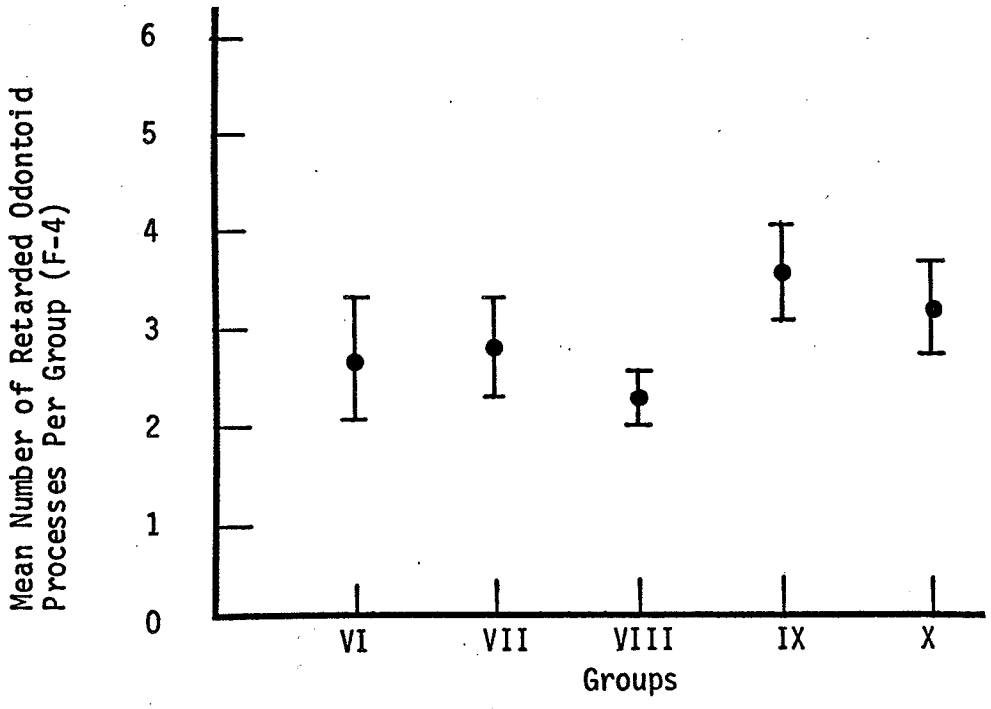


TABLE 8

Mean Number of Duplicated Thoracic Vertebral Centra Per Group (F-5)
Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	2.30	0.72
II	Fast	9-11	4.00	0.68
III	Fast	11-13	0.10	0.10
IV	Control	-	0.90	0.31
V	Control	-	0.50	0.22

GRAPH 7

Experiment Number I

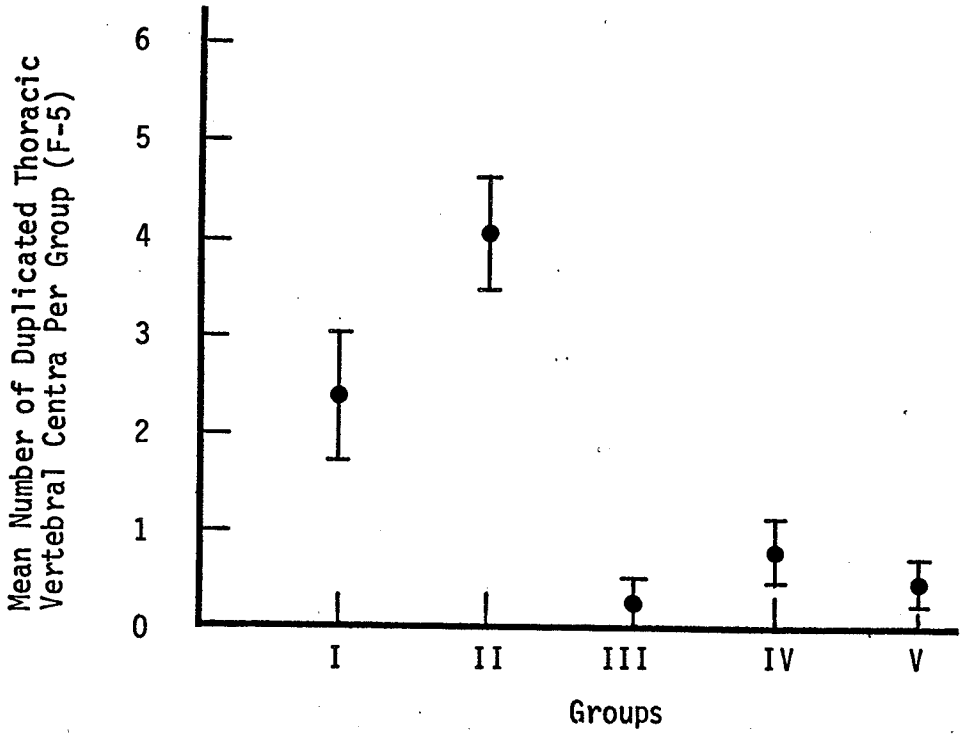


TABLE 9

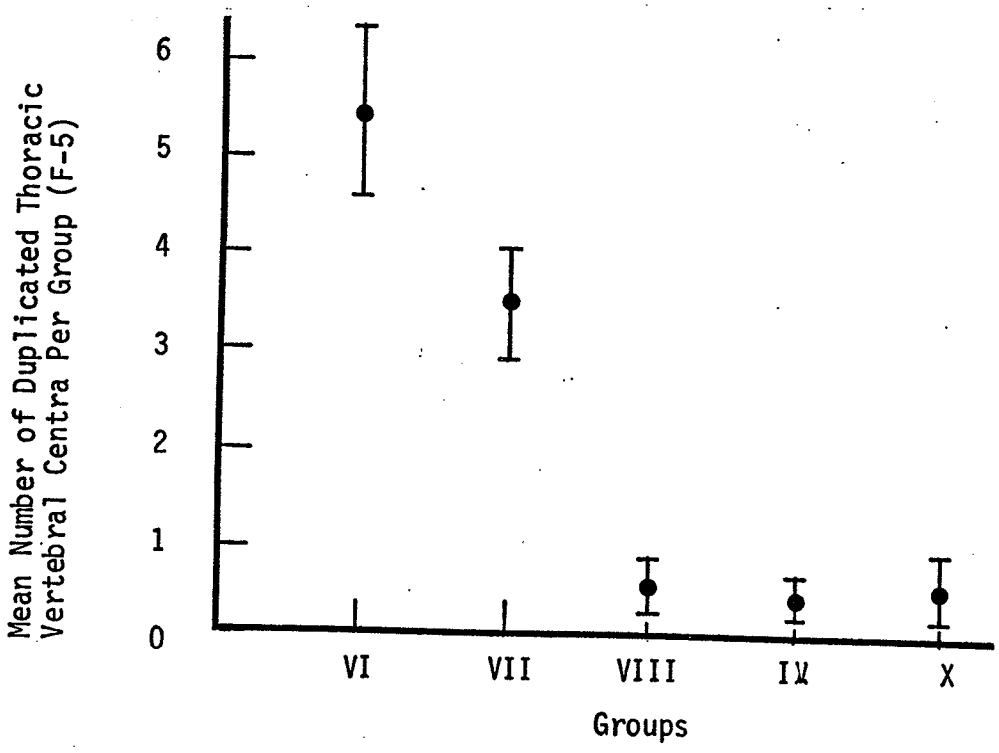
Mean Number of Duplicated Thoracic Vertebral Centra Per Group (F-5)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	5.30	1.03
VII	Fast	9-11	3.40	0.60
VIII	PZI	9-11	0.70	0.21
IX	Saline	9-11	0.50	0.22
X	Control	-	0.80	0.33

GRAPH 8

Experiment Number II



centra occurred significantly more often than normal in Group VI (fast plus PZI days 7-9) and Group VII (fast days 7-9). The number of duplicated vertebral centra observed in Group VI (fast plus PZI days 9-11) was also significantly higher than the number observed in Group VII (fast days 9-11). This could be interpreted as being a partial additive effect produced by the insulin.

Therefore, the most critical period of susceptibility to the production of duplicated vertebral centra is somewhere between days 7-11.

Factor 6. Number of Live Born Fetuses (Table A-2, See Appendix)

There was no statistically significant difference in the number of live born fetuses in any of the groups studied.

Factor 7. Number of Resorptions (Tables 10 and 11, Graphs 9 and 10)

In Experiment I (table 10, graph 9) there was no statistically significant effect of fasting upon the resorption rate.

In Experiment II (table 11, graph 10) Group VI (fast plus PZI days 9-11) produced a statistically significant increase in the resorption rate. Since protamine zinc insulin alone (Group VIII) showed no effect, the resulting increase in resorption rate was probably due to the increased hypoglycemia produced by the combination of fasting and insulin. It is evident that this treatment endangers the viability of the embryo. The resorption rate of all other Groups in Experiment II was not significantly

TABLE 10

Mean Number of Resorptions Per Group (F-7)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	0.70	0.21
II	Fast	9-11	0.50	0.27
III	Fast	11-13	0.50	0.17
IV	Control	-	0.30	0.15
V	Control	-	0.20	0.13

GRAPH 9

Experiment Number I

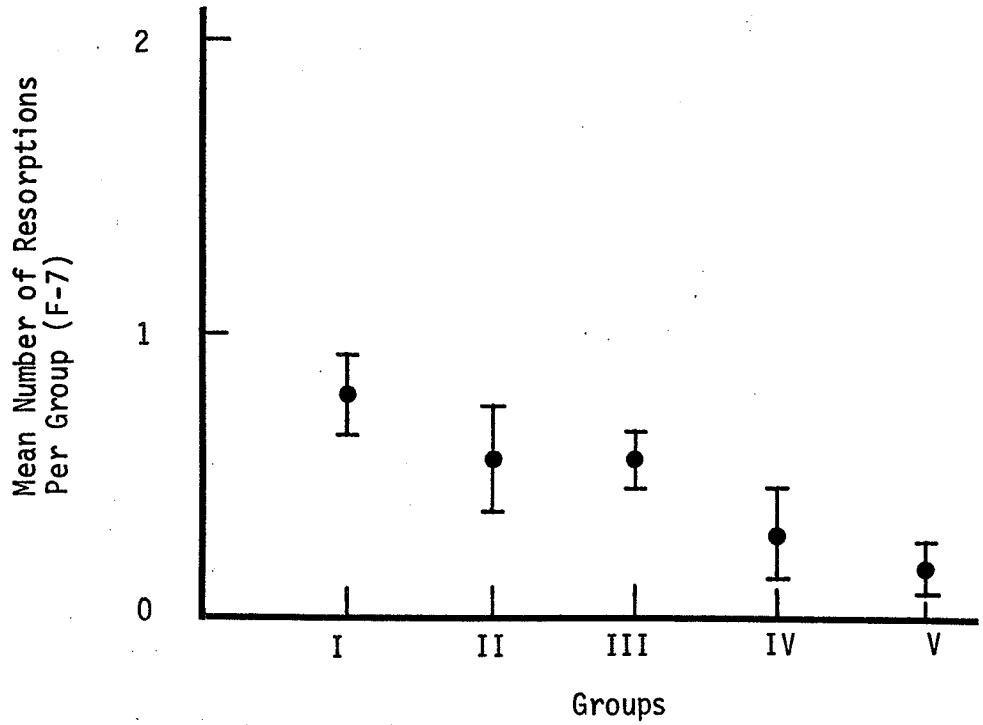


TABLE 11

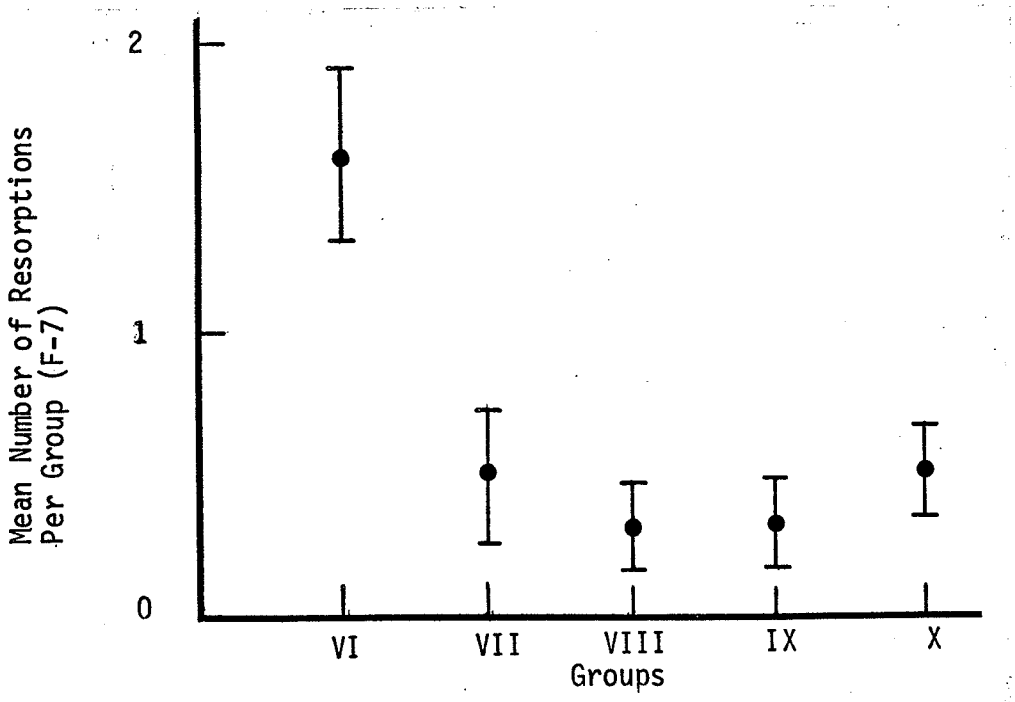
Mean Number of Resorptions Per Group (F-7)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	1.60	0.37
VII	Fast	9-11	0.40	0.22
VIII	PZI	9-11	0.30	0.15
IX	Saline	9-11	0.30	0.15
X	Control	-	0.40	0.16

GRAPH 10

Experiment Number II



different from the normal (Group X).

