EFFECTS OF AFLATOXIN ON PREGNANT HAMSTERS AND HAMSTER FETUSES

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CHAPTER I

INTRODUCTION

Within the past 13 years there has been an increasing awareness of the multitude of problems associated with the contamination of feeds and food by fungi and fungal toxins (28). A large part of the research into this general problem of fungal toxins has been concerned specifically with aflatoxin, a toxic product of Aspergillus flavus.

Research on aflatoxin has concerned primarily 2 aspects of its toxicity; acute poisoning resulting in death, and chronic toxicity leading to neoplasia. The literature in these 2 particular areas has been extensively reviewed (2,3,10,16,48), especially as it pertains to natural or experimental aflatoxicosis in domestic or laboratory animals.

A third possible effect of aflatoxin is on the developing fetus. Two studies (15,17) have incriminated aflatoxin as a teratogenic agent in mammals. In Oklahoma, congenital defects were seen in calves whose dams had consumed feed that contained high levels of aflatoxin. Because of the sparcity of work on the teratogenic effects of aflatoxin this study was undertaken to confirm previous (15,17) observations and to attempt duplication of the hypoplastic pancreases and cirrhotic livers seen in calves. Detailed gross and histologic examinations were done to determine the frequency of defects in organogenesis and other possible lesions.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Investigation of the effects of aflatoxin at the molecular level are basic to understanding its morphologic effects. Studies *in vivo* and *in vitro* provide several related explanations of aflatoxin's action.

Wogan (62) and Nabney (46) indicated that metabolism of aflatoxin took place in numerous organs, and little was excreted unchanged.

On the cellular level aflatoxin affected DNA synthesis, RNA metabolism, and protein synthesis (62,63). Effects on DNA synthesis involved an interaction of aflatoxin with DNA, however the exact mechanisms were not determined. The effects on RNA metabolism resulted from inhibition of RNA polymerase. In addition to effects on nuclear RNA, polysomal disaggregation and reaggregation of cytoplasmic RNA also occurred. The synthesis of proteins by the liver appeared to be inhibited at a specific stage but the exact locus was unknown. Total liver protein synthesis was not affected markedly indicating that alterations in protein synthesis could only be secondary to effects on RNA synthesis. Wogan stated that on the basis of present evidence, the biochemical changes induced by single doses of aflatoxin are associated with the acute toxicity of the compound instead of its carcinogenic effects.

Clifford *et al.* (14) indicated that the toxic action of the

aflatoxins was related to their interaction with DNA and that the degree of interaction determined the degree of toxicity. They proposed that the interaction in some way prevented DNA transcription. These workers mentioned that the mechanism of aflatoxin carcinogenesis was unclear. They proposed that inhibition of nuclear RNA synthesis resulted from the toxin interacting with DNA, preventing transcription. Pong and Wogan, however, reiterated that the action of aflatoxin B_1 was on RNA polymerase (50). Nuclear alterations in rat liver cells were correlated with the time course of RNA polymerase inhibition. Monneron (44) produced helical polysomes in rat liver cells, and proposed the possibility of transcription of abnormal RNA.

The effects of aflatoxin on various cell systems has been investigated *in vitro*. Sporn *et al.* (58) indicated that DNA was bound by aflatoxin and Legator (36) reported suppression of both DNA synthesis and mitosis within a few hours after adding aflatoxin to cultured embryonic lung cells. Legator stated that aflatoxin affects biologic systems in a manner similar to alkylating agents, and like these agents, is carcinogenic and mutagenic. In HeLa cells, aflatoxin B_1 inhibited synthesis of ribosomal and heterodisperse RNA (30). The mechanism of action of the toxin on RNA synthesis was considered by these authors to be related to its inhibitory effect on the maturation of the 45S ribosomal RNA precursor.

Expression of the molecular effects of aflatoxin have included acute toxic syndromes which vary somewhat in different animals, and chronic toxicity leading to the production of tumors in some species. A number of case reports of aflatoxicosis in animals have been published. Experimental production of the disease in chickens (57), dogs

(47,13,4), pigs (55), monkeys (51), guinea pigs (9) and rats (8), indicated that the primary target organ was the liver, with bile duct proliferation, necrosis of parenchyma, and fatty livers mentioned as common lesions. The LD_{50} of aflatoxin for hamsters was determined by Wogan (62), although there was no mention of the lesions produced.

The carcinogenic effects of aflatoxin in various species have been reviewed by Newberne and Butler (48). McD. Herrold (43) gave hamsters aflatoxin intragastrically and intraperitoneally for periods of 10.0-11.0 and 6.0-8.5 months respectively. Liver lesions included focal areas of hemorrhagic necrosis, hemosiderin deposits, megalocytosis and bile duct proliferation. Other lesions included shortening of small intestinal villi, cytomegalic changes in cells of the proximal tubules of the kidneys, hyperplasia and atypical acinar epithelium in Harderian glands and cell nests in the peridontal membrane of the lower incisor teeth.

The mutagenic and teratogenic effects of aflatoxin have been studied in several types of experimental systems. Maher and Summers (40) reported that aflatoxin induced mutations in DNA in an *in vitro* system. According to Maher and Summers the ability of DNA to act as an RNA template was altered. Utilizing plants, Lilly (39) produced abnormal anaphase, chromosome fragments and inhibition of mitosis following aflatoxin treatment.

Surviving embryos of fertile hen's eggs injected with aflatoxin B_1 (approximately LD_{50}) had a depression in growth after 12 days of development, but no gross morphologic abnormalities (54). In sheep given aflatoxin with their feed, Lewis *et al.* (38) reported lowered fertility but did not mention any fetal abnormalities. Hintze *et al.* (32)

fed swine a ration containing 450 ppb of aflatoxin B_1. They noted no effect on the growth performance of the pigs. These results contrasted with those of Cardeilhac et al. (12) who noted slower rates of weight gain and stunting by weaning time in pigs from sows fed crude aflatoxin, as compared with control pigs. Abortion was one of the clinical signs observed in a natural outbreak of mycotoxicosis in swine. However, the affected sows also had hemorrhagic enterocolitis, and the relationship of aflatoxin to abortion was not entirely proven (5).

The effects of aflatoxin on pregnant rodents of several species have been studied. Butler and Wigglesworth (11) gave oral doses of aflatoxin to pregnant rats early in pregnancy and noted no abnormalities in placentas, no malformed fetuses and no effect on fetal growth. If given later in gestation (day 16) there were severe effects on the pregnant female and impairment of fetal growth. These experiments indicated no direct action of aflatoxin on the fetus. Working with mice, Legator (37) noted that a mixture of aflatoxins B_1 and G_1 given to pregnant females increased postimplantation loss of embryos.

DiPaolo et al. (15) gave aflatoxin B_1 dissolved 1 mg/ml in triethylene glycol to hamsters, rats and mice intraperitoneally. The hamsters were injected on days 8 and 13 of pregnancy. A dose of 4 mg/kg given on day 8 produced the greatest number of malformed fetuses. In mice, doses of 8-12 mg/kg resulted in 90% resorption of fetuses. The authors concluded that the teratogenic effect varied according to species and stage of fetal development at the time of exposure.

Elis and DiPaolo (17) injected pregnant hamsters intraperitoneally with aflatoxin. They found that 2 mg/kg did not produce gross malformations of the fetuses, that 4 mg/kg produced the greatest number of

gross abnormalities and 6 mg/kg was the LD_{50} for the pregnant females. Malformations noted early in gestation included anencephaly while those in fetuses examined at 13 days of gestation were less severe, and included microencephaly and umbilical hernia. An aflatoxin-DNA mixture was less teratogenic than aflatoxin alone. Elis and DiPaolo saw neither deviation in chromosome number or chromosomal aberrations in treated animals. Injections of aflatoxin did cause necrosis in fetal liver within 8 hours, and hemorrhage and diffuse hepatic degeneration within 24 hours.

CHAPTER III

MATERIALS AND METHODS

Materials

Animals used were virgin female golden hamsters (Mesocricetus auratus) 3-6 months old. Commercially prepared, purified and crystallized aflatoxin B_1[1] was purchased and dissolved in either purified triethylene glycol[2] or glycerin.[3] Bouin's solution (30 gm Picric Acid, 2250 cc distilled H_2O, 750 cc 40% formaldehyde, 150 cc Acetic acid) was used to fix fetuses, and 70% ethyl alcohol was used to store them following fixation. Ten percent acetate buffered formalin was used for fixation of adult tissues.

General Methods

Acquisition, Husbandry and Breeding

Animals were purchased from a commercial supplier[4] and were shipped by commercial air freight. They were placed in the colony for 2 weeks prior to any experimental manipulation. All animals were caged individually in a closed room with a timed light sequence giving 13 hours of light per day. They were fed Purina Laboratory Chow and supplemented with fresh green vegetables once a week. Estrus was determined by Orsini's method (49) and timed matings were obtained by the method of Ferm (25).

Inoculation and Sampling Procedures

All inoculations were intraperitoneal using a tuberculin syringe and a 20 gauge disposable needle.

Day 1 of pregnancy was considered to be the 24 hour period beginning at 1:00 a.m. the day following placement of the female with the male. Inoculations were given at 8:00 a.m. on either day 8 or 9. Female hamsters were killed at 4:00 p.m. at intervals of 0.5, 1 and 3 days after inoculation, and on day 15 of pregnancy. The uterus was removed, gestation sacs counted, and the uterus was placed either directly in Bouin's fluid (gestation day 8 or 8.5) or in warm tap water in a Petri dish.

Those placed in warm tap water had the individual gestation sacs opened and the fetuses examined under a dissecting microscope. Fetuses of 9 days gestation and older were classified as viable if the heart was beating, and dead if not. Fetuses of 8.0 or 8.5 days gestation were classified as viable if they were morphologically normal. The number of gross external malformations per fetus was determined and each one described. Then fetuses were placed in Bouin's fluid for 48 hours, rinsed in running tap water and brought through 2 changes of 70% ethyl alcohol. Uteruses placed directly in Bouin's fluid were handled similarly.

A complete necropsy was performed on the female, and representative tissues were fixed in 10% formalin.

Histologic Technique

Following fixation, all fetuses and adult tissues were dehydrated in alcohol, embedded in paraffin, cut at 6 microns and stained with

hematoxylin and eosin. Fetuses at 8 and 8.5 days of gestation were left within gestation sacs. The sacs were embedded so that fetuses were cut in either transverse or sagittal sections. Fetuses of 9, 9.5, 10, 11 and 12 days gestation were embedded whole and oriented to allow either whole body sagittal or horizontal sectioning. Fetuses of 15 days gestation were sectioned grossly into 2 sagittal or 3 transverse portions and those portions embedded. Serial sections were cut from each block.

Experimental Design

Females were randomly divided into 4 experimental groups. For the first 3 experiments, individuals of a group were assigned randomly as outlined in Tables I and II.

Triethylene glycol was used as the diluent in experiment 1 and 10 mg of aflatoxin was dissolved in 10 ml of triethylene glycol giving a concentration of 1 mg aflatoxin/1 ml triethylene glycol. Aflatoxin was inoculated on the basis of 4 mg/kg. Controls were inoculated with 4 ml of triethylene glycol/kg. In experiment 2 glycerin was used instead of triethylene glycol as the diluent. Aflatoxin was inoculated on the basis of 4 mg/kg and controls inoculated with 4 ml/kg of glycerin. In experiment 3 glycerin was also the diluent, but the aflatoxin was dissolved 2 mg/ml and given at a dose of 6 mg/kg. Controls received 2 ml/kg of glycerin.

Animals in Experiment 4 were divided into 3 sub-groups, 6 animals per group (Table III).

Statistical analysis of results was done at the Oklahoma State University Computer Center using the Statistical Analysis System of the

Department of Statistics, North Carolina State University. Variables analyzed included the percentage of: viable fetuses (PVIAB), dead fetuses (PDEAD) and the total number of fetuses (PTOT). These were computed as a ratio of the total number of a particular variable to the total number of gestation sacs for controls or aflatoxin inoculated animals in each experiment. Other variables included the percent of: viable fetuses with abnormalities (PVWL), dead fetuses with abnormalities (PDWL) and the total percent of fetuses with abnormalities (PTWL). These percentages were calculated for each experiment as a ratio of the total number of abnormalities to the total of the viable or dead fetuses or sum of the viable and dead fetuses respectively. In addition, the percent of fetuses with malformations of the head (PHL) and spine (PSL), growth retardation (PGR) and miscellaneous lesions (PM) were determined as a ratio of the total number of the particular abnormality to the total number of fetuses (alive or dead) in a particular experiment. Factors in the analyses of variance for the above variables included: treatment, day of inoculation, day of examination, and interactions between these sources.

TABLE I

DESIGN OF EXPERIMENT 1

Day of Inoculation	Day of Examination	Number of Animals Inoculated	Number of Controls
8	9, 11, 15	6	6
9	10, 12, 15	6	6

TABLE II

DESIGN OF EXPERIMENT 2 AND EXPERIMENT 3

Day of Inoculation	Day of Examination	Number of Animals Inoculated	Number of Controls
8	8.5, 9, 11, 15	8	4
9	9.5, 10, 12, 15	8	4

TABLE III

DESIGN OF EXPERIMENT IV

Day of Inoculation	Day of Examination	Number of Animals Inoculated	Procedure
8	9, 11, 15	6	No Treatment
8	9, 11, 15	6	Intraperitoneal puncture by needle only.
8	9, 11, 15	6	4 ml/kg sterile physiologic saline intra-peritoneally.