Factor - 8. Retarded Fifth Sternebral Centre (Tables12 and 13, Graphs 11 and 12, Figure 8)

In Experiment I (table 12, graph 11) ossification of the fifth sternebra was significantly delayed in Group II (fast days 9-11) but fasting on days 7-9 and 11-13 had no significant effect, although ossification was not as advanced as in the control Groups IV and V.

In Experiment II (table 13, graph 12) Group VI (fast plus PZI days 9-11) and Group VII (fast days 9-11) showed a statistically significant delay in ossification. However, insulin plus fasting (Group VII) did not significantly increase the effect of fasting. Insulin alone (Group VIII) and saline (Group IX) treatments had no significant effect on the ossification rate of the fifth sternebra.

The ossification rate of the fifth sternebra was most susceptible to fasting and fasting plus insulin on days 9-11 of gestation.

Factor - 9. Number of Fifth Sternebra Misshapen (Table A-3 See Appendix, Figure 9)

Some fifth sternebral centra were well ossified but were not concentric in shape. No treatment had a significant effect on the number of misshapen fifth sternebra present, compared with the control Groups IV, V and X.

TABLE 12

Mean Number of Retarded Fifth Sternebra Per Group (F-8)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	1.10	0.43
II	Fast	9-11	2.70	0.67
III	Fast	11-13	1.60	0.56
IV	Control	-	0.90	0.31
V	Control	-	0.60	0.22

GRAPH 11

Experiment Number I

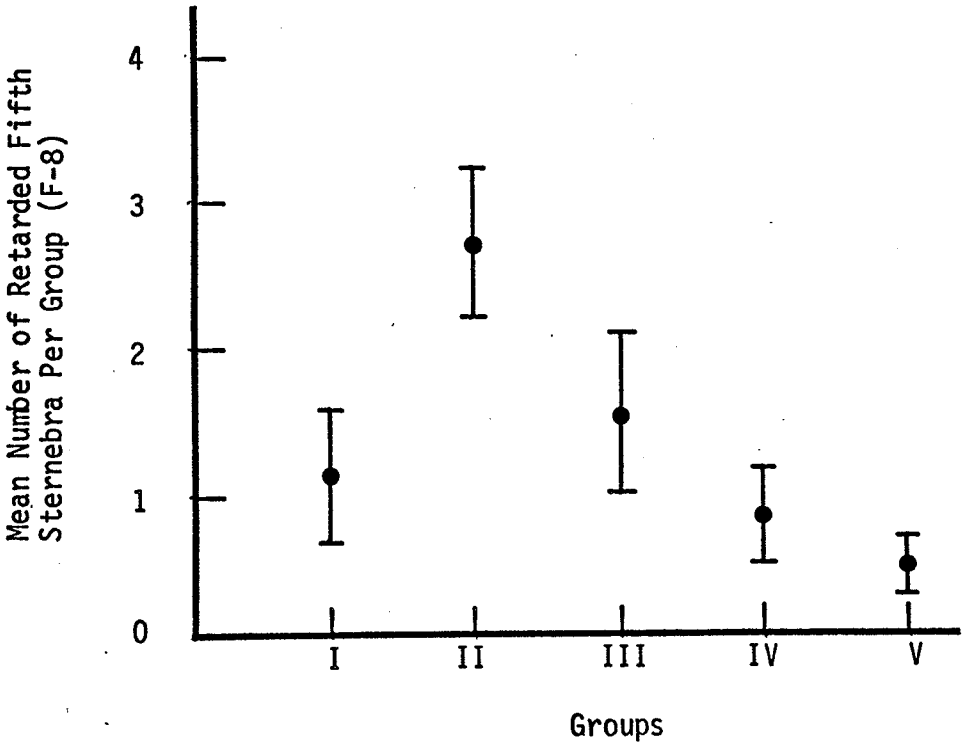


TABLE 13

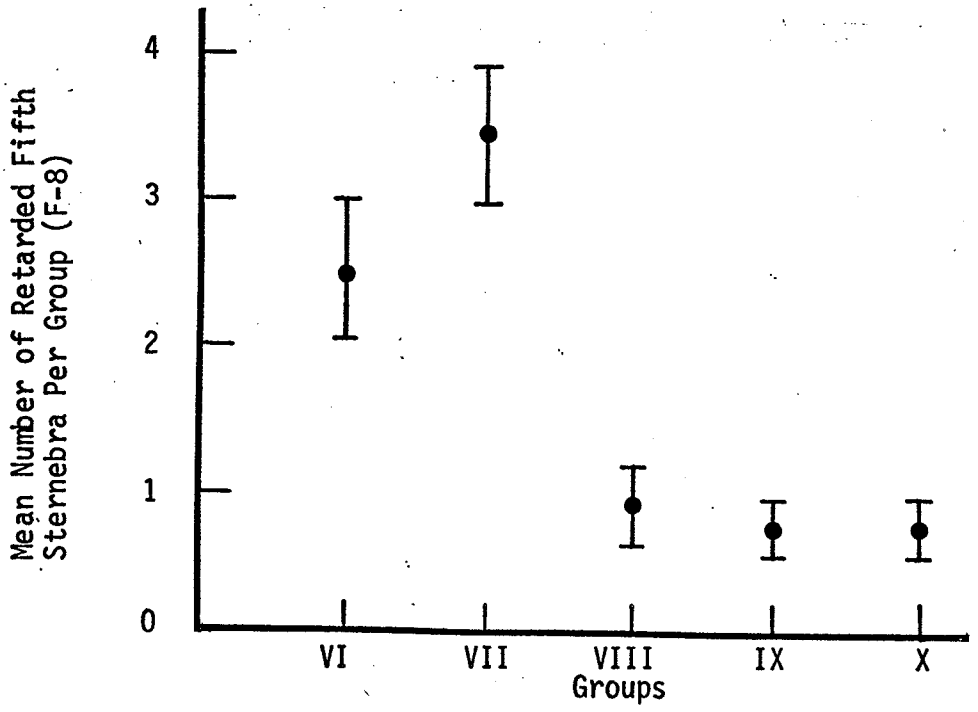
Mean Number of Retarded Fifth Sternebra Per Group (F-8)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	2.40	0.45
VII	Fast	9-11	3.40	0.45
VIII	PZI	9-11	1.00	0.26
IX	Saline	9-11	0.80	0.20
X	Control	-	0.80	0.29

GRAPH 12

Experiment Number II



Factor - 10. Sternebrae Misshapen, Excluding the Fifth Sternebra (Tables 14 and 15, Graphs 13 and 14, Figure 9)

Although the fifth sternebral center was not significantly affected by the treatments, other sternebral centra were affected.

The number of misshapen sternebrae was increased significantly by treatments occurring on days 9-11 (Group II, Group VI, Group VII) with the exception of Group VIII (PZI) and Group IX (saline). Fasting on other days had no statistically significant effect on the number of misshapen sternebrae.

I believe the misshapen appearance of the centra is due to partial delay in ossification, thereby allowing one part of the center to ossify faster than the other.

The fifth sternebral center is the last center to ossify (day 20). If the misshapen appearance is a naturally occurring phenomena, demarking partially delayed ossification, it would be reasonable to assume that it would not differ from "normal" at the time of examination on day 21.

Since the other sternebrae ossify first, it is easier to note a delay in their ossification than in the fifth sternebral center that is just beginning ossification at or near the time of examination. If the fetuses were not examined until a day later than usual, it is probable that some abnormal delay in ossification of the fifth sternebral center would be observed. This possibility will be checked in future experiments.

TABLE 14

Mean Number of Sternebrae Misshapen Per Group
Excluding the Fifth Sternebra (F-10)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	0.90	0.14
II	Fast	9-11	2.20	0.35
III	Fast	11-13	0.80	0.16
IV	Control	-	0.20	0.11
V	Control	-	0.30	0.13

GRAPH 13

Experiment Number I

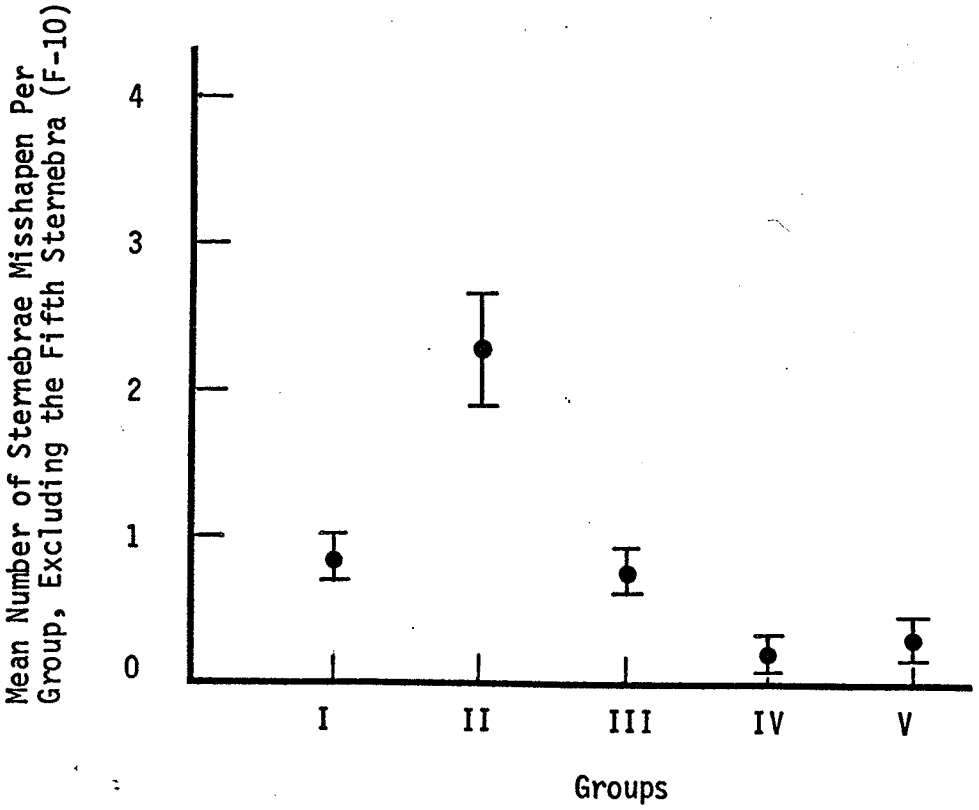


TABLE 15

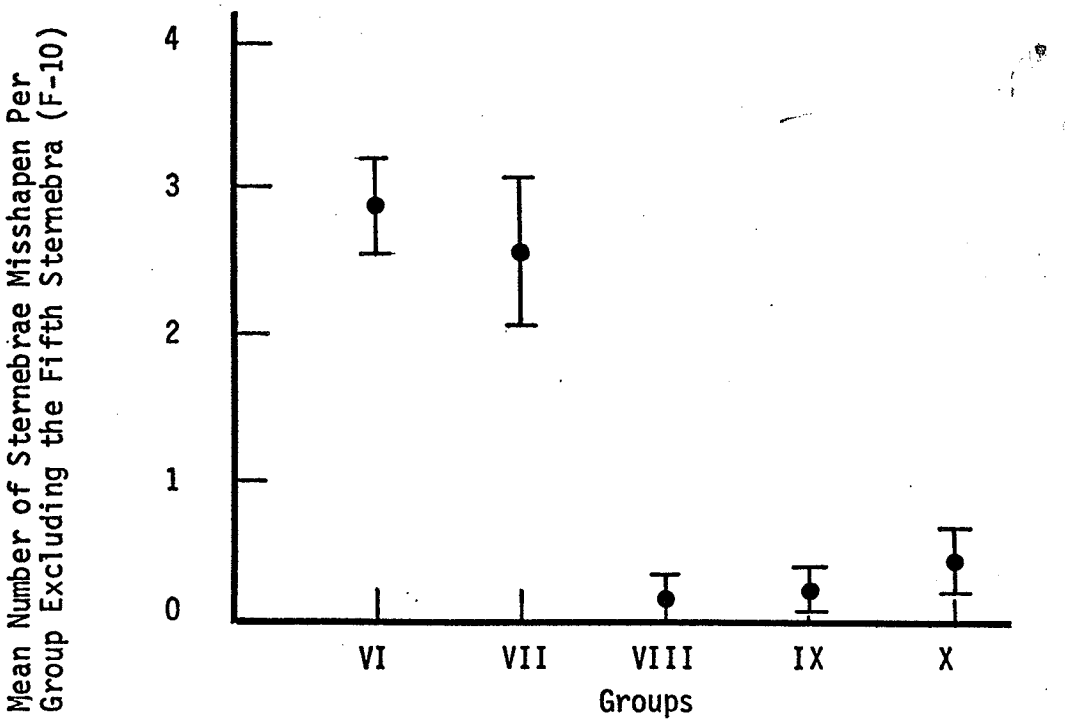
Mean Number of Sternebrae Misshapen Per Group
Excluding the Fifth Sternebra (F-10)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	2.90	0.46
VII	Fast	9-11	2.50	0.58
VIII	PZI	9-11	0.10	0.10
IX	Saline	9-11	0.20	0.13
X	Control	-	0.40	0.22

GRAPH 14

Experiment Number II



Factor - 11. Lumbar Supernumerary Ribs (Tables 16 and 17, Graphs 15 and 16, Figure 10)

In Experiment I (table 16, graph 15) the number of lumbar supernumerary ribs was statistically significant for Group II (fast days 9-11). Groups I and III (fasting days 7-9 and 11-13) had no significant effect on the number of supernumerary ribs present.