FOOTNOTES

[1]Calbiochem, LaJolla, California.

[2]Fisher Scientific Co., Fairlawn, New Jersey.

[3]Mallinkrodt Chemical Works, St. Louis, Missouri.

[4]Lakeview Hamster Colony, Newfield, New Jersey.

CHAPTER IV

RESULTS

Effects of Aflatoxin on Pregnant Female Hamsters

Controls

Of the 46 adult control animals from the 4 experimental groups there were 4 with clinical problems and/or gross lesions. These 4 animals had all been inoculated with triethylene glycol (4 ml/kg) on day 8 of gestation. One died 1 day after inoculation and 1 had clinical signs including anorexia, depression, dehydration and clear, dark yellow urine for 5 days following inoculation. At necropsy these 2 animals and 2 others without clinical signs had slightly enlarged kidneys that were pale purple-tan instead of the normal red-purple.

Histologically, kidney lesions were minimal, consisting of degenerative changes in convoluted tubules, including swollen, eosinophilic epithelial cells, pyknotic nuclei and necrosis. Some tubules contained necrotic debris.

Experiment 1

Of the 12 animals inoculated, 2 died approximately 6-8 hours prior to the time they would have been killed. Clinical signs included dehydration, anorexia and clear, yellow urine. These 2 and the 5 other animals of this treatment group had gross and histologic lesions of the

liver and kidney.

Grossly the lesions varied from slightly enlarged, pale livers to livers with moderate or marked lobulation. The most severely affected livers were friable. Histologically the lesion was one of periportal cellular degeneration which varied in severity. The animals with minimal gross changes had minimal cellular degeneration characterized by cytoplasmic eosinophilia and clearing and margination of nuclear chromatin. Some parenchymal cells were pyknotic. This mild periportal degeneration was not distinguished easily. The more severely affected livers had periportal fatty change and parenchymal degeneration including necrosis. These areas were extensive, adjacent periportal areas being connected by a zone of degenerate parenchyma giving the lobule a definite delineation.

The kidneys of all affected animals were slightly enlarged, appeared edematous and varied from pale-red-tan to light tan. Histologically there was a variable degree of tubular epithelial degeneration and necrosis with some tubules plugged by necrotic debris and others having flattened regenerative epithelium, giving the tubules a slightly dilated appearance.

Experiment 2

All pregnant females survived the required time following inoculation. Several became listless and somewhat anorectic and 2 had a unilateral mucopurulent conjunctivitis. Grossly, only the livers and kidneys appeared similar to those of animals inoculated with 4 mg/kg aflatoxin in triethylene glycol.

Hepatic lesions varied with the day of examination, from slightly

pale and enlarged 1 day after inoculation, to minimal or marked lobulation with scattered dry, yellow foci from 0.1 to 0.3 mm in diameter when examined 3 days following inoculation and on day 15 of gestation. No difference in the type of lesion was noted in animals whether on day 8 or day 9 of gestation.

Histologically, the severity of the renal lesions was related to the day of sampling postinoculation. At 0.5 day following inoculation very little morphologic change was present. At 1.0 day following inoculation there was minimal tubular necrosis in the kidneys characterized by flattening of tubular epithelial cells and some eosinophilic material in tubular lumens (Fig. 1). By 3 days after inoculation there were severe necrotic changes in cortical tubular epithelium and numerous tubules contained homogenous eosinophilic material (Fig. 2). In many focal areas only tubular basement membrane and a few necrotic epithelial cells remained (Fig. 3).

Histologic changes present in the liver 0.5 day after treatment were limited to immediate periportal areas and consisted of some clearing of nuclear chromatin in hepatocytes. One day after inoculation there was considerable involvement of periportal hepatocytes. The affected cells were shrunken and had homogenous eosinophilic cytoplasm and margination of nuclear chromatin (Fig. 4). A few livers had a minimal amount of bile duct proliferation (Fig. 5). By 3 days postinoculation portal areas were connected by well demarcated areas of fatty degeneration and necrosis (Fig. 6). The lesion at this stage correlated well with the gross appearance of moderate lobulation. Beyond 3 days following inoculation there was a variable degree of massive necrosis in many parts of many livers (Fig. 7, 8). In some areas only islands of

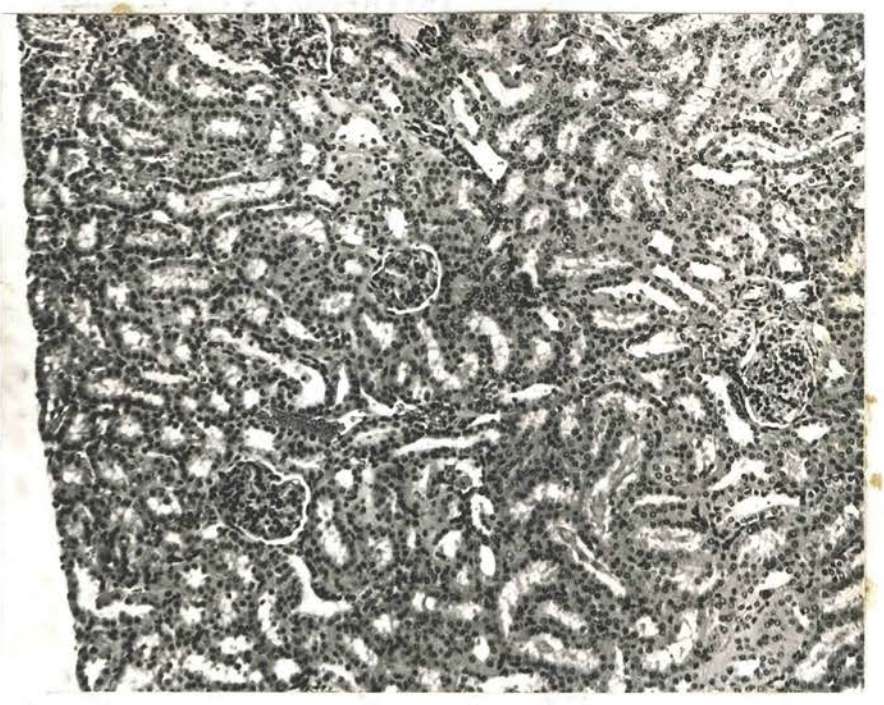

Figure 1. Minimal Nephrosis 1 Day Postinoculation with Aflatoxin, 4 mg/kg in Glycerin. Some Tubules Contain Eosinophilic Debris (H&E x 30)

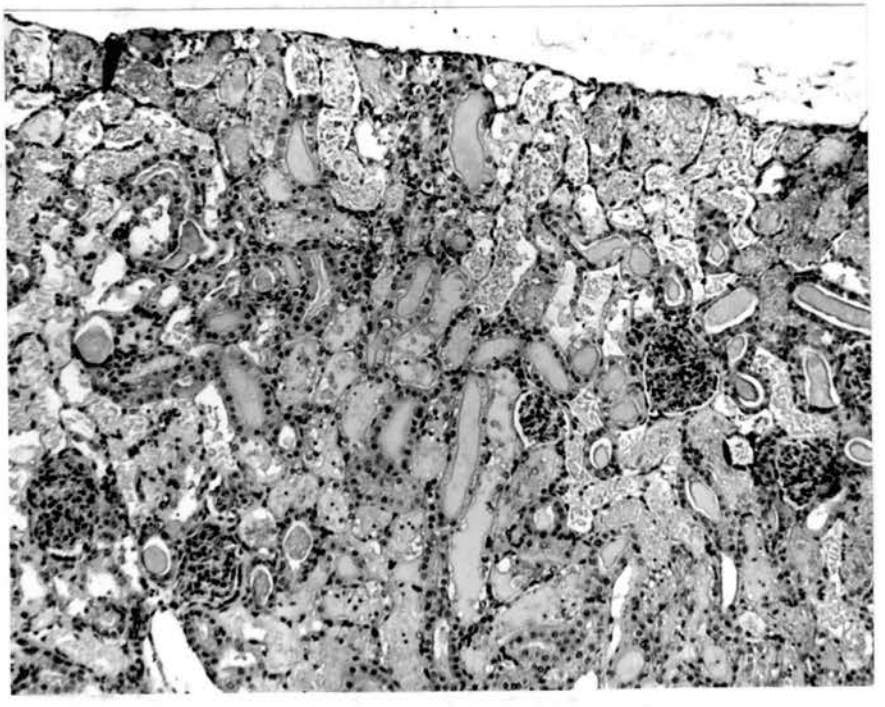

Figure 2. Severe Tubular Necrosis and Cast Formation in Kidney of Hamster 3 Days Post-inoculation With 4 mg/kg Aflatoxin in Glycerin (H&E x 30)

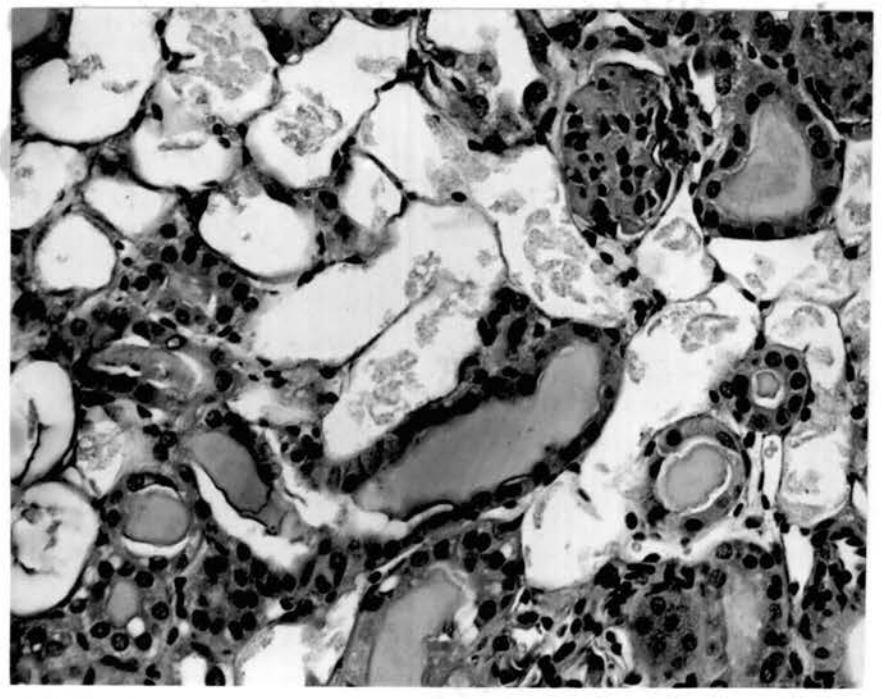

Figure 3. Higher Magnification of Kidney in Fig. 2. Note Complete Necrosis of Tubular Epithelium in Focal Area (H&E x 200)

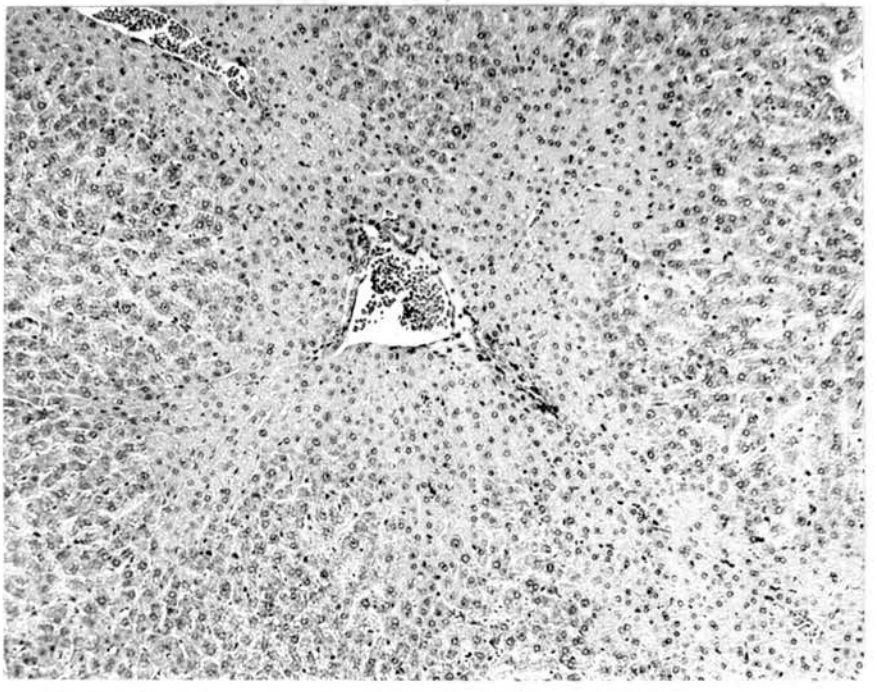

Figure 4. One Day Postinoculation With 4 mg/kg Aflatoxin in Glycerin. Well Demarcated Periportal Degeneration Characterized by Loss of Cytoplasmic Boundaries, Eosinophilia of Cytoplasm and Clearing of Nuclear Chromatin (H&E x 75)