In Experiment II (table 17, graph 16) the number of supernumerary ribs present for both Group VI (fasting plus PZI days 9-11) and Group VII (fast days 9-11) were increased significantly over the control values (Group X). Group VI had a significantly greater number of supernumerary ribs than were found in Group VII. Since insulin (Group VIII) and saline (Group IX) had no significant effect, the result of insulin plus fasting would seem to be due to the increased hypoglycemia as shown in Table 22. Therefore, the 0.4 units of protamine zinc insulin produced a partial additive effect to fasting in regard to this anomaly.

All supernumerary ribs were located on the first lumbar vertebra.

TABLE 16

Mean Number of Supernumerary Ribs Per Group (F-11)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	0.70	0.42
II	Fast	9-11	6.30	1.84
III	Fast	11-13	2.40	0.60
IV	Control	-	1.60	0.76
V	Control	-	1.50	0.58

GRAPH 15

Experiment Number I

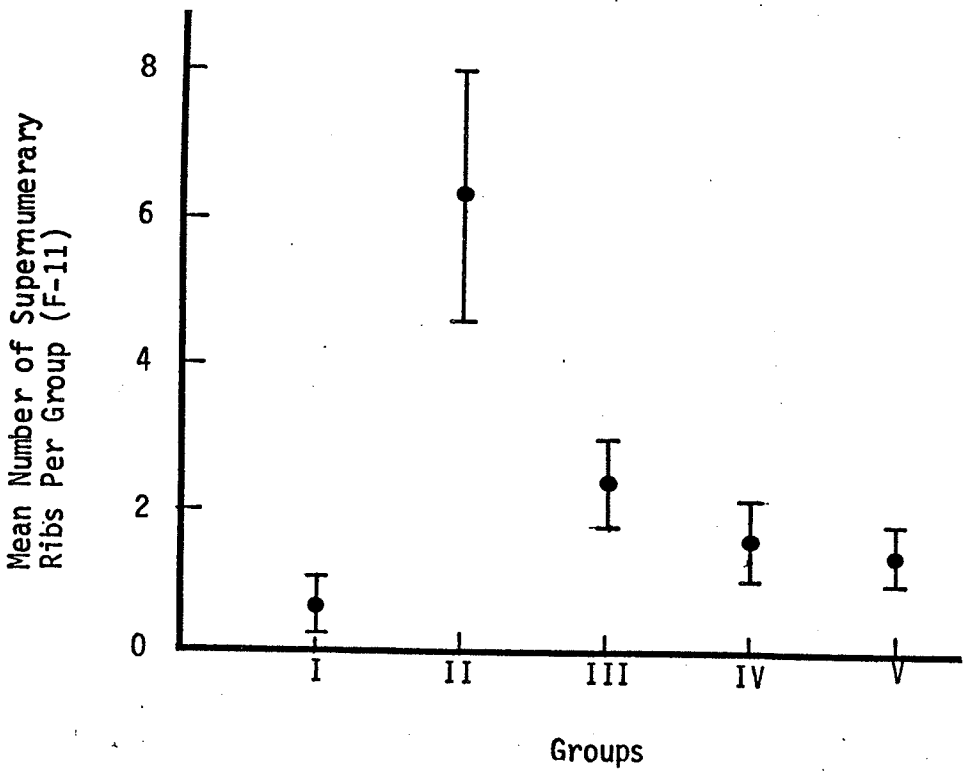


TABLE 17

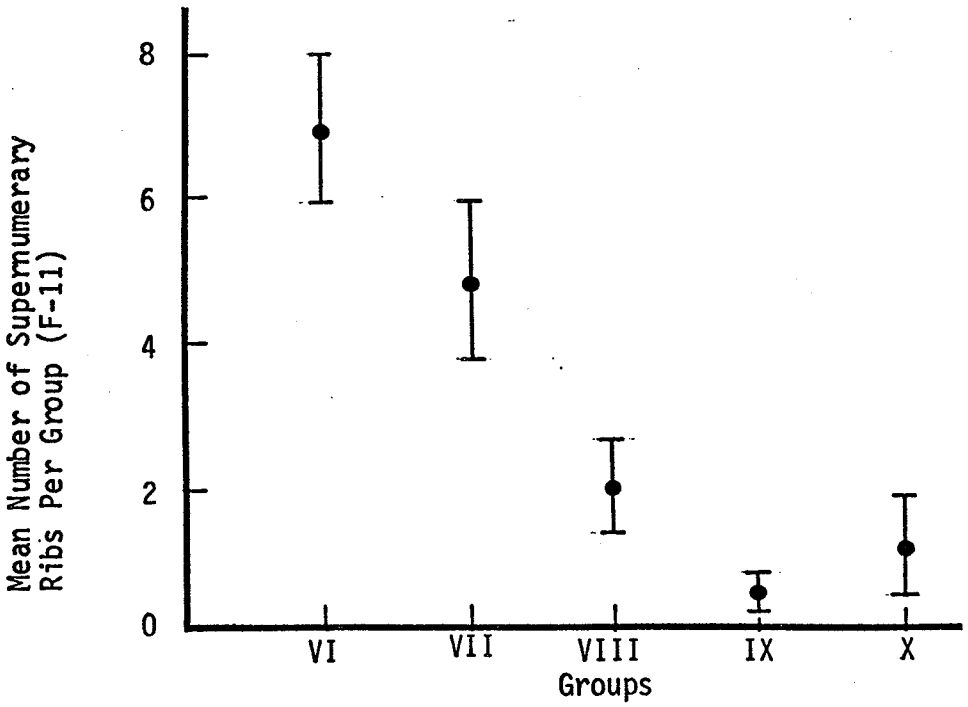
Mean Number of Supernumerary Ribs Per Group (F-11)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	6.70	1.30
VII	Fast	9-11	4.80	1.10
VIII	PZI	9-11	2.00	0.70
IX	Saline	9-11	0.70	0.33
X	Control	-	1.40	0.92

GRAPH 16

Experiment Number II



Factor - 12. Asynchronous Cervical Vertebral Centra (Tables 18 and 19, Graphs 17 and 18, Figure 5)

Cervical vertebral centra ossify in order in the caudal to rostral direction. When ossification proceeded sporadically, resulting in absence of some centra, it was considered to be an anomaly.

Asynchronous cervical vertebral centra were not observed in any control groups, or in Group VIII (PZI alone) and Group IX (saline). They were observed, however, in all other treatment groups. Therefore this condition seems to be produced by a level of hypoglycemia greater than that produced by insulin injection alone and the period of susceptibility is fairly long. This condition was noted only in cervical vertebrae.

TABLE 18

Mean Number of Asynchronous Vertebral Centra (F-12)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	0.30	0.10
II	Fast	9-11	0.40	0.15
III	Fast	11-13	0.10	0.11
IV	Control	-	0.00	0.00
V	Control	-	0.00	0.00

Graph 17

Experiment Number I

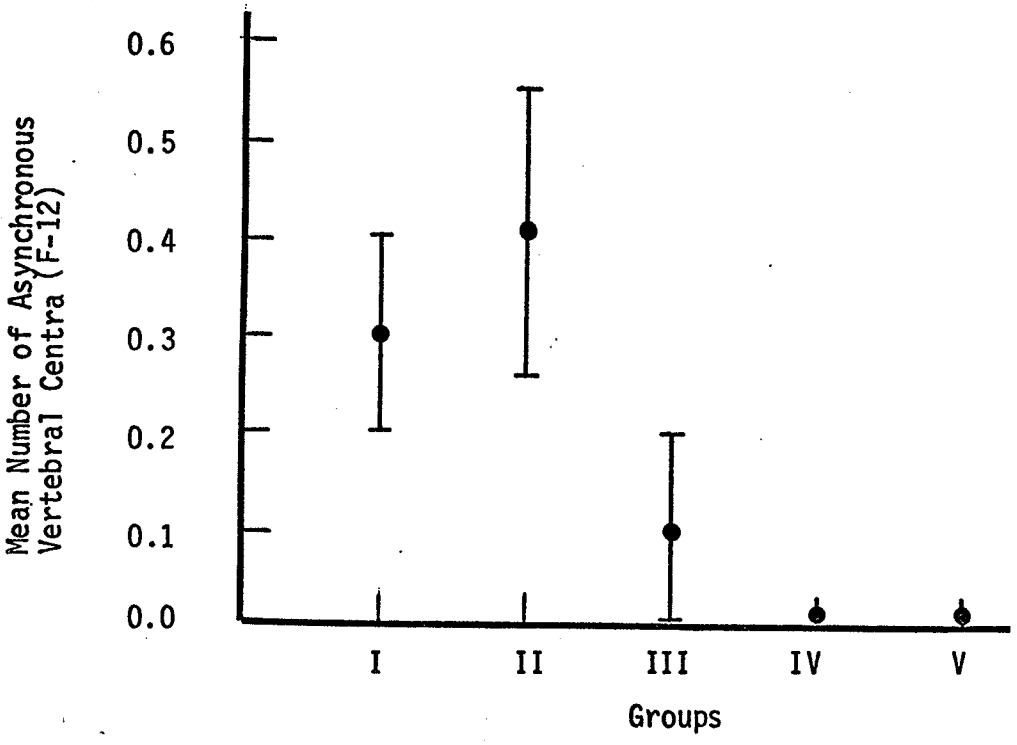


TABLE 19

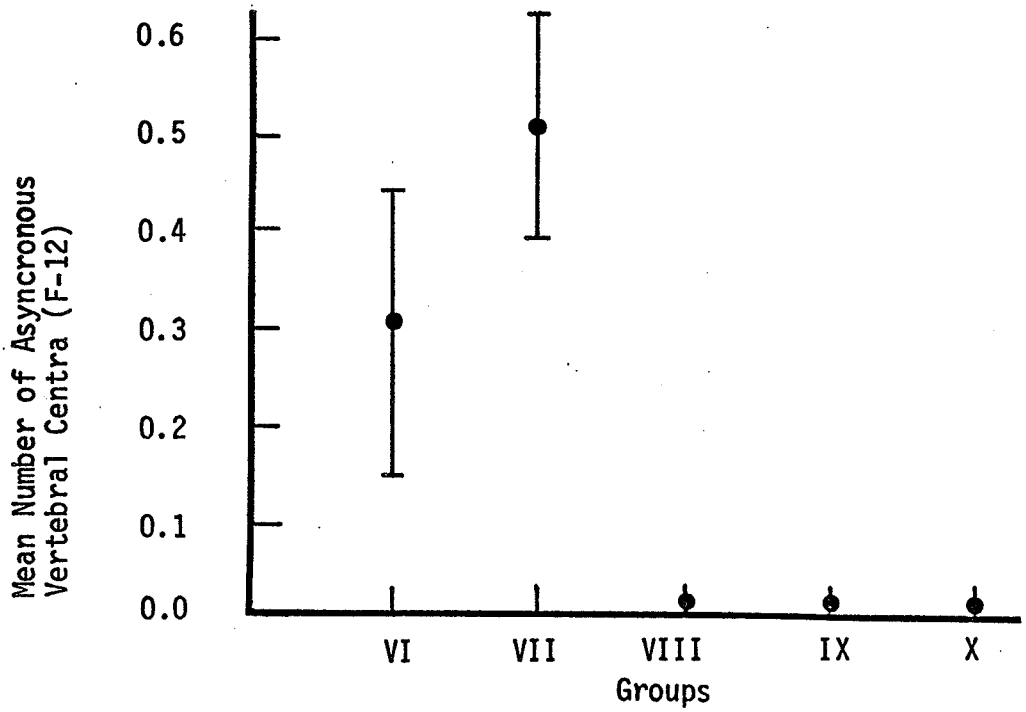
Mean Number of Asynchronous Vertebral Centra (F-12)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	0.30	0.13
VII	Fast	9-11	0.50	0.17
VIII	PZI	9-11	0.00	0.00
IX	Saline	9-11	0.00	0.00
X	Control	-	0.00	0.00

GRAPH 18

Experiment Number II



Factor - 13. Abnormal Anterior Arch of the Atlas
(Tables 20 and 21, Graphs 19 and 20, Figure 11)

In Experiment I (table 20, graph 19) there was a statistically significant decrease in the rate of ossification of the anterior arch of the Atlas in Group II (fast days 9-11). The other two periods of fasting showed no significant difference from the control Groups IV and V.

In Experiment II (table 21, graph 20) both Group VI (fast plus PZI days 9-11) and Group VII (fast days 9-11) showed a statistically significant different rate of ossification but the two Groups did not differ from each other. Group VIII (PZI) and Group IX (saline) showed no significant difference from normal (Group X).

This factor represents a decrease in the rate of ossification and the susceptible period is days 9-11.

TABLE 20

Mean Number of Abnormal Anterior Arch of the Atlas Per Group (F-13)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	2.60	0.96
II	Fast	9-11	4.70	0.84
III	Fast	11-13	2.00	0.61
IV	Control	-	1.90	0.48
V	Control	-	1.90	0.55

GRAPH 19

Experiment Number I

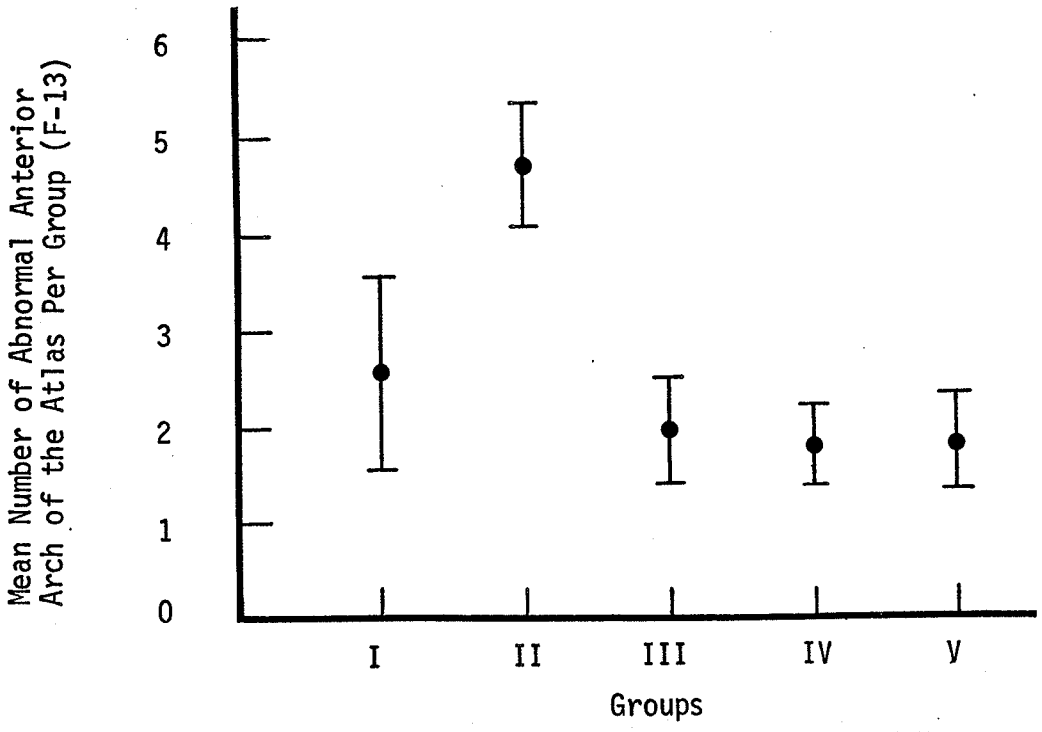


TABLE 21

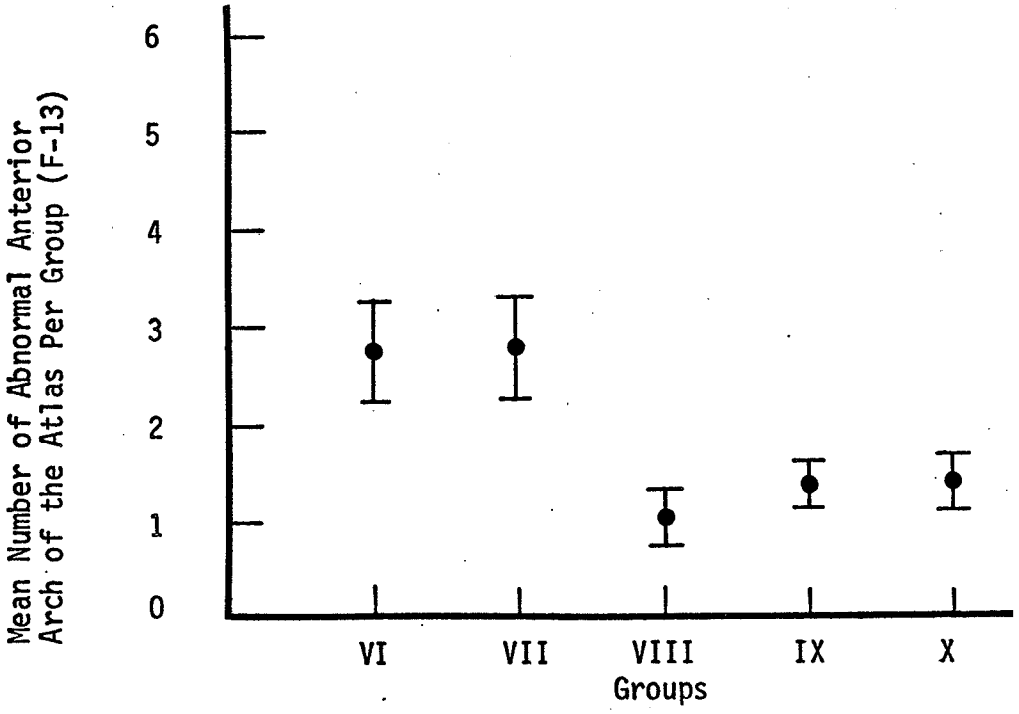
Mean Number of Abnormal Anterior Arch of the Atlas Per Group (F-13)

Experiment Number II

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
VI	Fast+PZI	9-11	2.80	0.63
VII	Fast	9-11	2.80	0.61
VIII	PZI	9-11	1.10	0.23
IX	Saline	9-11	1.40	0.27
X	Control	-	1.40	0.34

GRAPH 20

Experiment Number II



Effect of Acute Fasting on Maternal Blood Glucose

By fasting pregnant female rats on days 9-11 the maternal blood glucose was lowered by approximately 25 percent (p less than .05), within 24 hours. As shown in graph 22, the blood glucose then began to show a gradual increase, probably due to the effects of gluconeogenesis. When food was returned to the animals their blood glucose levels returned to "normal" within 12 hours.

Effect of Acute Fasting plus Protamine Zinc Insulin on Maternal Blood Glucose

The combination of fasting and 0.4 units of protamine zinc insulin on days 9-11 of pregnancy lowered the maternal rat blood glucose by approximately 22 percent (p less than .05) within 12 hours. After a second injection of insulin at 24 hours the blood glucose level dropped to approximately 42 percent (p less than .05) below control values by 48 hours. When the food was returned to the animals at the end of 48 hours, the blood glucose levels returned to "normal" within 12 hours (graph 23).

As shown in graph 21, this combined treatment depressed maternal blood glucose levels for the longest duration of time and to a lower value than any of the other treatments used.

This treatment produces approximately the same level of blood glucose depression, but for a much shorter duration than

TABLE 22

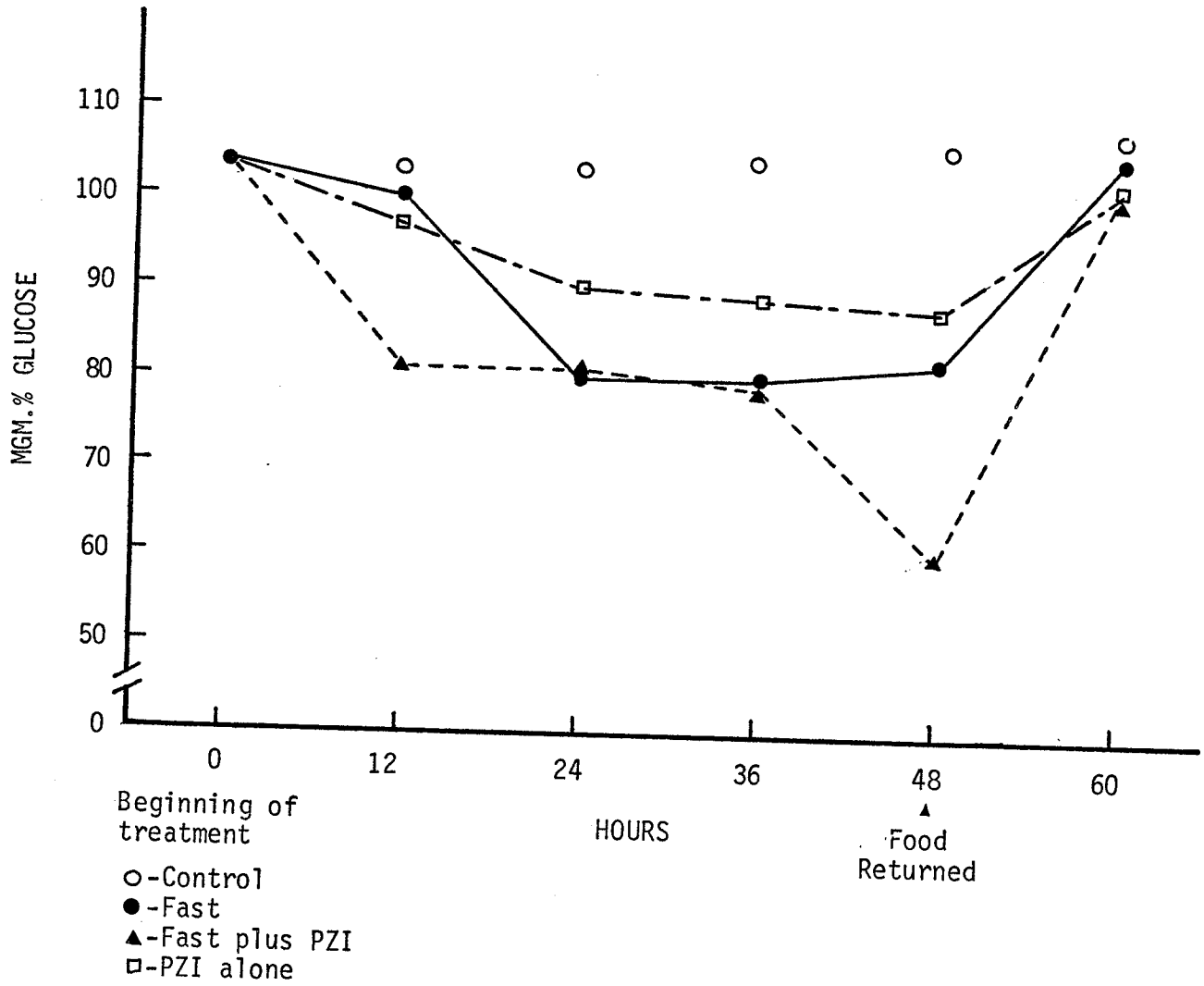
Effect of a Treatment on the Blood Glucose of Pregnant Rats

TREATMENT	HOURS (Mean Blood Glucose)(mgm%)					
	0	12	24	36	48	60
NORMAL	104±4.5*	103±4.0	104±3.0	105±3.0	105±3.3	106±4.0
FAST	104±4.5	100±5.0	79±2.5	80±2.7	82±9.0	105±1.5
FAST+ PZI**	104±4.5	81±1.6	81±8.2	79±4.8	60±5.0	102±4.4
PZI	104±4.5	97±2.7	90±2.1	89±3.2	88±3.1	102±4.3