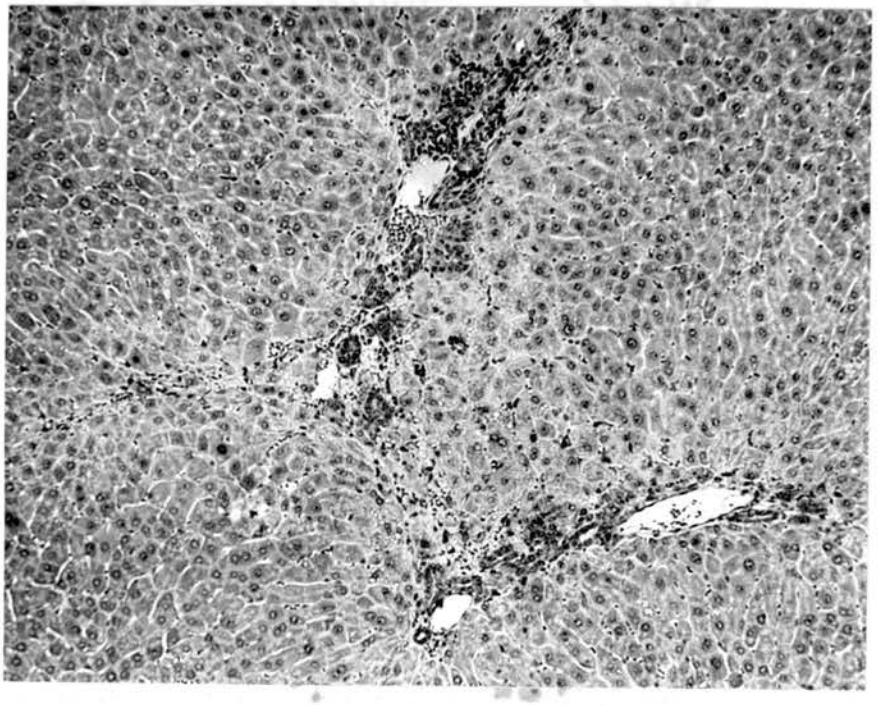

Figure 5. Biliary Hyperplasia 1 Day Postinoculation With 4 mg/kg Aflatoxin in Glycerin (H&E x 75)

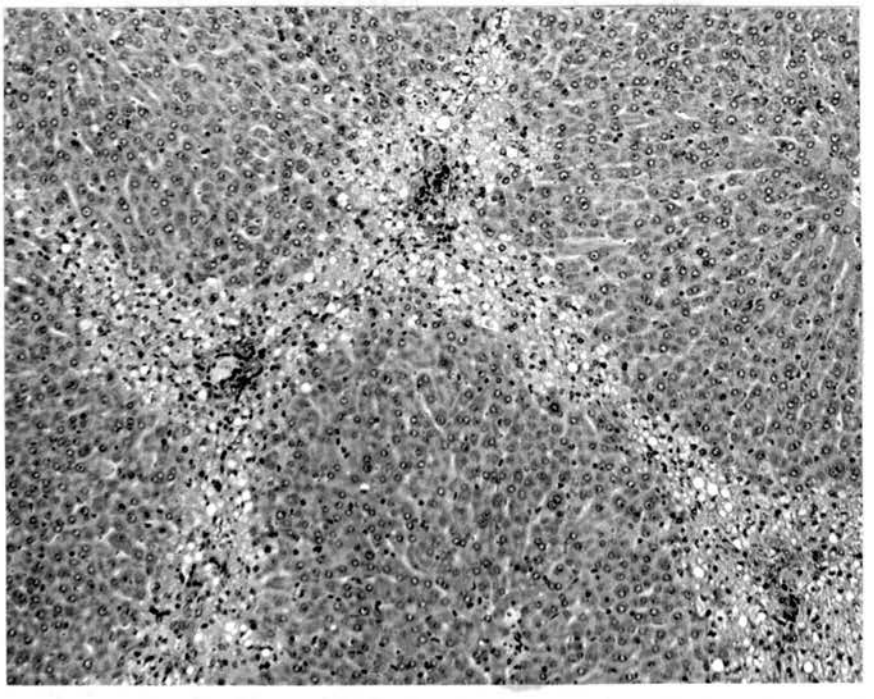

Figure 6. Zones of Fatty Degeneration Connecting Portal Areas in Liver of Hamster 3 Days Postinoculation With 4 mg/kg Aflatoxin in Glycerin (H&E x 30)

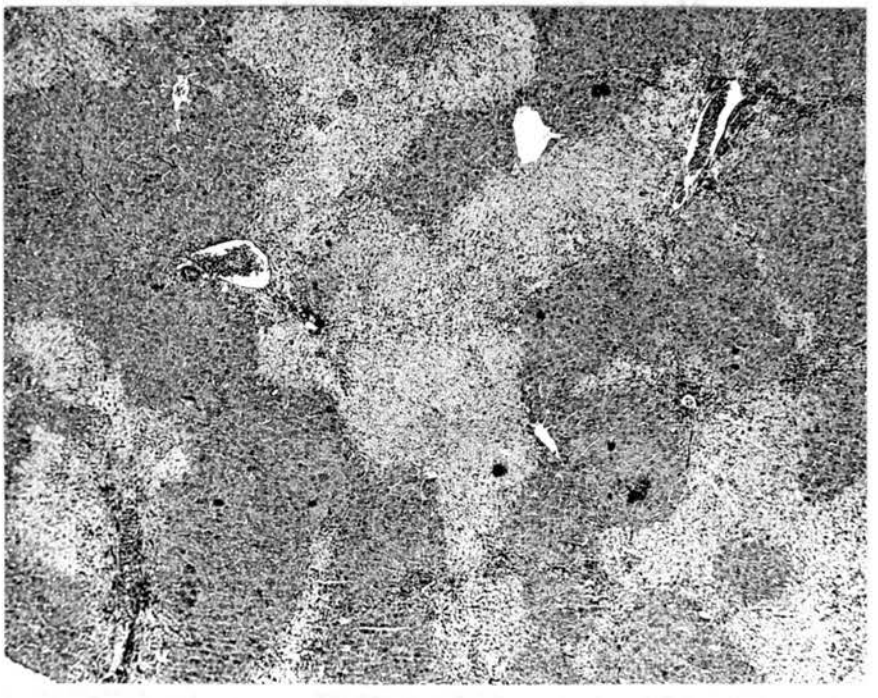

Figure 7. Massive Necrosis in Liver From Animal Killed at Day 15 of Gestation. 4 mg/kg Aflatoxin in Glycerin (H&E x 30)

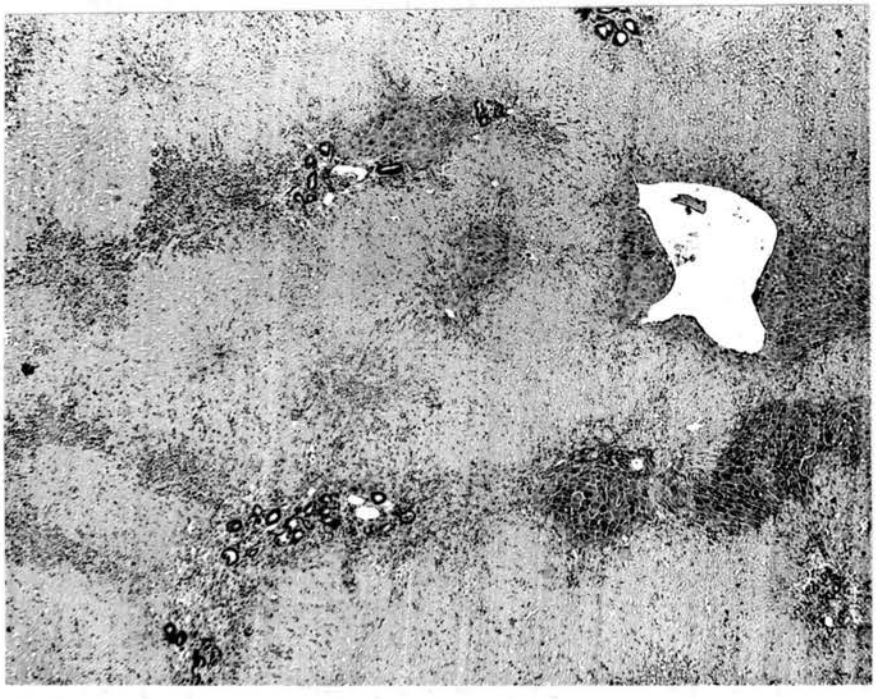

Figure 8. Severe Degree of Massive Liver Necrosis. 4 mg/kg Aflatoxin in Glycerin, Day 15 of Gestation (H&E x 200)

liver parenchyma remained clustered around central veins (Fig. 8). At this stage there was a sharp zone of demarcation between relatively normal parenchyma and necrotic liver (Fig. 9).

Serum glutamic-pyruvic-transaminase (SGPT) determinations were done terminally on 2 of the clinically ill animals. Values were 300 Sigma Frankel (SF) units and 1200 SF units respectively. Blood Urea Nitrogen (BUN) levels on these animals were 20 and 65 mg%, with the higher level corresponding to a greater degree of illness and to more severe morphologic kidney lesions.

Experiment 3

Five animals were killed prior to the schedule called for in the experimental design. These animals appeared clinically normal from 2-4 days after inoculation and in each case the onset of signs was sudden. Major clinical signs included clear, dark yellow urine, anorexia, depression and convulsions. When convulsions occurred, the animals were killed rather than have fetuses undergo postmortem degeneration after death of the dam.

Qualitatively, gross lesions in all aniamls were similar to those described previously, and included progressive liver and kidney necrosis. The animals more severely affected clinically had a slight to moderate amount of fluid in the peritoneal cavity, and there was hyperemia of serous membranes of intestines, pancreas and the mesenteries. Changes in the liver and kidneys were more severe immediately following treatment. Kidneys were slightly to extremely pale, and many had a "scotch grain" appearance on the surface (Fig. 10). The liver had accentuation of lobular pattern and white-yellow areas of

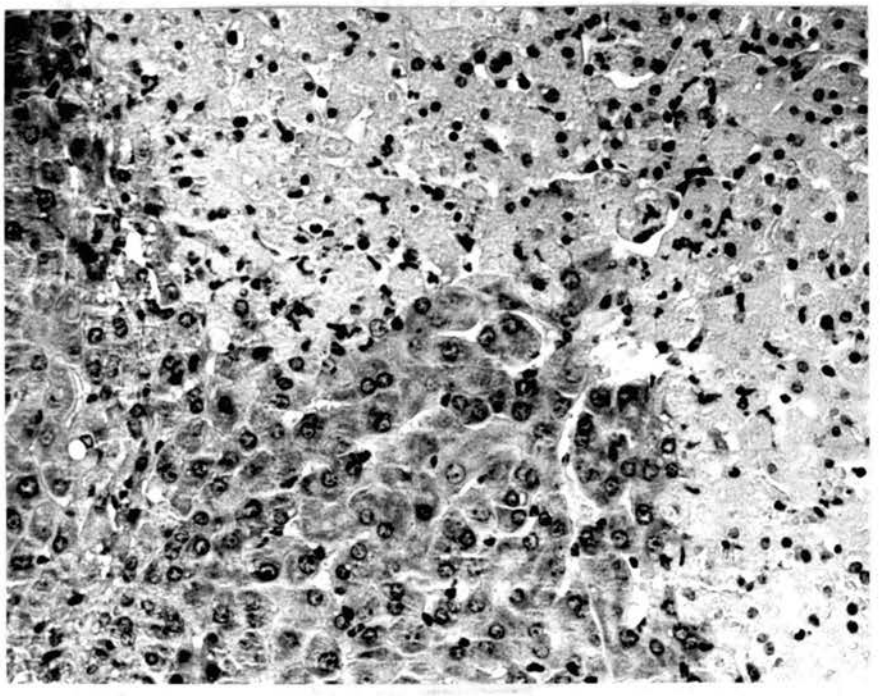

Figure 9. Higher Magnification of an Area of Fig. 13. Note Sharp Demarcation Between Normal and Necrotic Parenchyma (H&E x 200)

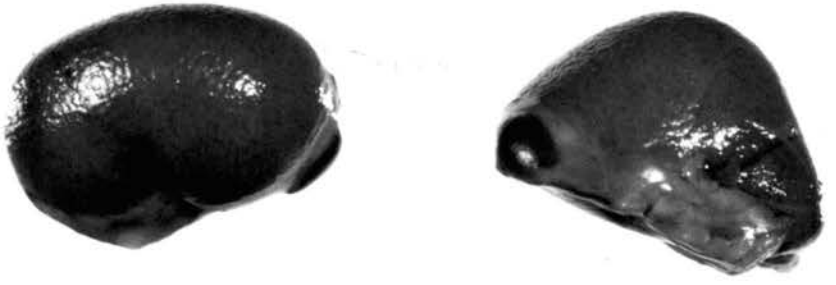

Figure 10. Finely Nodular Appearance of Kidneys 3 Days Postinoculation With 6 mg/kg Aflatoxin in Glycerin

variable size and distribution (Figs. 11, 12, 13).

Histologic lesions of the liver were nearly identical to those described for animals given 4 mg/kg aflatoxin, except that there was a greater degree of hyperemia and hemorrhage in necrotic areas (Fig. 14).