* = Mean ± Standard Deviation

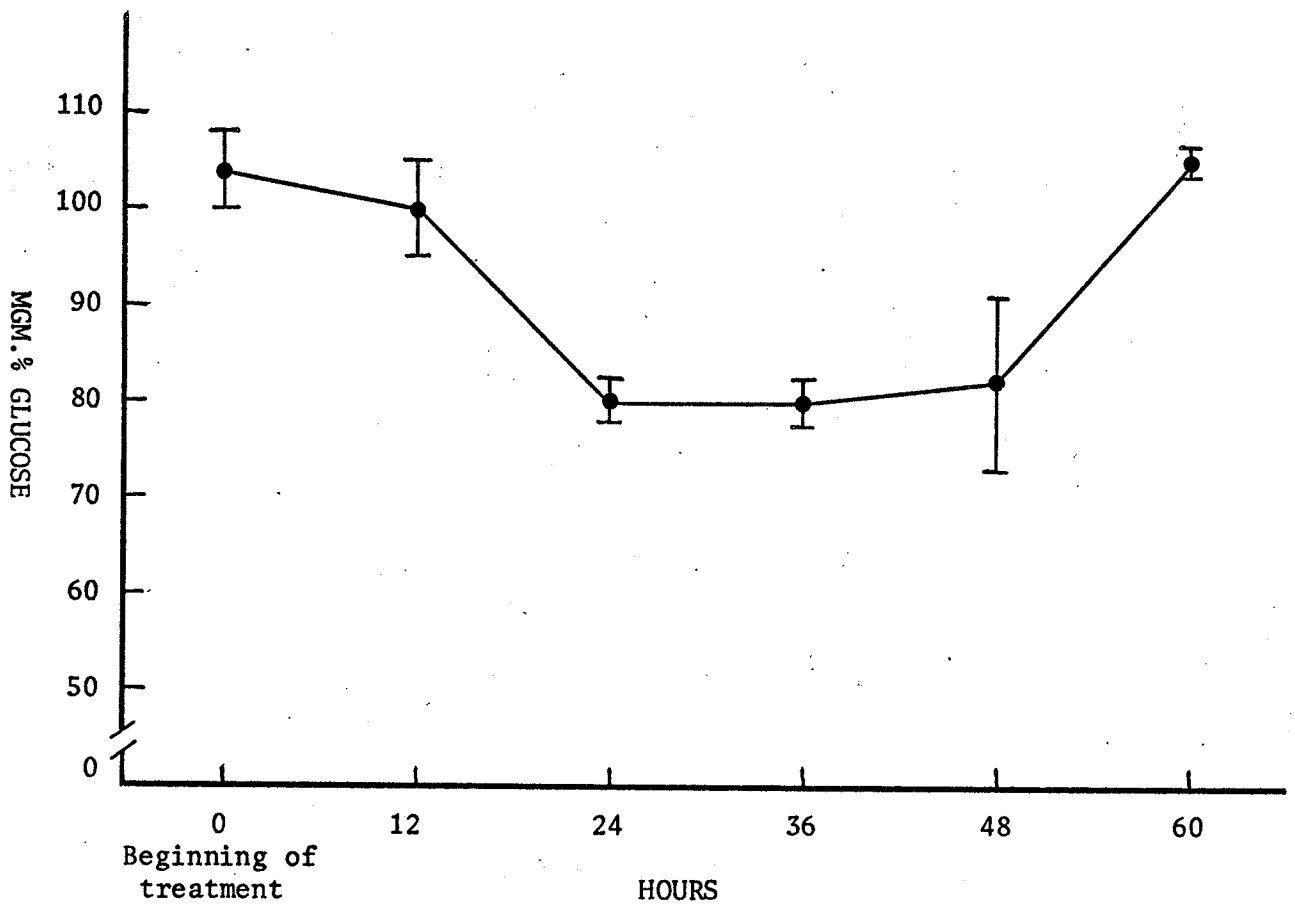
** = PZI = Protamine Zinc Insulin

Effects of Various Treatments on Maternal Blood Glucose (Days 9-11)



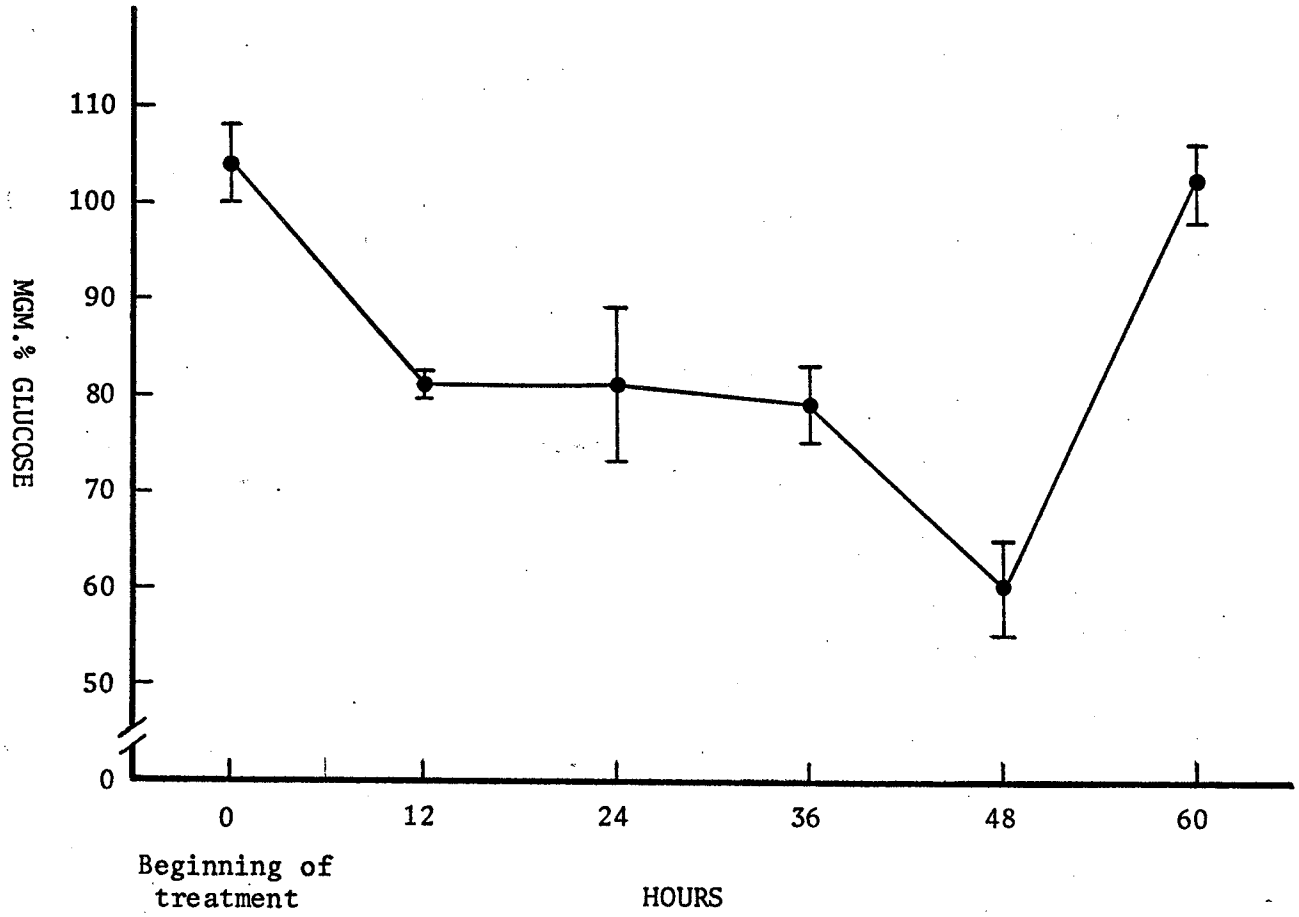
GRAPH 21

Effect of Fasting on Maternal Blood Glucose (Days 9-11)



GRAPH 22

Effect of Fasting and Insulin on Maternal Blood Glucose (Days 9-11)



GRAPH 23

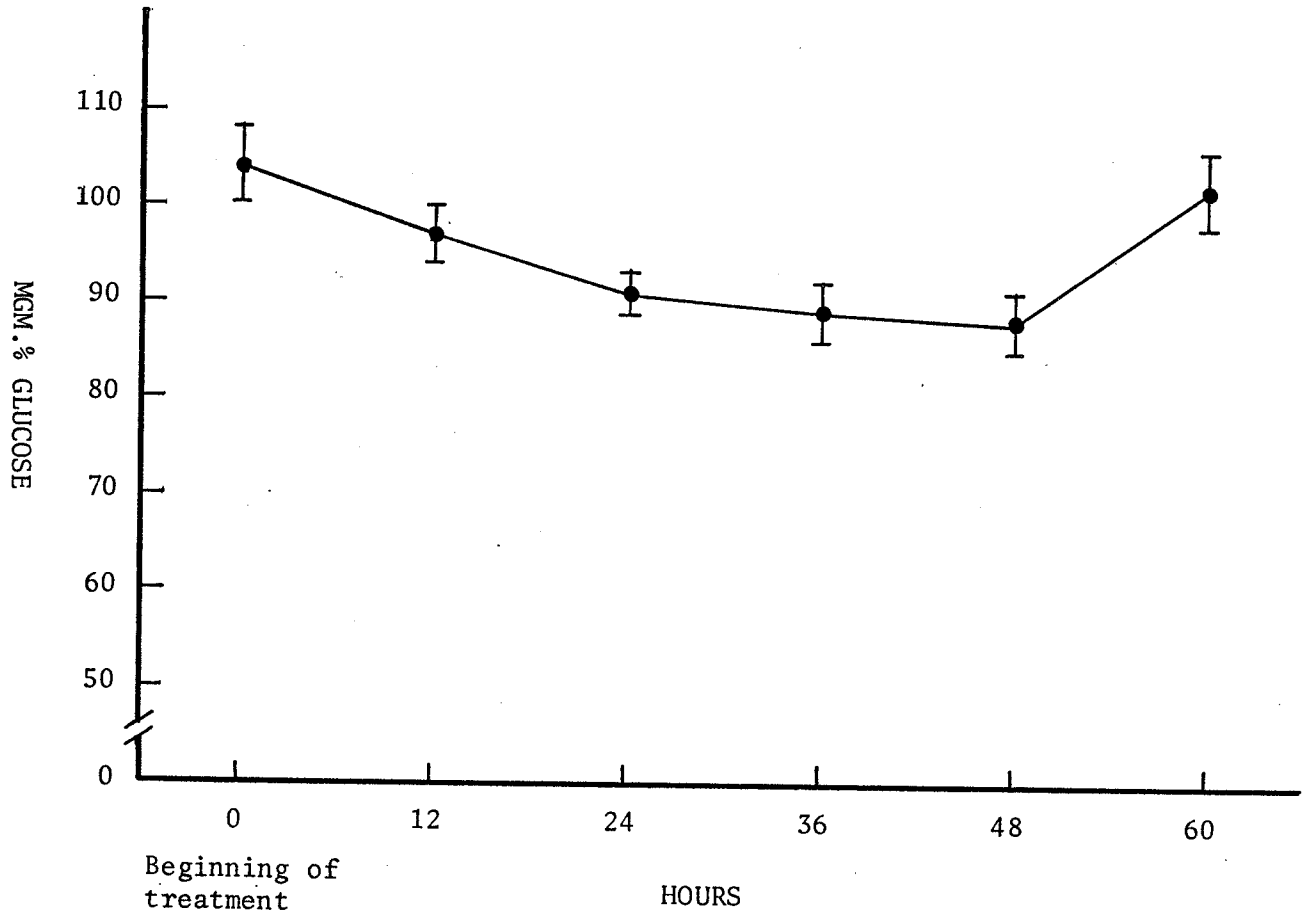
was observed in mice, as McClure (1961) produced by only fasting mice.

Effect of Protamine Zinc Insulin on Maternal Blood Glucose

By injecting a total of 0.4 units of protamine zinc insulin into pregnant female rats on days 9 and 10, a drop of approximately 14 percent (p less than .05) was observed in maternal blood glucose within 24 hours (graph 24). The second injection of insulin (0.2 units) at 24 hours had no significant affect on the blood glucose level. This injection was probably successful in placing a partial block on gluconeogenesis but without the fasting state, it produced very little effect on the maternal blood glucose level. The effect of the insulin was lost within 60 hours after the time of the first injection.

As shown on graph 21, protamine zinc insulin injections alone, produced the smallest depression of any of the treatments used.

Effect of Insulin on Maternal Blood Glucose (Days 9-11)



GRAPH 24

Retrospective Criticism of Analysis

The fetuses in this research project were examined in groups and the examiner was aware of the treatment, or lack of it, their mothers had received.

Although it is believed this knowledge had no effect on the results, in any future research of this nature it would be advisable to have an impartial observer choose the fetuses at random, thereby preventing the examiner from knowing the identity of fetuses and thus eliminating any subconscious bias in recording observations.

Summary of Results

(1) Groups III and VII were given the same treatment but were carried out at different times of the year. All the significant factors recorded for Group III were also recorded for Group VII, however, Group III had two additional significant factors; decreased fetal length and absent cervical vertebral centra. This result could be due to a possible seasonal variation in the rats.

(2) In Experiment II, insulin in combination with fasting (Group VI), significantly increased the number of supernumerary ribs and duplicated vertebral centra over the number which occurred when the animals were only fasted (Group VII). Insulin produced an additive effect in the production of these two anomalies when administered with fasting.

(3) The results show a definite decrease in the fetal ossification rate for animals that were fasted, with the most severe retardation occurring when the pregnant animals were fasted on days 9 to 11 of gestation.

(4) In Experiment II control Groups VIII (PZI alone) and IX (saline), did not differ from normal (Group X) for any of the factors studied.

TABLE 23

Summary of Statistically Significant Observations on Rat Embryos after Maternal Fasting and Insulin Injections

Experiment	Group	Factors (Observations)$^{\psi}$									
		1	3	4	5	7	8	10	11	12	13
I	I (fast days 7-9)			+	+					+	
	II (fast days 9-11)	+	+		+		+	+	+	+	+
	III (fast days 11-13)		+	+						+	
II	VI (fast+PZI days 9-11)				+*	+	+	+	+$^{\phi}$	+	+
	VII (fast days 9-11)				+		+	+	+	+	+

*-Significantly greater than value in group VII
ϕ-Significantly greater than value in group VII

ψ1. Fetal Length
3. Absent Cervical Vertebral Centra
4. Retarded Odontoid Processes
5. Duplicated Thoracic Vertebral Centra
7. Number of Resorptions
8. Retarded Fifth Sternebra
10. Sternebrae Misshapen, Excluding the Fifth Sternebra
11. Supernumerary Ribs
12. Asynchronous Cervical Vertebral Centra
13. Abnormal Anterior Arch of the Atlas

V DISCUSSION

This series of experiments was undertaken to explore the effects, if any, of hypoglycemia on fetal skeletal development in the rat. In 1965, Runner stated that normal development in mouse embryos and probably in all mammalian embryos, is dependant on carbohydrate metabolism. He worked with mice which have unusually low carbohydrate stores, allowing a stress such as fasting to produce an immediate effect on carbohydrate metabolism.