The kidneys, especially from animals clinically ill, were more severely affected than the kidneys from animals in experiments 1 and 2. There was widespread tubular necrosis and diffuse homogenous eosinophilic casts in tubules of both the cortex and medulla (Fig. 15). SGPT values for 2 severely ill and euthanized animals were 1200 and 4300 SF units. BUN values were 60 and 65 mg% respectively.

The uteri and placentae from all treatment groups were normal.

Effects of Aflatoxin on Fetuses

For each experiment a number of variables were recorded. These variables included the percent of viable fetuses (PVIAB), dead fetuses (PDEAD), total number of fetuses (PTOT), dead (PDWL), viable (PVWL), and total number of fetuses with abnormalities (PTWL), and the categories of abnormality including malformations of the head (PHL) and spine (PSL), growth retardation (PGR), and miscellaneous lesions (PM). The results of each experiment were analyzed using an analysis of variance (AOV) for each variable, and appropriate F tests. P values are given as (P=x) or ($x<P<y$).

Experiment 1

Table IV gives the mean percent of PVIAB and PDEAD for all combinations of treatment, day of inoculation and day of examination. The mean percent for PVIAB, PDEAD and resorptions as an effect of aflatoxin

Figure 11. Moderate Lobular Pattern and Focal Pale Areas in Liver From Hamster Inoculated With 6 mg/kg of Aflatoxin in Glycerin; 3 Days Post-inoculation

Figure 12. Marked Lobulation and Demarcated White Necrotic Areas in Liver From Hamster Inoculated With 6 mg/kg Aflatoxin; 4 Days Postinoculation

Figure 13. Diffuse, Severe Hepatic Necrosis. 6 mg/kg Aflatoxin, 5 Days Postinoculation

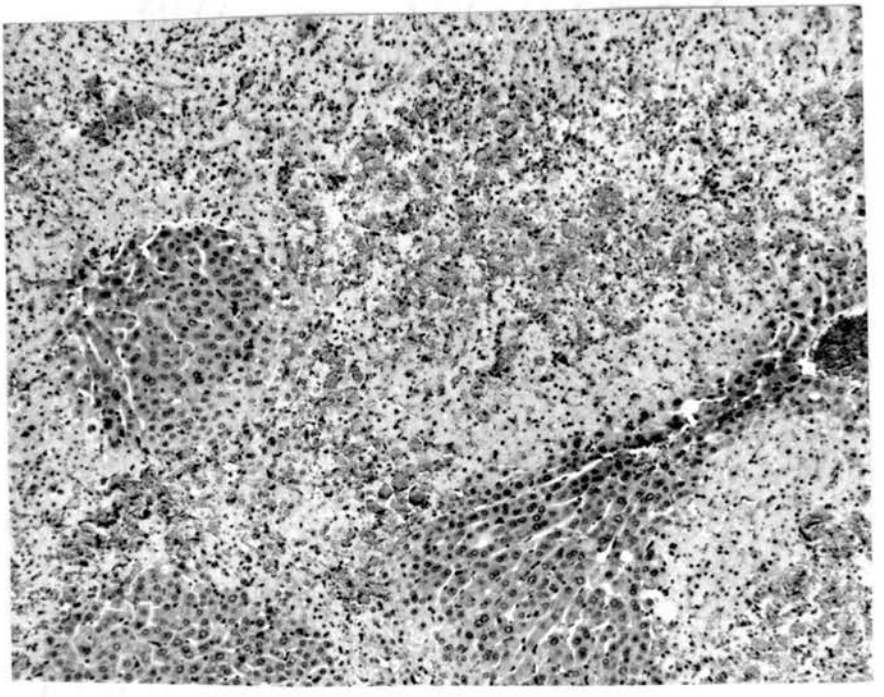

Figure 14. Hemorrhage in Necrotic Areas of Liver in Fig. 13 (H&E x 30)

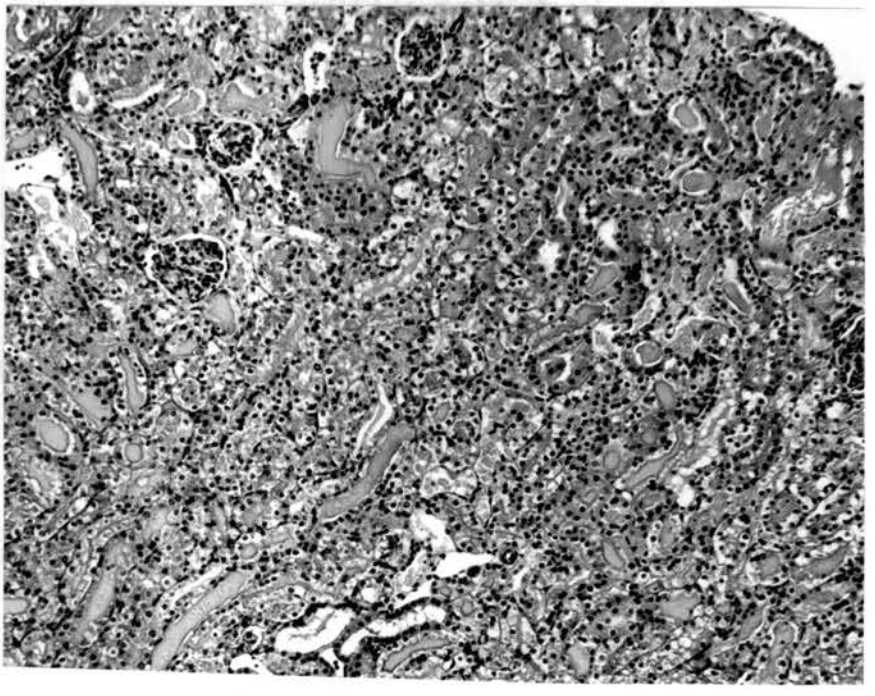

Figure 15. Diffuse, Severe Necrotic Changes and Eosinophilic Casts in Kidney Tubules. 6 mg/kg Aflatoxin in Glycerin. Three Days Postinoculation (H&E x 30)

TABLE IV

EXPERIMENT 1: AFLATOXIN EFFECTS ON HAMSTER FETUSES

Number of Females Inoculated	PDEAD	PVIAB	% Resorptions	Inoculated/Control	Day Inoculated	Day Examined
2	12	84	4	control	8	9
2	0	50	50	control	8	11
2	0	38	62	control	8	15
2	18	71	11	inoculated	8	9
2	34	50	16	inoculated	8	11
2	0	25	75	inoculated	8	15
2	0	75	25	control	9	10
2	3	81	16	control	9	12
2	0	100	0	control	9	15
2	50	49	1	inoculated	9	10
2	0	50	50	inoculated	9	12
1	0	0	100	inoculated	0	15

inoculation vs. controls is shown in Figure 16. The difference between the control and inoculated group means for PVIAB and PDEAD was significant in both cases ($0.10<P<0.25$). Resorptions were a prominent portion of the percent of nonviable fetuses in both inoculated and control groups. The PTOT varied with the day of examination ($0.10<P<0.25$), decreasing in a linear fashion with increasing days after inoculation.

Figure 17 illustrates the effect of aflatoxin inoculation on PVWL, PDWL, PTWL, PHL, PSL, PM and PGR. Significance ($0.10<P<0.25$) was noted for the difference in mean percent of control and inoculated for the variable PTWL. Some difference in means between the 2 groups is noted for PM but the most prominent difference between means is that of PGR ($0.10<P<0.25$). All observations of PHL and PSL (control and inoculated groups) were made on days of examination 9 or 10.

Experiment 2

Figure 18 illustrates the mean percent of PVIAB, PDEAD and resorptions as a function of aflatoxin inoculation. Sources of variation affecting the PVIAB included treatment ($P=0.10$), day of examination ($0.025<P<0.05$) and the day of examination x day of inoculation interaction ($0.01<P<0.025$). This effect was primarily due to the low PVIAB (8) seen in the inoculated group for day 8 inoculation and day 9 of examination. The PTOT varied only with the day of inoculation ($0.10<P<0.25$), in contrast to Experiment 1 where it varied with the day of examination.

The effect of aflatoxin on the variables PVWL, PDWL, PTWL, PHL, PSL, PGR and PM is shown in Figure 19. Although the mean percents for PVWL, PDWL and PTWL were all higher in the inoculated group, only the

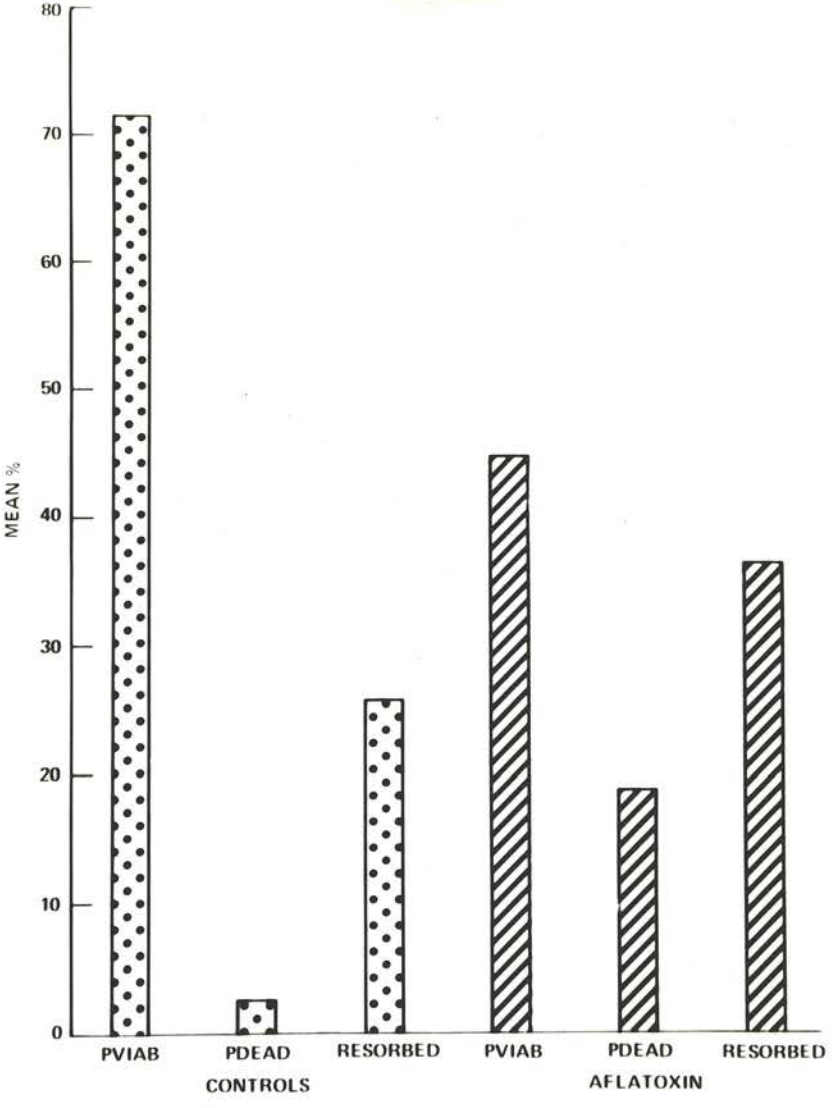

Figure 16. Comparison of Mean Percent of PVIAB, PDEAD, and Resorptions in Inoculated and Control Groups From Experiment 1

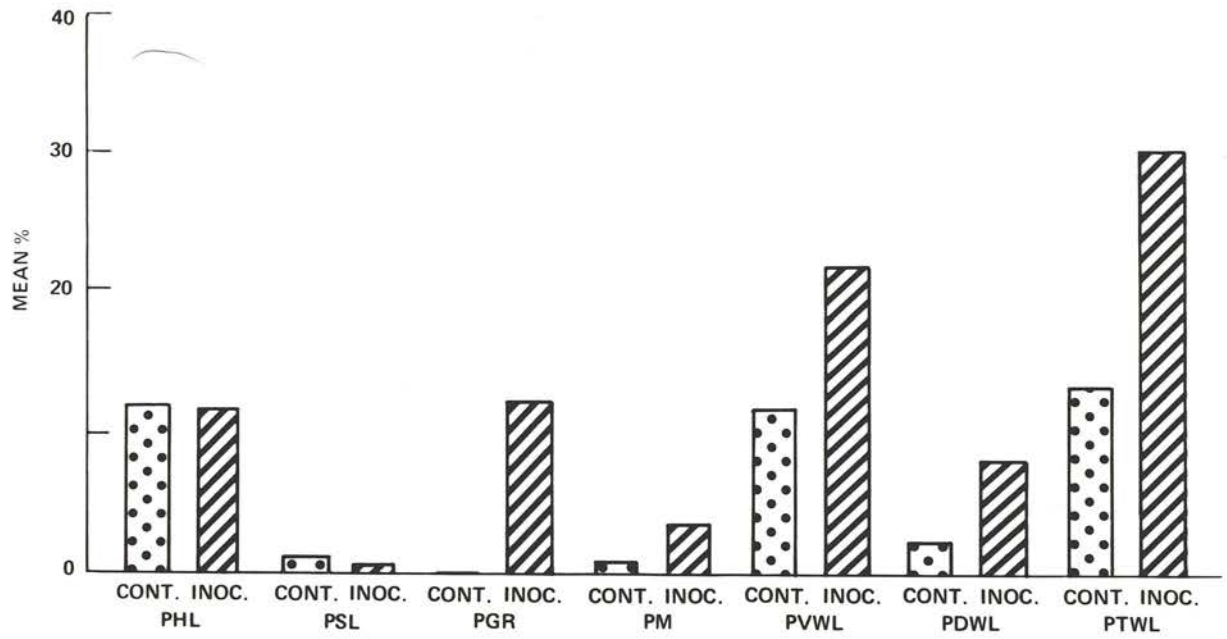

Figure 17. Comparison of the Mean Percent for the Variables PHL, PSL, PGR, PM, PVWL, PDWL and PTWL of Aflatoxin and Control Groups From Experiment 1