The rat was chosen for this series of experiments because, like the human, it has large resources of substrates capable of maintaining near normal carbohydrate metabolism under stress.

With Runner's work in mind, we decided to try and observe the effects of acute fasting on fetal development in the Holtzman rat, by duplicating the times of fasting that Runner had used in 1959. As Runner predicted, the maternal blood glucose levels during the fast in rats, did not compare to the severe 50 percent drop he observed for mice.

It was then decided to try and produce the 50 percent drop in blood glucose observed in mice. We chose protamine zinc insulin as a hypoglycemic agent for two reasons, the first reason being that insulin does not cross the placental barrier and therefore cannot reach the fetus as shown by Goodner and Freinkel (1961) and Clark et al (1968). Secondly, protamine zinc insulin is long acting and therefore the number of

injections needed to produce the desired effect was reduced to a minimum, thereby alleviating as much trauma to the animal as possible.

All other hypoglycemic agents encountered in my search of the literature have been shown to have some direct teratological effect on the embryo and for this reason they were rejected for the present investigation.

Environment

In planning this series of experiments, an attempt was made to remove as many environmental variables as possible. The pregnant animals were housed in an environment chamber in which the temperature was maintained at 75°F ± 2° as recommended as optimum by Dwornik (1969). The chamber was equipped with a good ventilation system which supplied a continuous flow of fresh air to the animals, thereby protecting the animals from possible hypoxia, thought to be a possible teratogen by Grabowski (1963). Relative humidity was maintained at 50 percent ± 20 percent. The rather large fluctuation was due to fluctuation in the ambient humidity of the outside room air occurring over the summer and winter periods. A relative humidity of 50 percent is recommended as optimum for rats by several animal care manuals.

To synchronize the ovulatory time of rats in order to ensure that all fetuses would be the same age ± 2 hours, the

lights in both the animal room and the environment chamber were set for 12 hours on and 12 hours off basis. This light-dark sequence has been shown to work successfully in synchronizing ovulation in rats by Everett and Sawyer (1950).

Fasting as a Teratogen

Fasting was shown to be a strong teratogen in mice by Runner (1959) but in the present study it has been found to be only a mild teratogen in rats. It is not possible to state what the effect of acute fasting would have on the human embryo since Fraser (1964) has said, "One cannot reliably predict from the teratogenic behavior of a drug in one species, what it will do in another". However, in view of these effects in animals one should caution against such fasting during the critical period of development in man (3-8 weeks).

The main effect of acute fasting found on the rat embryo was a general retardation of ossification in the axial skeleton, however, an increase in anomalies was also observed; accessory ribs on lumbar vertebrae and duplicated thoracic vertebral centra. In accordance with Runner's (1965) hypothesis, this fasting effect appears to result from the depression of maternal blood glucose by approximately 25 percent over a 48 hour period, (table 22). One animal from Group II was severely malformed, (figure 12). Externally, the fetus was severely stunted and the tail was absent. Internally, the lumbar vertebrae were

absent and there were many duplicated vertebral centra and all sternebral centers were very misshapen. There were also severe adhesions of the gastro-intestinal tract. Although only one such fetus was obtained in the present series, its significance should not be overlooked since such a fetus has never been observed in control litters in this laboratory, comprising over 5000 fetuses. Many more animals would be required to determine a frequency for this condition.

Fasting rats for a 48 hour period over the six day period tested, produced no significant effect on the number of resorptions, or the littering rate (number of fetuses per litter). It may be concluded, therefore, that fasting is a subletal teratogen in rats and the fetuses studied were from non-depleted litters. Runner (1959) obtained the same results and reached the same conclusion when mice were fasted for short periods of time during organogenesis.

Vitamin Deficiency

The question arises as to whether all effects of the treatments in this study resulted from hypoglycemia. Much has been published about the effects of vitamin deficiency on embryonic development of rats during the same time period studied in these experiments. Vitamin deficiency, e.g. B12, niacin, PGA etc., during short periods in the second week of gestation, has been shown to produce severe abnormalities in rats, Johnson (1964), but very few vitamin deficiencies have produced effects on skeletal development in the rat. Riboflavin and PGA deficiency have been shown, however, to produce skeletal abnormalities in rats, Nelson (1953) but these abnormalities do not correspond to those observed in the present experiments, e.g. in PGA deficiency, a large percentage of animals had cleft palates and club feet. Neither of these conditions was observed in any fetuses examined in our laboratory. The anomalies we observed were similar to those observed by Runner (1959) in fasted mice.

To produce conclusive results on acute vitamin deficiency, Asling (1969) administered vitamin antimetabolites which blocked the action of any vitamins, stored or produced by intestinal bacteria, on metabolism. This work probably explains why acute vitamin deficiency is not teratogenic in acute fasting experiments.

It should also be noted that stress on the animals' metabolism is much greater when all food intake is terminated,

than when a single vitamin is removed from the diet for a 48 hour period. The more severe stress of fasting would probably enhance the adaptation of the animals' metabolism much quicker than would occur if only a single vitamin was removed from the diet.

Effect of Acute Fasting on Abortion

In Experiment I, Group I (fast days 7-9) a large number of abortions occurred. Thirty-one pregnant rats were used in this experiment to obtain ten animals that maintained pregnancy, under these conditions. This is an abortion rate of approximately 2:1, one that does not compare with any other experiments we performed. The greatest number of abortions that occurred in any of the other groups was three (Group III, fast days 9-11).

Implantation occurs on day seven in the rat, thus the fast period we used encompasses the period of implantation and early formation of the placenta. There are at least two possible explanations for the high abortion rate observed in Group I, either direct or indirect. The direct effect would be on the implanting blastocysts. At the time of implantation, fasting hypoglycemia resulting in a decrease in the amount of glycogen present in the decidua could reduce the amount of substrate available for the blastocyst below the level needed for survival. This could result in a failure of implantation and result in embryonic mortality.

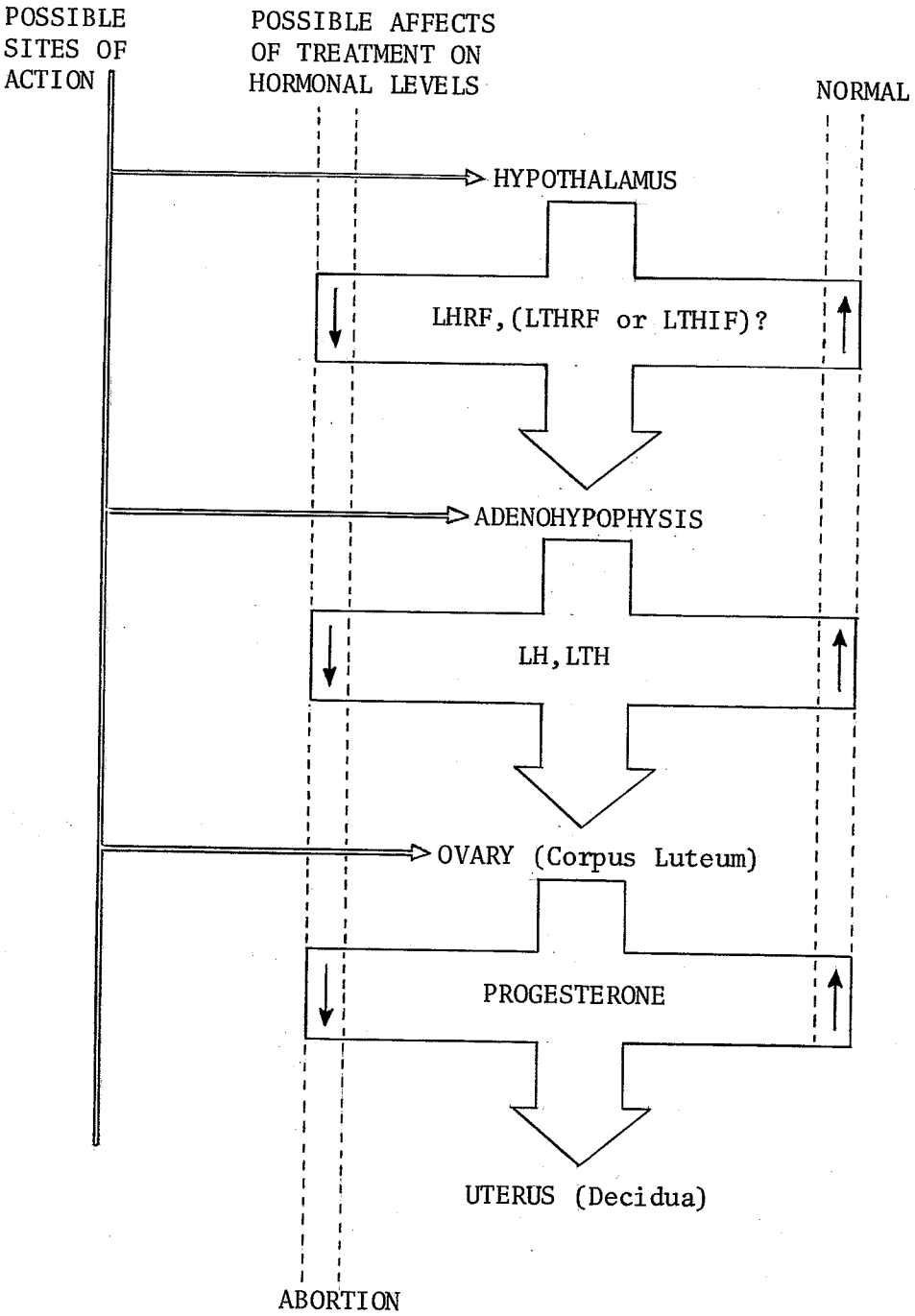
A possible indirect cause of abortion during Group I might be hormonal, resulting in a breakdown, or non-maintainance of the decidua, thereby causing abortion and secondary embryonic mortality. Hypoglycemia resulting from fasting could possibly affect the normal hormonal maintainance of pregnancy at any one or a combination of the following points: (1) hypothalamus (2) adenohypophysis or (3) ovary.

Critchlow and Sawyer (1955) observed an increase in electrical activity in the hypothalamus during secretion of gonadotropin from the adenohypophysis. Fasting hypoglycemia could possibly affect this function by decreasing electrical activity of the nerve cells due to decreased production of chemical transmitter substance, (McIlwain 1959; Puskarev 1964). This effect could lower the amount of lutenizing hormone releasing factor (LHRF) produced by the hypothalamus, which would lower the lutenizing hormone (LH) release by the adenohypophysis. Low LH levels would lower the output of progesterone by the corpus luteum, thereby causing a decidual breakdown.

Fasting hypoglycemia may also cause a hormonal disturbance at lower levels, as shown in figure 3. For example, it may directly affect the adenohypophysis decreasing output of both LH and luteotropic hormone (LTH). Although no results are available about the affect of acute fasting on the pituitary, chronic fasting has been shown to reduce drastically adenohypophseal output (Mullinos and Pomerantz, 1940).

FIGURE 3

Possible Etiology of Abortions in Rats
Fasted over Days Seven to Nine of Gestation



Fasting may affect the corpus luteum directly. Estrogen and progesterone, which are steroids produced by the corpus luteum, require nicotine adenosine diphosphate (NAD) for their biosynthesis (Villee, 1961). Fasting hypoglycemia could reduce the amount of available NAD enough to lower the levels of both estrogen and progesterone, even under strong LTH and LH stimulation.

Any one, or a combination, of the aforementioned proposed hormonal disturbances could result in a disruption of progesterone levels which are required to maintain the decidua.

It must be noted, however, that the rat placenta does not become fully functional in producing hormones until day twelve (Greenwald and Johnson, 1968). This would mean that the amount of placental gonadotropin in the circulation would be negligible during days 7-9. Presence of placental gonadotropin on day twelve could explain in part why the abortion rate decreased in groups fasted at a later time.

In the rat, prolactin (LTH) is the major luteotropic hormone (Everett, 1956). The control of prolactin release by the adenohypophysis remains a controversial question as to the existence of either luteotropic hormone releasing factor (LTHRF) or luteotropic hormone inhibiting factor (LTHIF).

A homologous transplanted adenohypophysis in the rat produced only LTH (Everett, 1954; 1956; Nikitovitch and Everett, 1958). This would suggest that the rat hypothalamus does not produce a LTHRF, but it does not discount the presence of a

LTHIF. If LTHIF is present, the effect of low blood glucose levels on the hormonal control shown in figure 3 must be below the hypothalamus.

Fasting plus Insulin as a Teratogen

Days 9-11 were chosen as the time to administer insulin because this was the most critical time for the effect of acute fasting on bone development (table 23).

Insulin was used to lower the maternal blood glucose below that observed in the fasting experiment. This was done to try and duplicate,as close as possible, in the rat the work of McClure in mice (1961). McClure observed a drop of 50 percent in the blood glucose of mice, which occurred four hours after the beginning of the fast. In our rats a decrease of approximately 35 percent was observed, but for a much shorter duration than that which had been observed in McClure's mice (graph 23). Statistical analysis showed that the combination of fasting plus insulin did not significantly decrease the rate of ossification any more than was observed with fasting alone. However, it did have a partial additive effect on the frequency of two anomalies: lumbar supernumerary ribs and duplicated thoracic vertebral centra.

Use of a dose of protamine zinc insulin larger than 0.4 units in the fasting rat, resulted in a high maternal mortality rate due to hypoglycemic shock. Therefore 0.4 units of protamine

zinc insulin was found to be the optimum dose for this experiment.

Insulin in combination with fasting over days 9-11 produced a significant increase in the number of resorption sites (areas where embryos had begun development), while insulin alone, administered at the same times had no effect on gestation. It can be concluded that the level of hypoglycemia resulting from fasting and insulin in combination, produced a lethal effect on some embryos. It is impossible to determine from the present experiments if the cause of resorptions was due to a lethal malformation, nutritional depletion or hormonal disturbance.

Insulin and Saline as Teratogens

Protamine zinc insulin alone (Group VIII) had no effect on skeletal development, therefore, it may be concluded that insulin in the dosage used is not teratogenic in rats of the Holtzman strain. The hypoglycemic action of the dosage of protamine zinc insulin used was very small, as shown in Figure 15.

The saline control group (Group IX) contained no abnormal fetuses, therefore, saline is non-teratogenic in these rats. This also proves that fetal development was not affected by the procedure of injecting insulin into the mother twice during pregnancy.

VI SUMMARY OF RESULTS

The most critical time for the effects of fasting on fetal bone development in Holtzman rats has been determined to be within a 48 hour period from day 9 to day 11 of gestation. The major effect of acute fasting was a general retardation in the rate of ossification of the axial skeleton in the fetal rat. Fasting the pregnant rat on days 9-11 resulted in the production of three anomalies in her young: supernumerary lumbar ribs, duplicated thoracic vertebral centra and asynchronous cervical vertebral centra.

Combining fasting and insulin as a treatment produced no further decrease in the rate of fetal ossification and therefore there was no additive effect on overall ossification. Insulin in combination with fasting produced a partial additive effect, however, in the production of two anomalies: supernumerary lumbar ribs and duplicated thoracic vertebral centra. It is concluded that the action of fasting and fasting in combination with insulin on fetal bone development, probably acts via a single pathway in interrupting normal development in the rat. The common pathway likely begins with a reduction of available glucose for the embryo due to maternal hypoglycemia.

Stresses to carbohydrate metabolism in the Holtzman rat, in combination with a fasting state, can produce an additive developmental effect; therefore, nutritional control should be of prime concern in setting up of any teratological experiments conducted with Holtzman rats and probably other strains also.

BIBLIOGRAPHY

Asling, C.W.: Nutrition and teratogenesis. In: H. Nishimura and J. Miller, Ed.: Methods for Teratological Studies in Experimental Animals and Man. New York: Medical Examination Publishing Co. Inc., 1969. pp. 76-91.

Buchanan, K.D., Vance, J.E., and Williams, R.H.: Effect of starvation on insulin and glucagon release from isolated Islets of Langerhans of the rat. Metabolism 18: 155-162, 1969.

Clark, C.M., Cahill, G.F., and Soeldner, J.S.: Effects of exogenus insulin on the rate of fatty acid synthesis and glucose C^{14} utilization in the twenty day rat fetus. Diabetes 17: 362-368, 1968.

Corey, E.L.: Placental permeability to insulin in the albino rat. Physiological Zoology 5: 36-48, 1932.

Critchlow, B.V., and Sawyer, C.H.: Electrical activity of the rat brain corelated with neurogenic stimulation of the adenohypophysis. Fed. Proc. 14: 32-33, 1955.

Duraiswami, P.K.: Insulin induced skeletal abnormalities in developing chickens. Brit. M.J. 2: 384-390, 1950.

Dwornik, J.J.: Effect of podophyllin and temperature on skeletal development of the Holtzman rat. Ph.D. Thesis, University of Manitoba, 1969.

Everett, J.W.: Leuteotrophic function of autographs of the rat hypophysis. Endocrinology 54: 685-690, 1954.

Everett, J.W.: Functional corpora lutea maintained for months by autographs of rat hypophysis. Endocrinology 58: 786-796, 1956.

Everett, J.W., and Sawyer, C.H.: A 24 hour periodicity in the "LH release apparatus" of female rats disclosed by barbituate sedation. Endocrinology 47: 198, 1950.

Fraser, C.F.: Experimental teratogenesis in relation to congenital malformations in man. In: M. Fishbein, Ed.: Second International Conference on Congenital Malformations. New York: The International Medical Congress Ltd., 1964. pp. 277-287.

Goodner, C.J., Conway, M.J., and Werrbach, J.H.: Relation between plasma glucose levels of mother and fetus during maternal hypoglycemia, hyperglycemia and fasting in the rat. Pediat. Res. 3: 121-127, 1969.

Goodner, C.J., and Freinkel, N.: Carbohydrate metabolism in pregnancy. IV Studies on the permeability of rat placentae to I^{131} insulin. Diabetes 10: 383-392, 1961.

Grabowski, C.T.: Teratogenic significance of ionic fluid imbalances. Science 142: 1064, 1963.

Grant, D.R., and Moorehouse, J.A.: Pilot study for a diabetes detection program based upon rapid glucose microanalysis of postprandial capillary blood. Canad. M. A. J. 94: 1213-1219, 1966.

Greenwald, G.S., and Johnson, D.C.: Gonadotropic requirements for the maintenance of pregnancy in the hypophysectomized rat. Endocrinology 83: 1052-1064, 1968.

Johnson, E.M.: Nutritional factors in mammalian teratology. In: J. Wilson and J. Warkany, Ed.: Teratology: Principles and Techniques. University of Chicago Press, 1965. pp. 113-130.

Lichtenstein, H., Guest, G.M., and Warkany, J.: Abnormalities in offspring of white rats given protamin zinc insulin during pregnancy. Proc. Soc. Exper. Biol. & Med. 78: 384-402, 1951.

MacFarlane, W.V., Pennycuik, P.R., and Thrift, E.: Resorption and loss of foetuses in rats living at 35°C. J. Physiol. (Lond.) 135: 451-459, 1957.

McClure, T.J.: Temporary nutritional stress and infertility in female mice. J. Physiol. 147: 221-225, 1959.

McClure, T.J.: Pathogenesis of early embryonic mortality caused by fasting pregnant rats and mice for short periods. J. Reprod. Fertil. 2: 381-386, 1961.

McClure, T.J.: Uterine pathology of temporarily fasted pregnant mice. J. Comp. Path. 71: 16-19, 1961.

McClure, T.J.: Fertility in mice caused by fasting at about the time of mating. III Pathogenesis. J. Reprod. Fert. 13: 393-403, 1967.

McIlwain, H.: Biochemistry of the Central Nervous System. London: Churchill, 1959.

Mullinos, M.G., and Pomerantz, L.: Pseudohypophysectomy, a condition resembling hypophysectomy, produced by malnutrition. J. Nutrition 19: 493, 1940.

Nelson, M.M., Wright, H.V., Baird, C.D.C., and Evans, H.M.: Effects of a 36 hour period of pteroylglutamic acid deficiency on fetal development in the rat. Proc. Soc. Exper. Biol. Med. 92: 554-568, 1956.

Nikitovitch-Winer, M., and Everett, J.W.: Comparative study of leutotrophin secretion by hypophyseal autotransplants in the rat. Effects of site and stages of the estrous cycle. Endocrinology 62: 522-532, 1958.

Pomerantz, L., and Mullinos, M.G.: Pseudo-hypophysectomy produced by inanition. Amer. J. Physiol. 126: 601, 1939.

Pushkarev, Y.P.: Effect of blood sugar levels on post-tetanic potentiation in cat synaptic ganglia (Translated title). Fiziologicheskii zhurnal SSSR imeni I M Sechenova 50: 329, 1964. Translated in: Fed. Proc. 24: T340, 1965.

Reece, R.P., and Turner, C.W.: Experimentally altering galactin content of the rat pituitary. Proc. Soc. Exper. Biol. Med. 36: 283-285, 1937.

Rinaldini, L.M.C.: Effect of chronic inanition on the gonadotropic contents of the pituitary gland. J. Endocrinology 6: 54, 1949.

Robson, J.M., and Sharaf, A.A.: Effect of ACTH and cortisone on pregnancy. J. Physiol. (Lond.) 116: 236, 1952.

Runner, M.N.: Inheritance of susceptibility to congenital deformity. Pediatrics 23: 245-251, 1959.

Runner, M.N.: General mechanisms of teratogenesis. In: J. Wilson and J. Warkany, Ed.: Teratology: Principles and Techniques. University of Chicago Press, 1965. pp. 95-112.

Runner, M.N., and Miller, J.R.: Congenital deformity in the mouse as a consequence of fasting. Anat. Rec. 124: 437-438, 1956.

Sobel, D.E.: Fetal damage due to E.C.T., insulin coma, chlorpromazine or reserpine. A.M.A. Arch. Gen. Psychiat. 2: 606-611, 1960.

Smithberg, M., Sanchez, H.W., and Runner, M.N.: Congenital deformity in the mouse induced by insulin. Anat. Rec. 124: 441, 1956.

Villee, C.A.: Ovarian Secretions. In: W.C. Young, Ed.: Sex and Internal Secretions. Volume 1. London: Williams and Wilkins Company, 1961. pp. 647.

Werner, S.C.: Failure of gonadotrophic function in the rat hypophysis during chronic inanition. Proc. Soc. Exper. Biol. Med. 41: 101, 1939.

Wickes, I.G.: Foetal defects following insulin coma therapy in early pregnancy. Brit. M. J. Oct: 1029-1030, 1954.

Witschi, E.: In: P. Altman and D. Dittmer, Ed.: Growth. Federation of American Societies for Experimental Biology, 1962. pp. 304-313.

Wood, F.C., Domenge, L., Bally, P.R., Renold, A.E., and Thorn, G.W.: Studies on the metabolic response to prolonged fasting. M. Clin. N. America 44: 1371-1380, 1960.

Wright, H.V., Asling, C.W., Dougherty, H.L., Nelson, M.M., and Evans, H.M.: Prenatal development of the skeleton in Long-Evans rats. Anat. Rec. 130: 659-672, 1958.

APPENDIX

TABLE A-1

Mean Weight of Fetuses Per Group (F-2)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	5.51	0.14
II	Fast	9-11	5.21	0.10
III	Fast	11-13	5.39	0.08
IV	Control	-	5.32	0.08
V	Control	-	5.11	0.17
		Experiment Number II		
VI	Fast+PZI	9-11	5.27	0.10
VII	Fast	9-11	5.14	0.08
VIII	PZI	9-11	5.23	0.16
IX	Saline	9-11	5.11	0.17
X	Control	-	5.31	0.07

TABLE A-2

Mean Number of Live Born Per Group (F-6)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	9.00	1.41
II	Fast	9-11	11.70	0.52
III	Fast	11-13	11.80	0.79
IV	Control	-	12.00	0.54
V	Control	-	11.30	0.42
		Experiment Number II		
VI	Fast+PZI	9-11	9.70	0.54
VII	Fast	9-11	11.00	0.47
VIII	PZI	9-11	9.60	0.43
IX	Saline	9-11	10.90	0.38
X	Control	-	10.10	0.35

TABLE A-3

Mean Number of Fifth Sternebra Misshapen Per Group (F-9)

Experiment Number I

Group Number	Treatment	Days of Gestation	Mean Number/ Group	Standard Error
I	Fast	7-9	0.70	0.30
II	Fast	9-11	2.30	0.76
III	Fast	11-13	1.80	0.49
IV	Control	-	1.20	0.36
V	Control	-	0.70	0.26
		Experiment Number II		
VI	Fast+PZI	9-11	1.80	0.33
VII	Fast	9-11	1.20	0.49
VIII	PZI	9-11	1.30	0.26
IX	Saline	9-11	1.20	0.25
X	Control	-	1.00	0.21

FIGURES

Figure 4

A vaginal smear after mating. Note the presence of spermatozoa.

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Figure 5

The control cervical vertebral column is on the left. Note on the right, the absence of cervical vertebral centra and the asynchronous pattern of ossification.

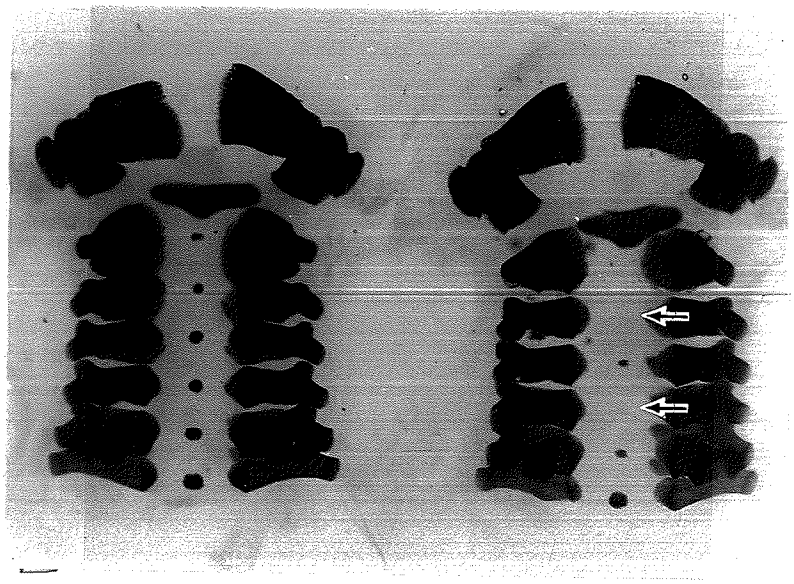

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Figure 6

The normal condition of two ossification centres of the odontoid process is on the left. Note on the right, only one ossification centre of the odontoid process is present.