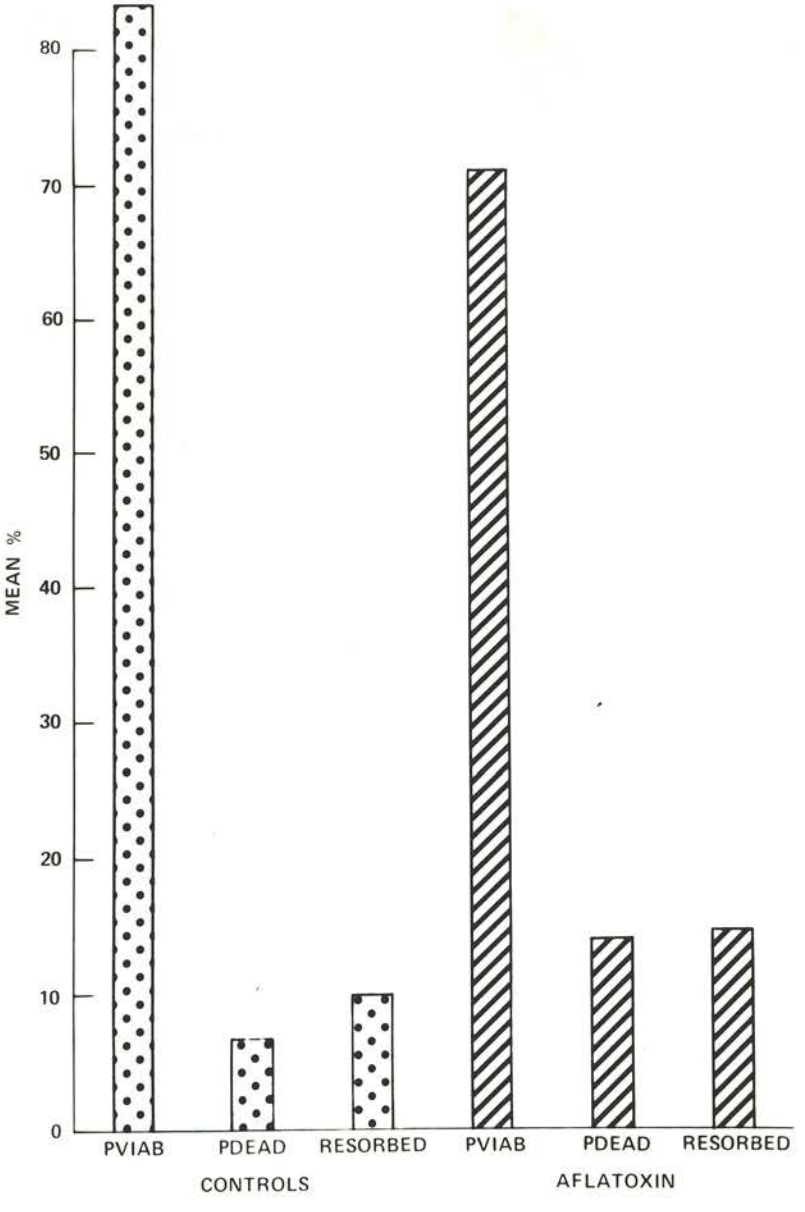

Figure 18. Comparison of PVIAB, PDEAD and Resorptions Between Inoculated and Control Groups in Experiment 2.

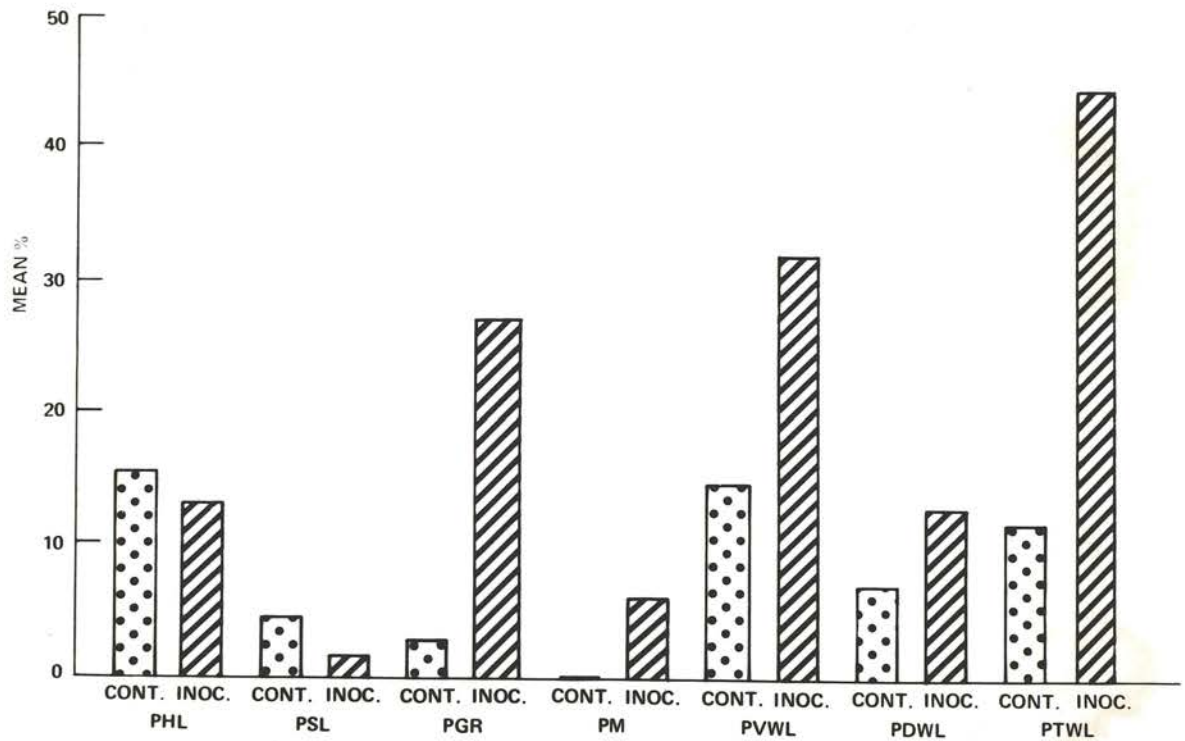

Figure 19. Effects of Aflatoxin Inoculation on the Mean Percent of the Variables PHL, PSL, PGR, PM, PVWL, PDWL, and PTWL in Experiment 2

PTWL had a statistically significant elevation ($0.10<P<0.25$). Considering the breakdown of type of abnormality, there is an elevation for the PM and PGR ($0.05<P<0.10$). Of interest is the reversal of the graph for PHL and PSL, with a slightly higher mean percent being present in the control groups.

Comparison of the treatment effect (Fig. 19) with the effect of day of inoculation (Fig. 20) shows that the PTWL was significant ($0.025<P<0.05$), because it was much higher for fetuses from animals inoculated on day 9 than day 8. The various types of abnormalities also have higher mean percents for day 9 inoculation, but the differences are not statistically significant.

Other sources of variation affecting the types of abnormality included the day of inoculation x day of examination interaction for PSL ($P<0.005$), with spinal malformation seen only on examination days 9.0, 9.5 or 10.0, and the day of examination for PGR ($0.10<P<0.25$) with the majority of growth retarded fetuses noted on days of examination 12 and 15.

Experiment 3

The effect of aflatoxin on the variables PVIAB ($0.025<P<0.05$), PDEAD ($0.10<P<0.25$) and resorptions is illustrated in Figure 21. The number of viable fetuses in the inoculated group was less on days of examination 11, 12 and 15. Aflatoxin effects on the mean percent for the variables PVWL, PDWL, PTWL, PHL, PSL, PGR and PM are shown in Figure 22. Significant differences between control and inoculated means were noted for PDWL ($P=0.10$) and PTWL ($0.005<P<0.01$). For the types of abnormality, the differences between control and inoculated

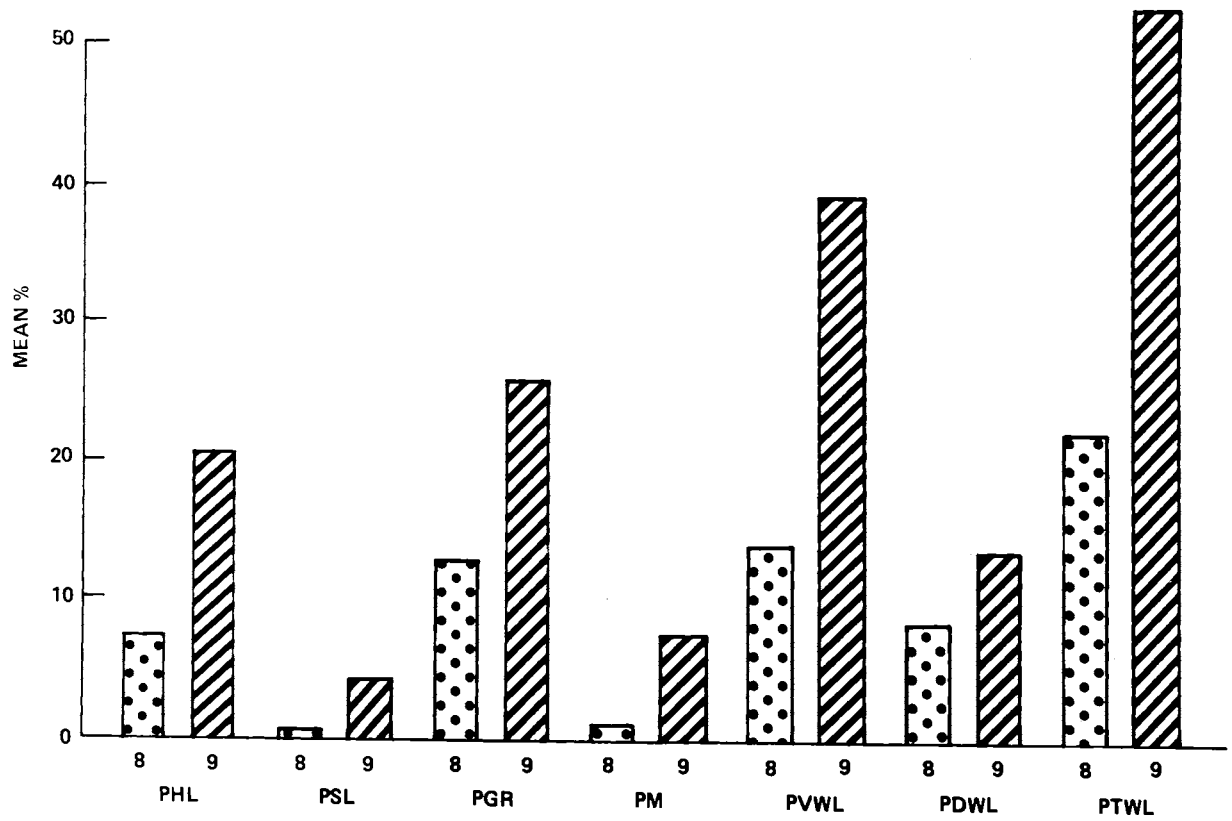

Figure 20. Effect of Day of Inoculation on the Mean Percent of the Variables PHL, PSL, PGR, PM, PVWL, PDWL, and PTWL in Experiment 2