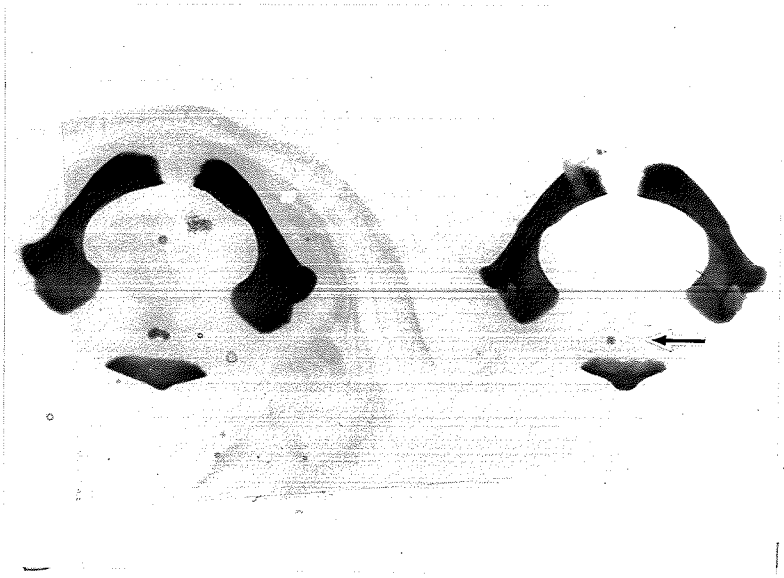

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Figure 7

The arrow indicates a duplicated thoracic vertebral centra.

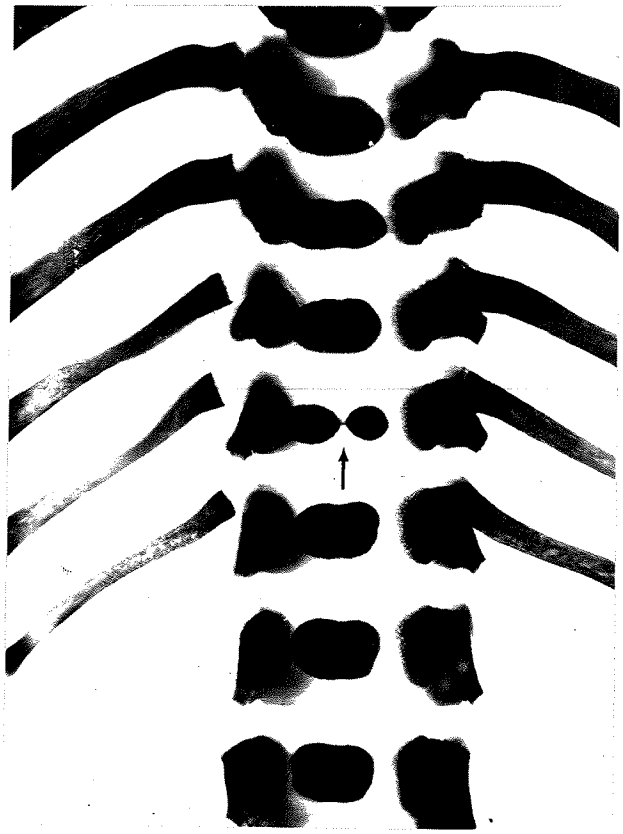

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Figure 8

On the left is the normal pattern of sternebral ossification. Note, on the right, the severely retarded fifth sternebral centre.

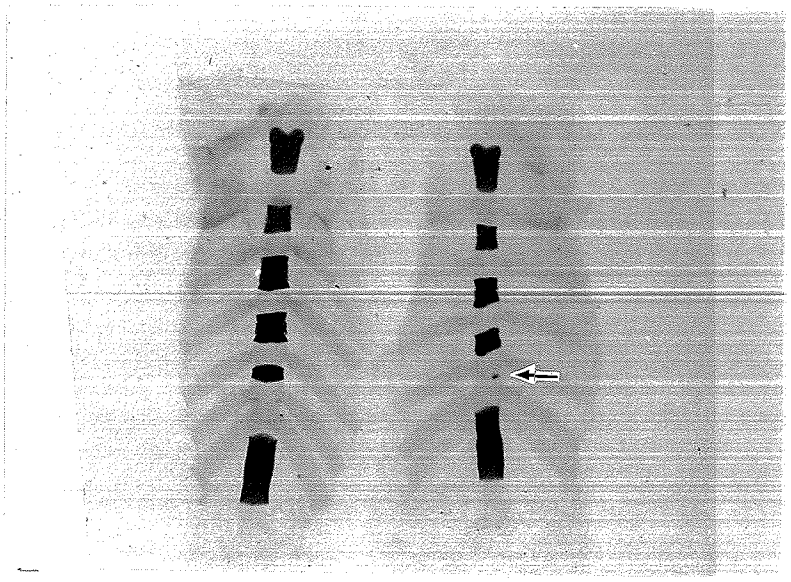

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Figure 9

On the left is the normal pattern of sternebral ossification. Note, on the right, that more than one sternebral centre is misshapen.

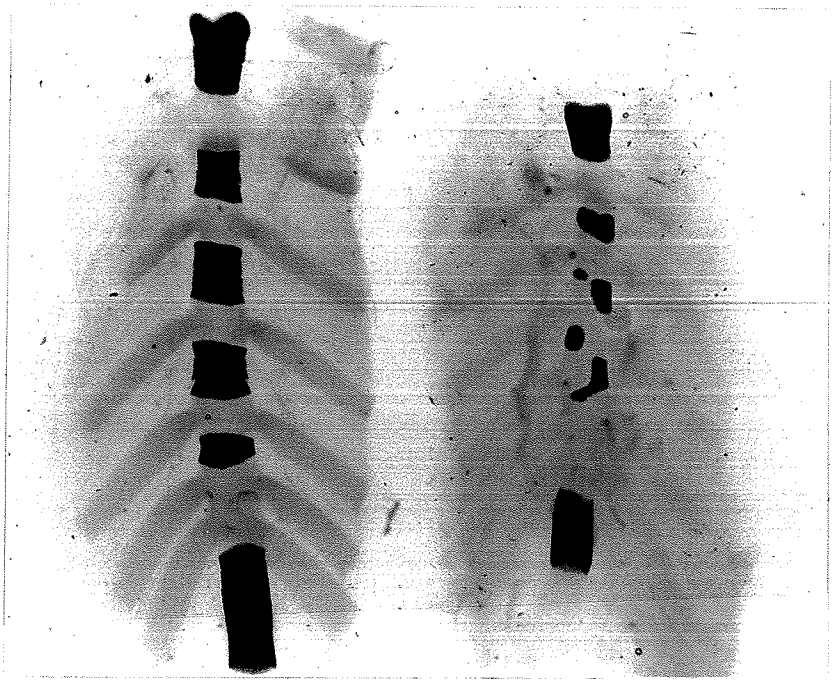

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Figure 10

The arrows indicate the presence of bilateral supernumerary ribs on the first lumbar vertebra.

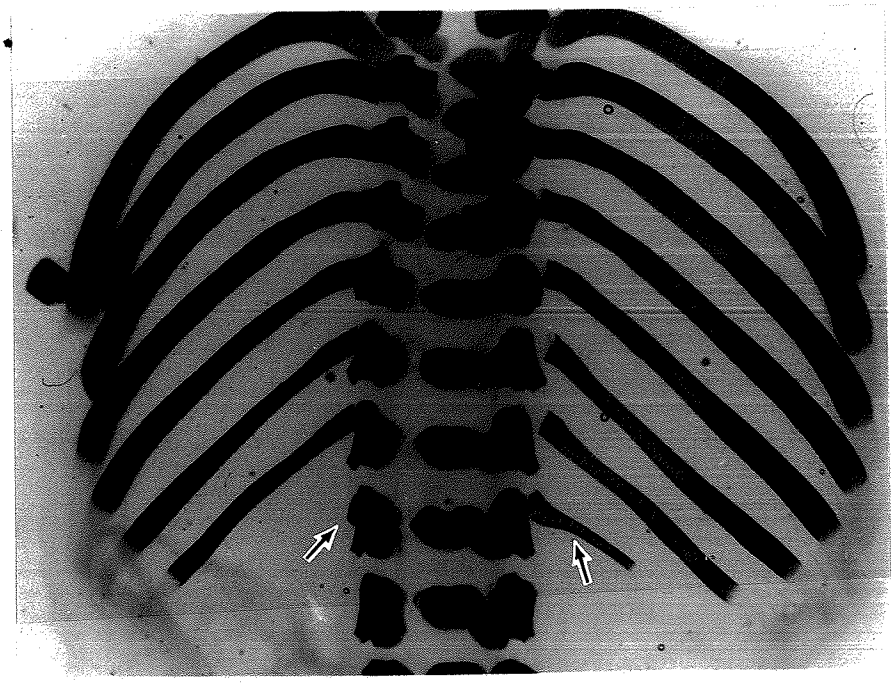

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Figure 11

The normal ossification of the anterior arch of the atlas is on the left. Note the retarded ossification of the anterior arch of the atlas on the right.

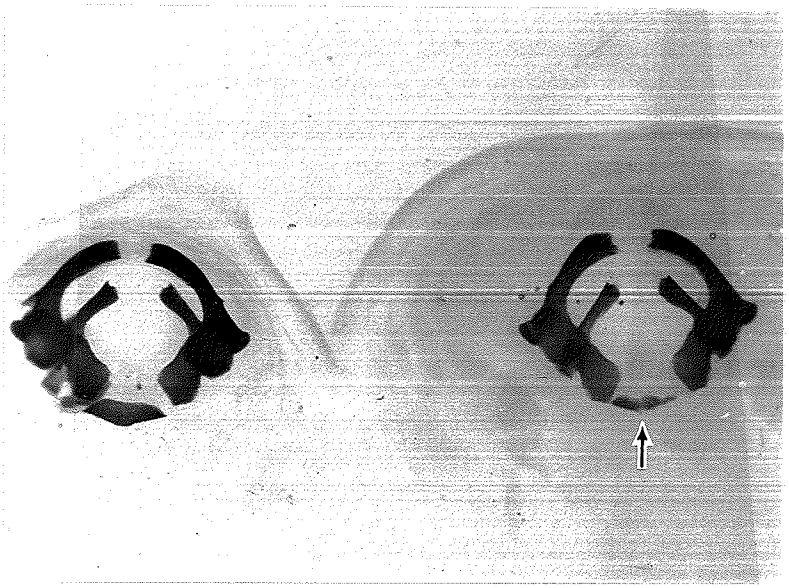

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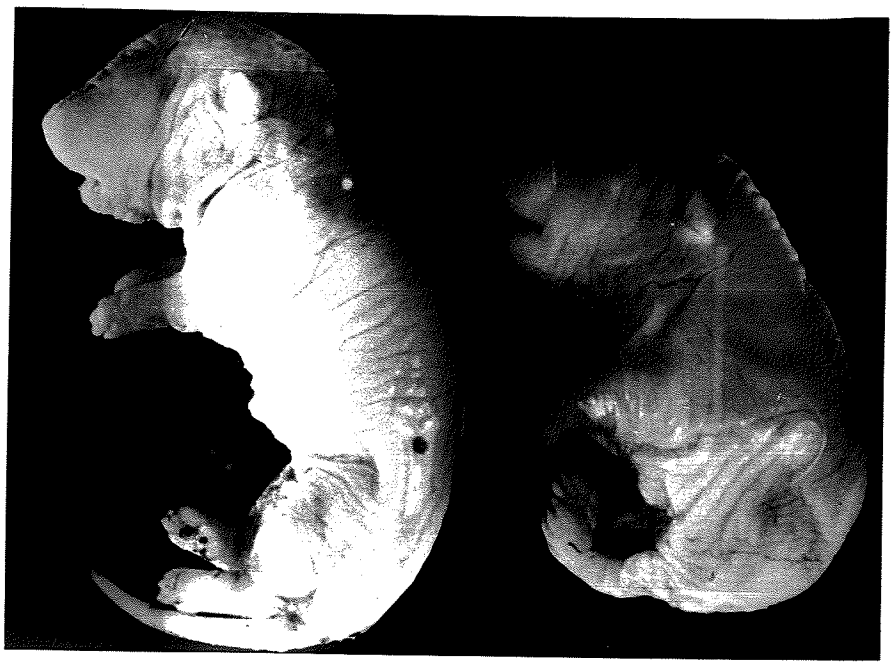
Figure 12 (a)

The normal fetus is on the left. On the right, note an abnormal fetus from Group II (fast days 9-11).

Figure 12 (b)

Alizarin staining technique on the above fetuses shows that the abnormal fetus (left) does not have any vertebrae below the thoracic region.



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