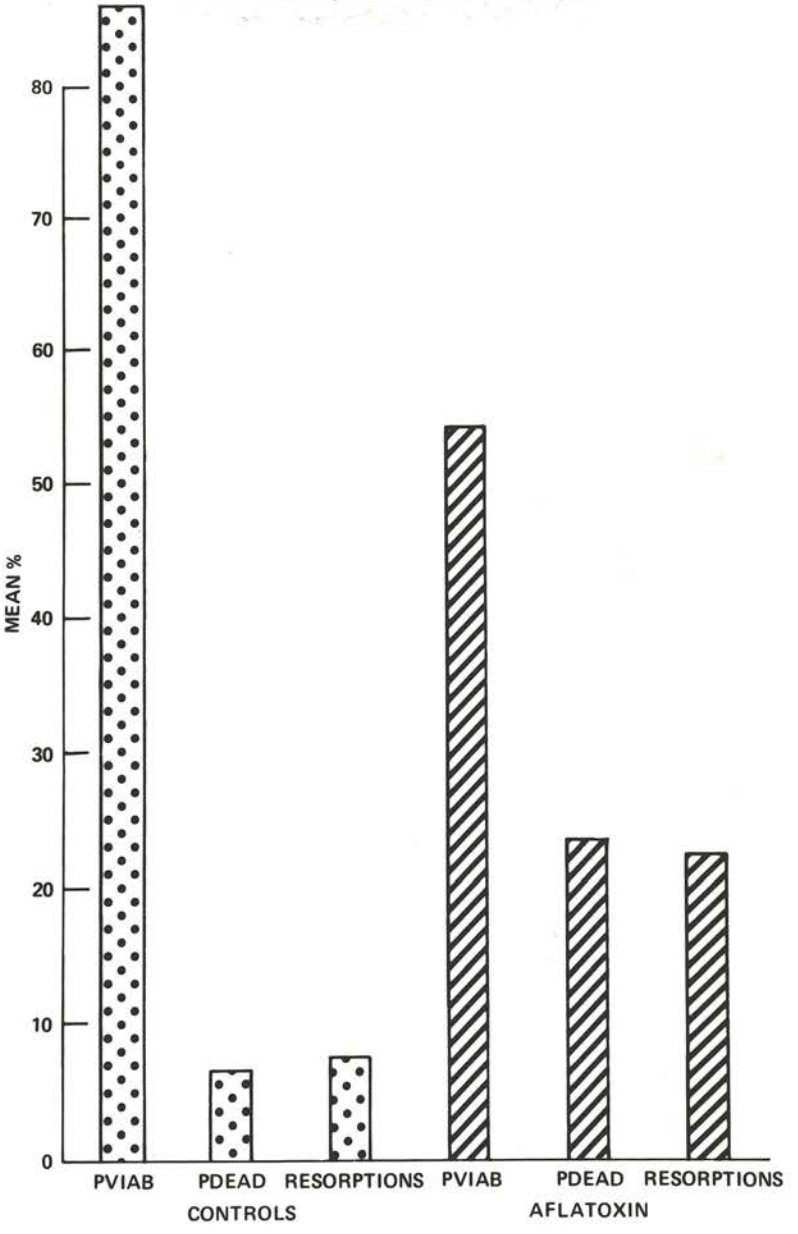

Figure 21. Comparison Between Inoculated and Control Groups of the Variables PVIAB, PDEAD and Resorptions in Experiment 3

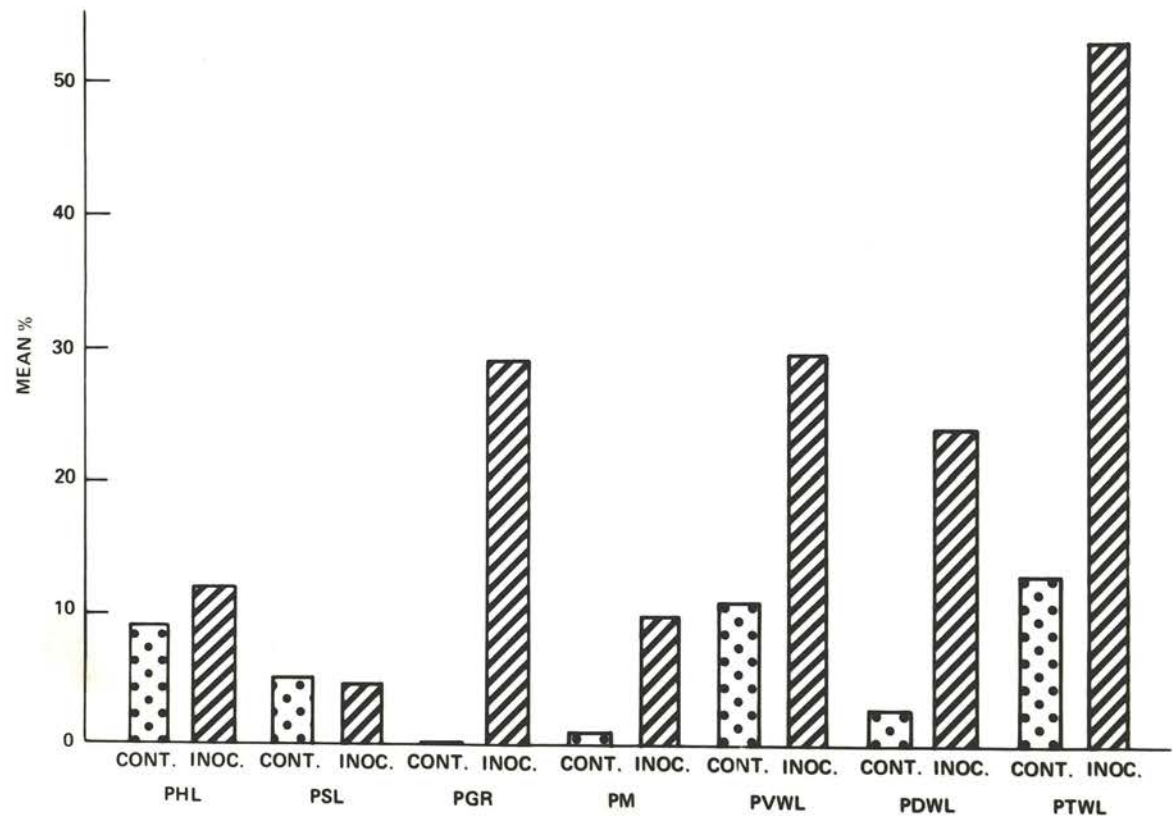

Figure 22. Effects of Aflatoxin Inoculation on the Mean Percent of PHL, PSL, PGR, PM, PVWL, PDWL and PTWL in Experiment 3

for PHL and PSL were similar to the differences between them in experiments 1 and 2. For PM the inoculated group had a higher mean percent and for PGR the inoculated group had a significantly ($0.005 < P < 0.01$), higher mean percent.

The day of examination also affected the PGR ($0.025 < P < 0.01$), with the highest number of affected fetuses seen on day 15 examination. The PSL was affected by the day of examination ($P=0.005$) with all malformations of the spine whether in control or inoculated groups being seen on examination days 9.0 or 9.5. The treatment x day of inoculation interaction also affected PSL ($P=0.005$). The larger numbers of spinal malformations were found in the aflatoxin treated group inoculated on day 9.

Experiment 4

Figure 23 illustrates the mean percent of several of the variables as function of day of examination. The P values for the source of variation "day of examination" for several variables are given in Table V. As seen in Figure 23 examination of fetuses on day 9 gave the highest mean PDEAD, PTWL and PHL, and the lowest mean PVIAB.

Comparisons Between Experiments

A higher mean percent of resorptions was seen in groups inoculated with aflatoxin in triethylene glycol or triethylene glycol alone than in groups inoculated with glycerin or aflatoxin in glycerin (Fig. 16, 18, 21). The pattern of mean percent for fetuses with abnormalities and for types of abnormality was similar in experiments 1, 2 and 3 (Fig. 17, 19, 22), with all variables usually having a greater mean percent in

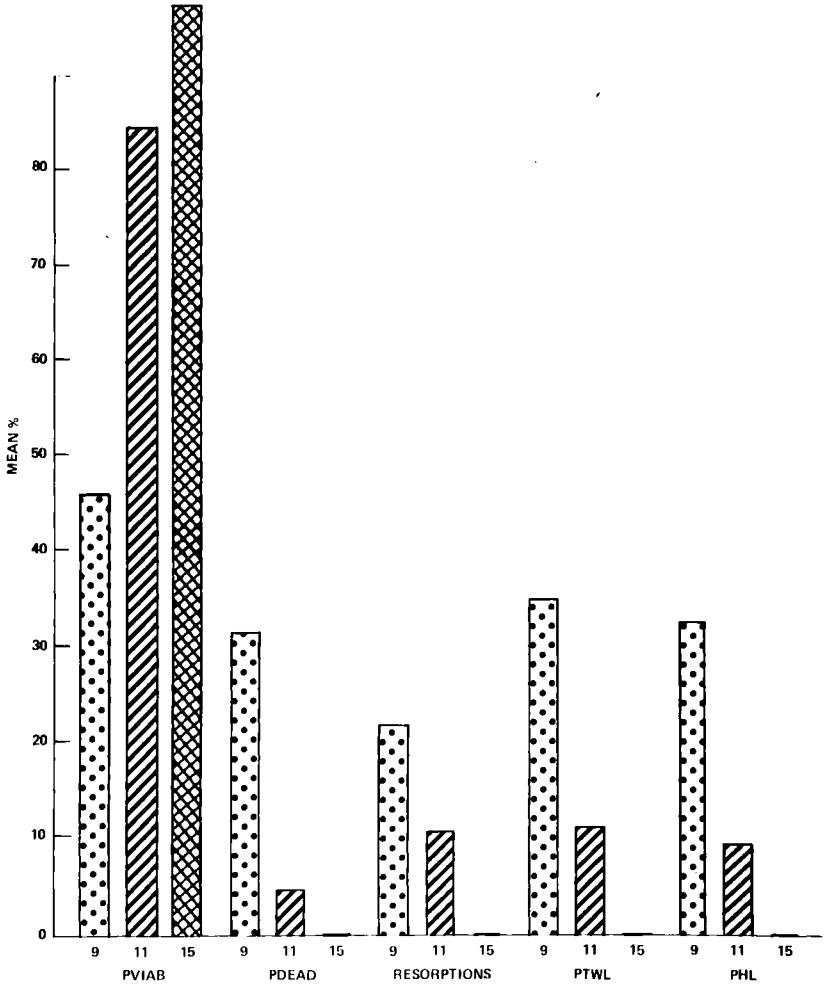

Figure 23. Effects of Day of Examination on PVIAB, PDEAD, PTWL and PHL in Experiment 4

TABLE V

EXPERIMENT 4: P VALUES FOR THE EFFECT OF DAY OF EXAMINATION ON PVIAB, PDEAD, PVWL, PDWL, PTWL AND PHL

VARIABLE	P
PVIAB	0.005-0.010
PDEAD	<0.005
PVWL	0.100-0.250
PDWL	<0.005
PTWL	0.005-0.010
PHL	0.005-0.010

the glycerin diluent animals, especially at the 6 mg/kg dose. The patterns of these variables among controls were also similar in experiments 1, 2 and 3.

There was a consistently significant difference in mean percent for type of abnormality between control and inoculated animals in all experiments (except 4) for the variable PGR, although it can be seen (Fig. 17, 19, 22) that there was also a consistent elevation of PM in aflatoxin inoculated animals, giving at least an indication of treatment effect. Although the category PM was considered to be made up of miscellaneous lesions, most of these appeared to involve the heart or vascular system as described in detail in the following section.

Morphologic Description

The morphology of the abnormalities that occurred in a given fetal age group was similar regardless of the dose of aflatoxin, type of diluent or type of control. These abnormalities are described as a group. Miscellaneous lesions present in a particular fetal age group are given separate descriptions. Normal fetuses of gestation day 9, 10, 12 and 15 are illustrated for reference (Fig. 24, 25, 26, 27).

Gestation Day 8.5. No gross or histologic lesion was noted in any fetuses from these groups.

Gestation Day 9 and 9.5. Dead fetuses were often partially resorbed, and no determination of antemortem abnormalities was made (Fig. 28). The histologic appearance of dead fetuses was characteristic (Fig. 29) with a general disorganization of structure and loss of sharp histologic detail.

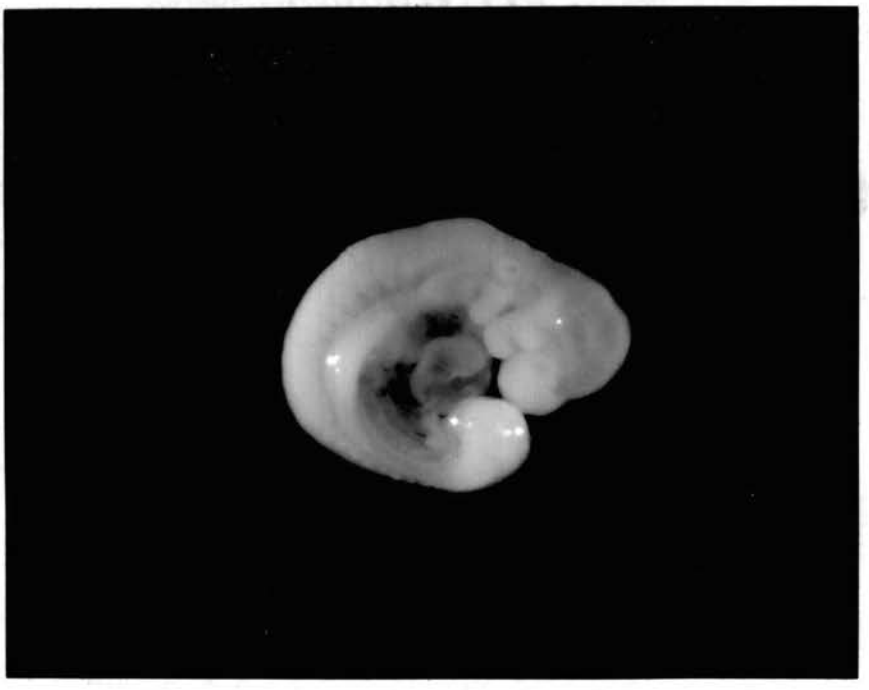

Figure 24. Normal Hamster Fetus, Day 9 of Gestation

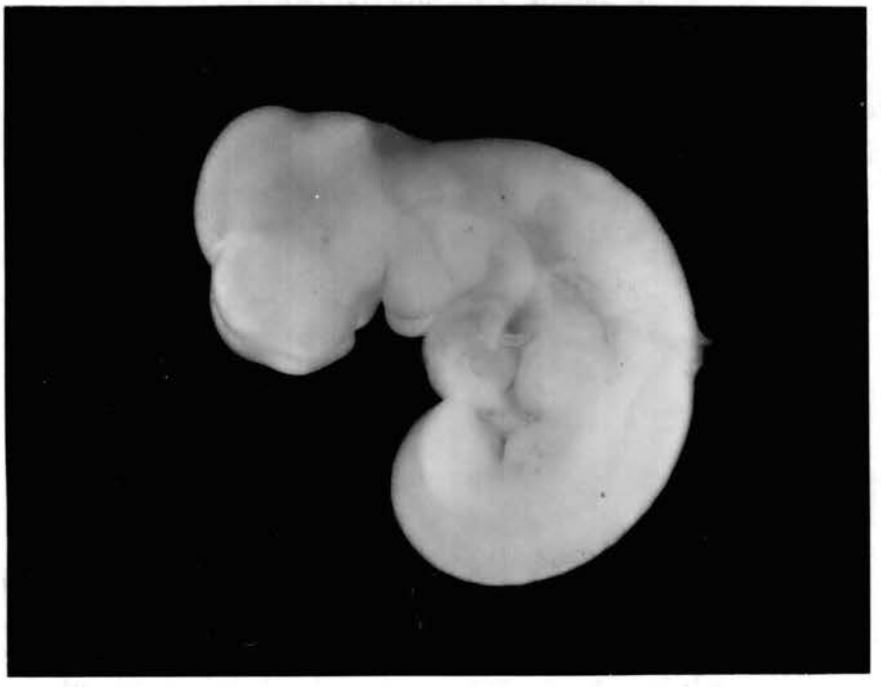

Figure 25. Normal Hamster Fetus, Day 10 of Gestation. The Projections Seen in the Midspinal Region Are Remnants of Fetal Membranes.

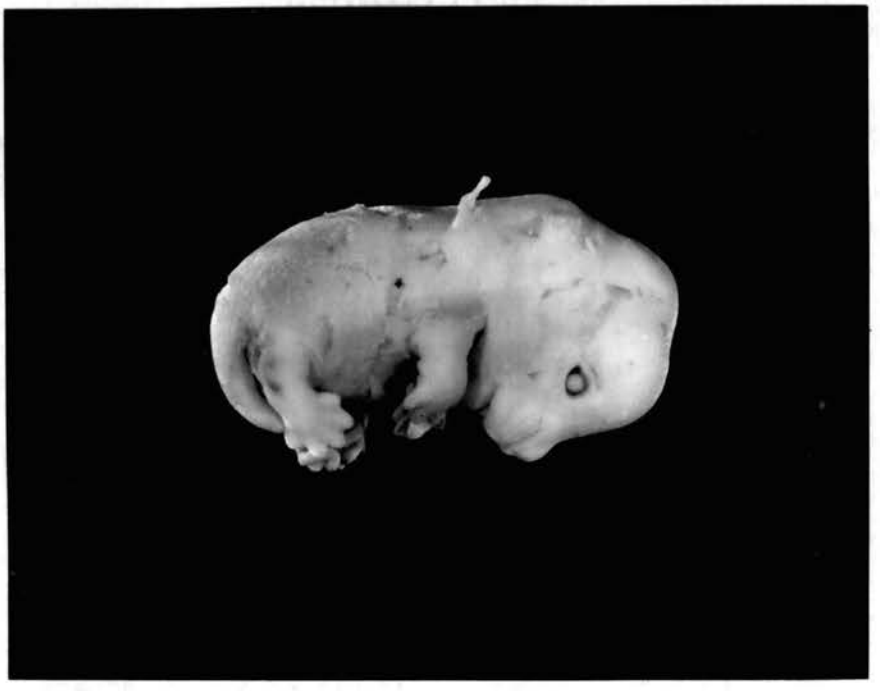

Figure 26. Normal Hamster Fetus, Day 11 of Gestation. The Skin Abrasions and Tags Are Artifacts

Figure 27. Normal Hamster Fetus, Day 15 of Gestation

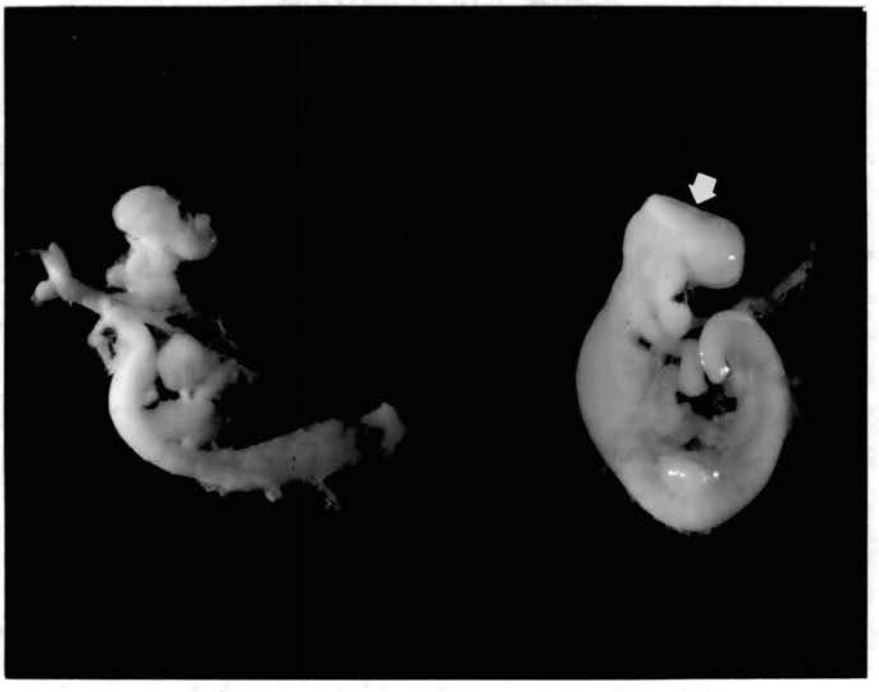

Figure 28. Gestation Day 9. Fetus on Left Was Dead and Undergoing Resorption. Fetus on Right Has Severe Cranioschisis (arrow)

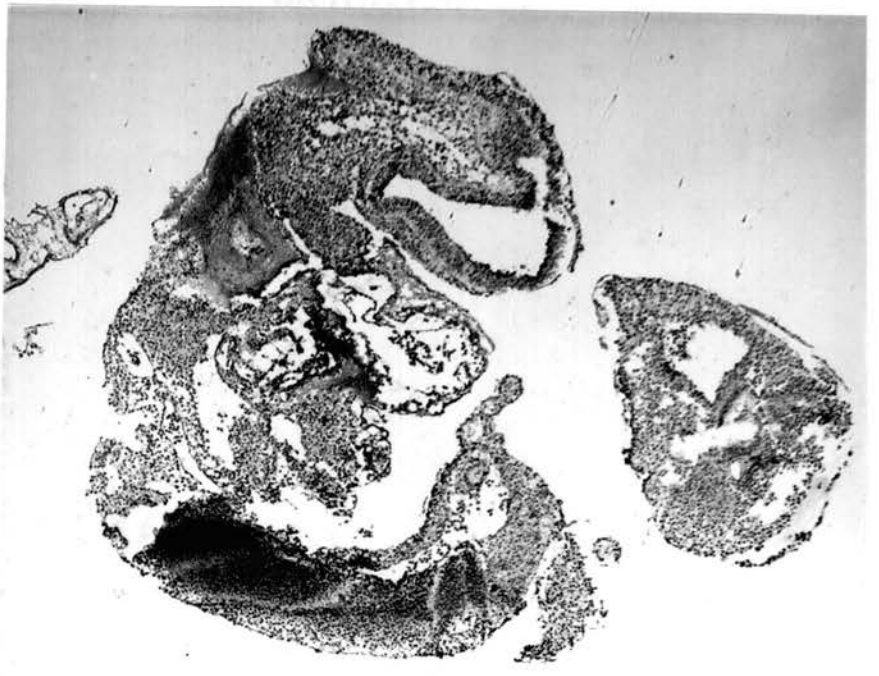

Figure 29. Dead Fetus, Gestation Day 9. Structural Detail Is Lacking (H&E x 30)

Minimal gross changes in live fetuses included a slight microcephaly characterized by a "doming" of the cranium, and occasional mandibular hypoplasia. These abnormalities were more noticeable in fetuses at day 9.5. A few fetuses failed to develop past 8.5 days, with little or no neural tube closure and no retroflexion (Fig. 30).

Neural tube lesions varied in size and location. In some fetuses there was a small opening in the cranial portion of the neural tube while others had no closure in the cranial portion of the fetus (Fig. 31). Histologically the less severe lesions were characterized by an outward rolling of neuroepithelium which was contiguous with the developing skin (Fig. 32, 33, 34). In more severe cases of craniochisis, the neuroepithelium was disorganized and lost some of its normal laminar appearance (Fig. 35).

Myeloschisis presented as variable sized openings in any region of the spinal cord (Fig. 36). In some fetuses there were 2 or more defects in closure. Histologically the neuroepithelium was similar in appearance to the lesion described for the brain (Fig. 37).

Miscellaneous histologic lesions seen in fetuses that were grossly normal included scattered foci of necrosis in neuroepithelium characterized by the accumulation of necrospherules and a few examples of thinning of the cranial neuroepithelium of the developing brain (Fig. 38).

Fetuses from 3 inoculated and 1 control animal had degrees of myocardial necrosis. This was characterized by condensation and increased eosinophilia in a linear pattern and an increase in thickness of the developing heart wall (Fig. 39). Necrospherules, possibly representing pyknotic nuclei, were present along the line of necrosis (Fig. 40).

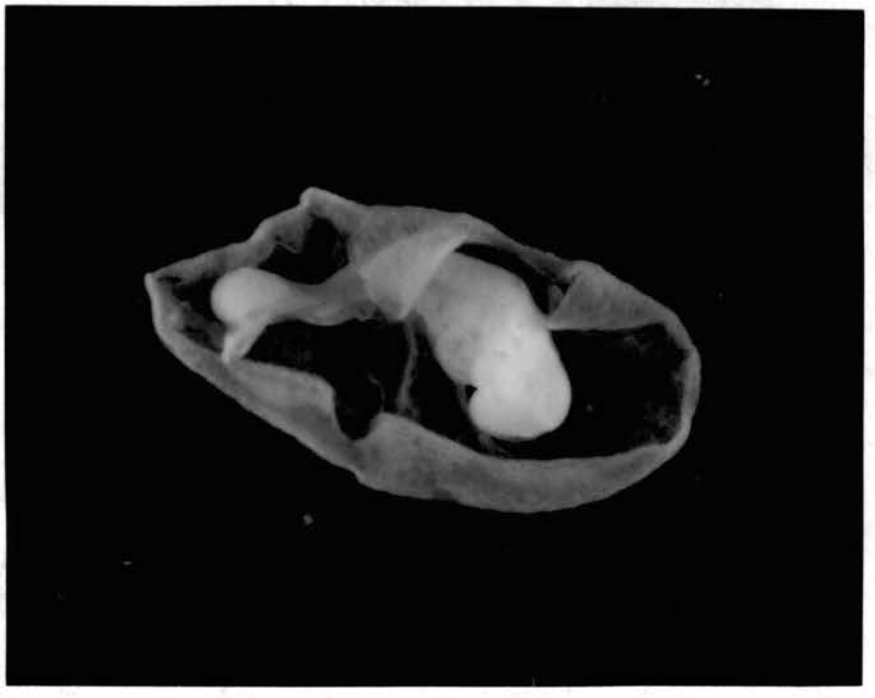

Figure 30. Underdeveloped Fetus on Day 9 of Gestation. Note Lack of Structural Detail and No Retroflexion

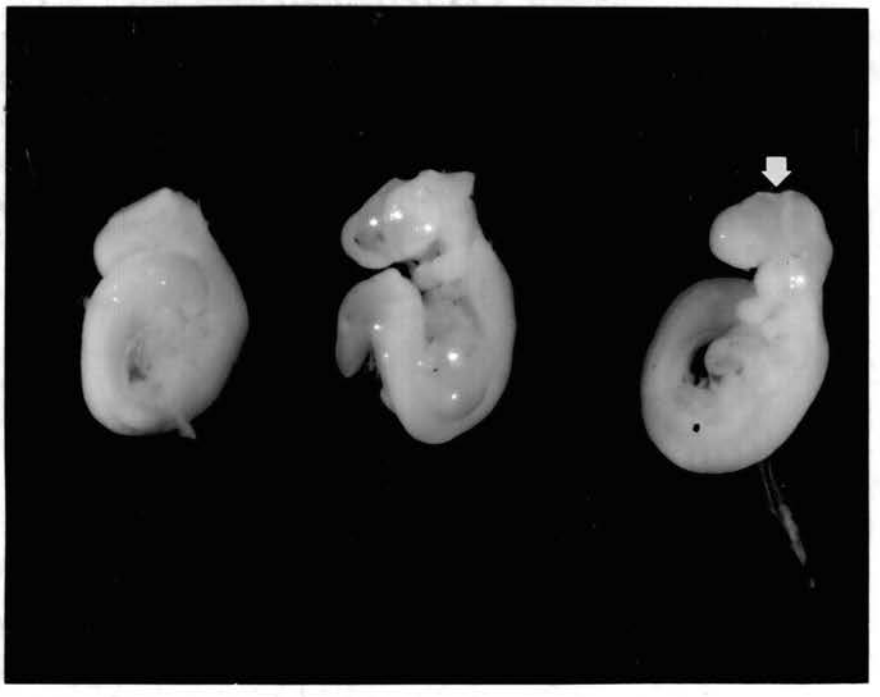

Figure 31. Variation in Degree of Cranioschisis in Fetuses at Day 9 of Gestation. The Fetus on the Right Has a Small Opening (arrow), the One on the Left is More Severe, and the Center Fetus Has a Failure of Cranial Closure

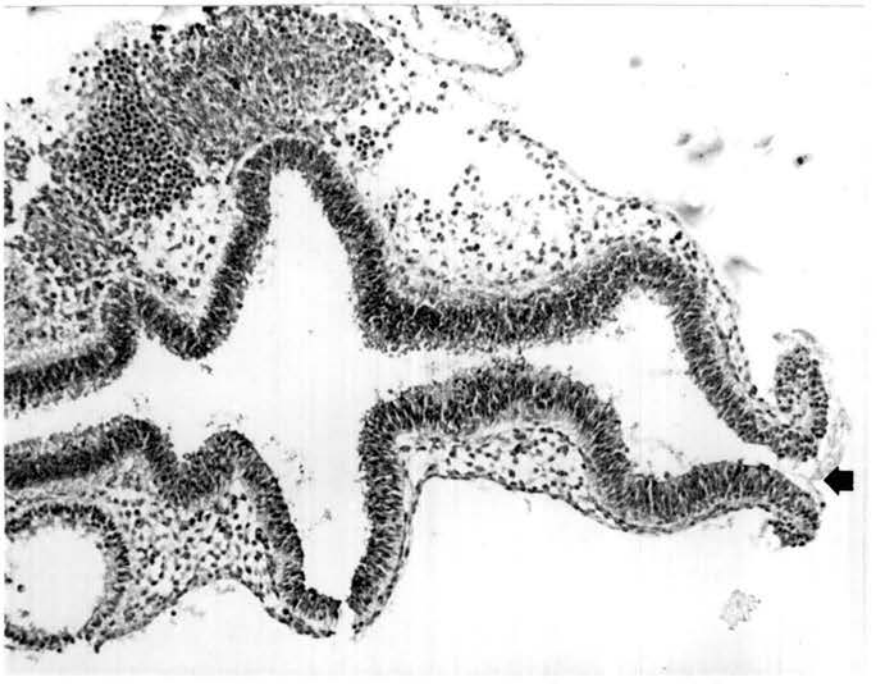

Figure 32. Longitudinal Section of 9 Day Fetus. Grossly There Was a Small Opening At the Cranial End of the Neural Tube (arrow) (H&E x 200)

Figure 33. Gross Section of Moderate Cranio-schisis in 9 Day Fetus (H&E x 30)

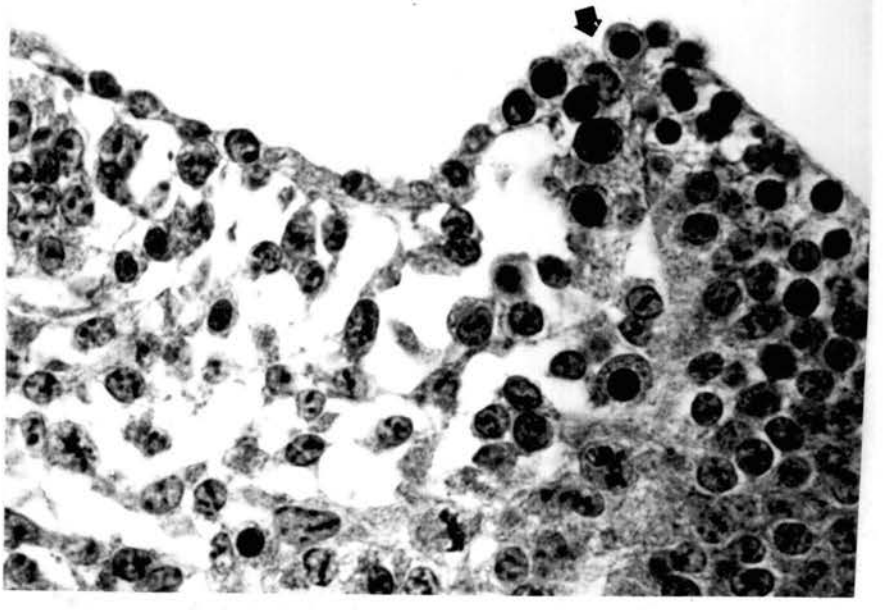

Figure 34. Detail of Border Between Neuroepithelium and Developing Skin. Arrow Indicates the Point of Transition (H&E x 200)

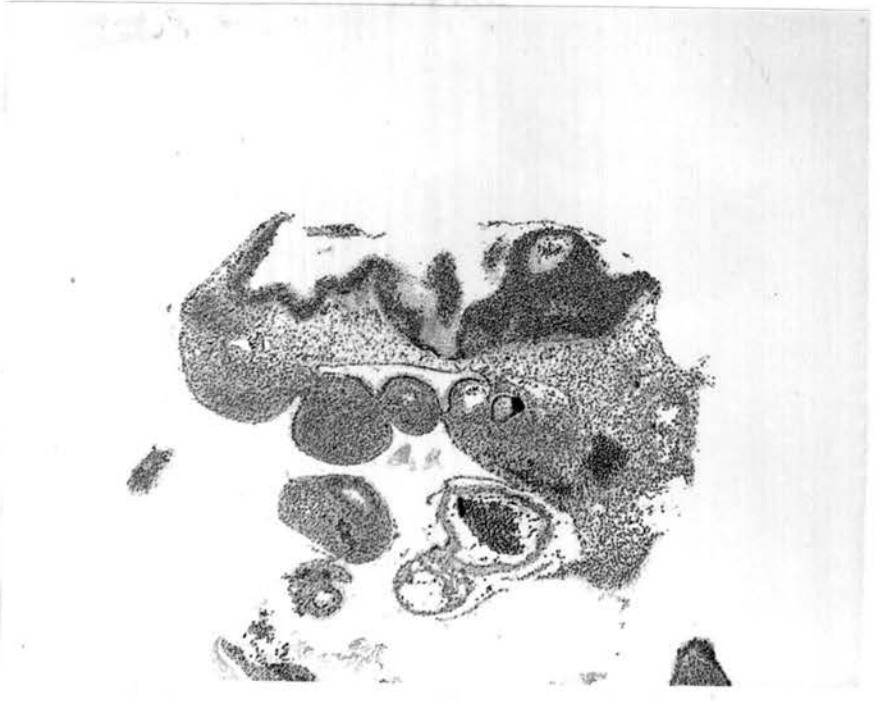

Figure 35. Disorganized Neuroepithelium in Day 9 Fetus With Failure of Cranial Closure (H&E x 30)

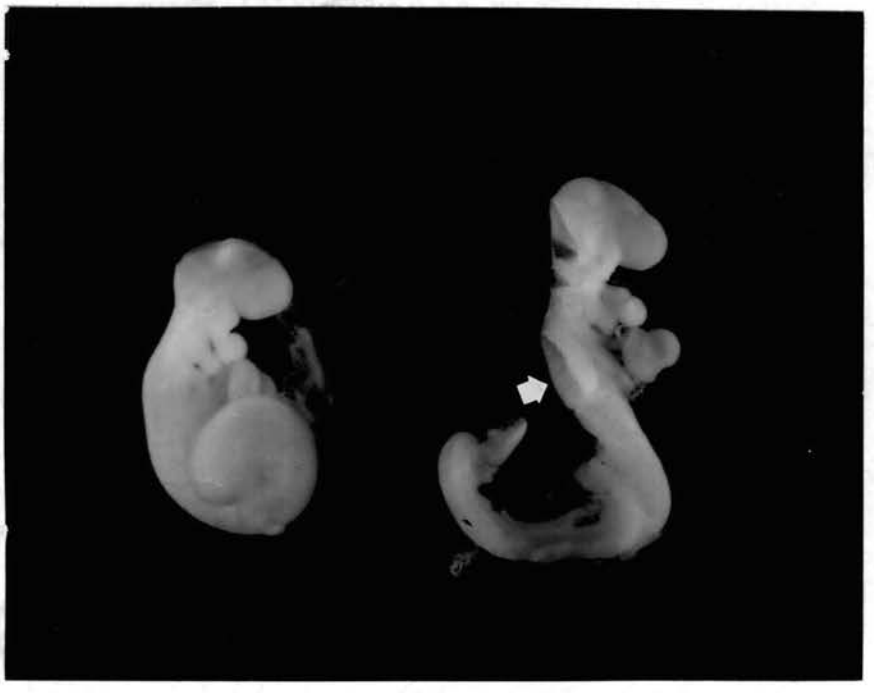

Figure 36. Large Opening in Thoracic Spinal Cord of a 9 Day Fetus (arrow). There Are Smaller Openings at the Base of the Skull.

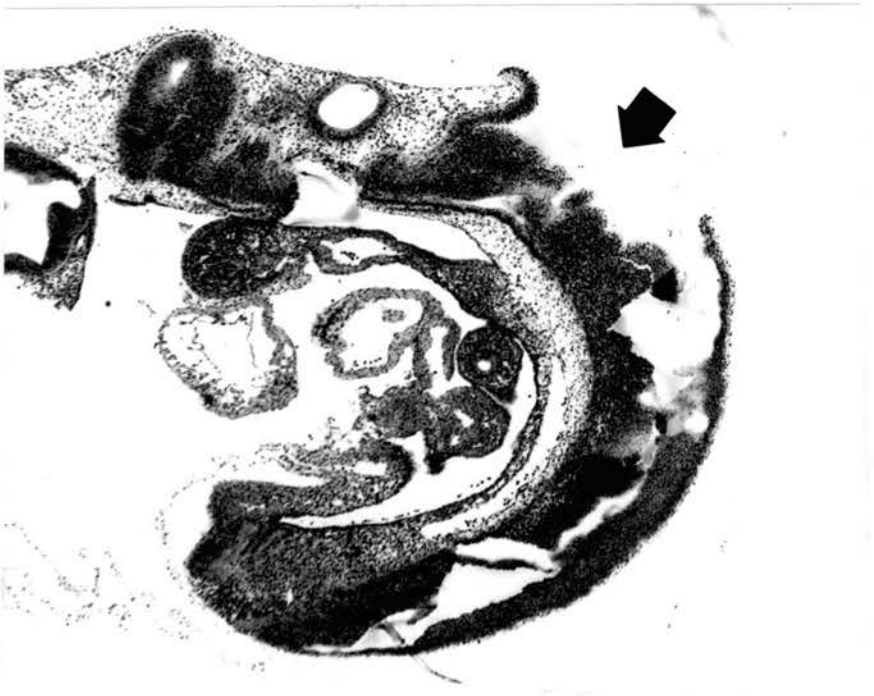

Figure 37. Histologic Appearance of Myeloschisis. Large Area of Thoracic Cord Involved (arrow) (H&E x 30)

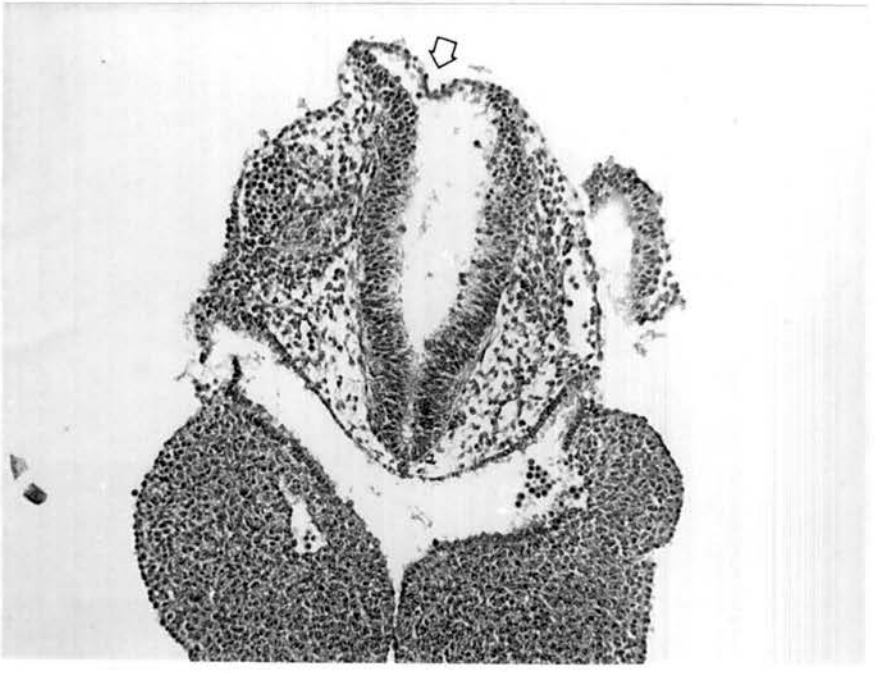

Figure 38. Thinning and Distortion of Neuroepithelium in 9 Day Fetus. The Ventricular Cavity Is Closed, But Very Little Neuroepithelium Is Present Below the Outer Epithelial Covering (arrow) (H&E x 30)

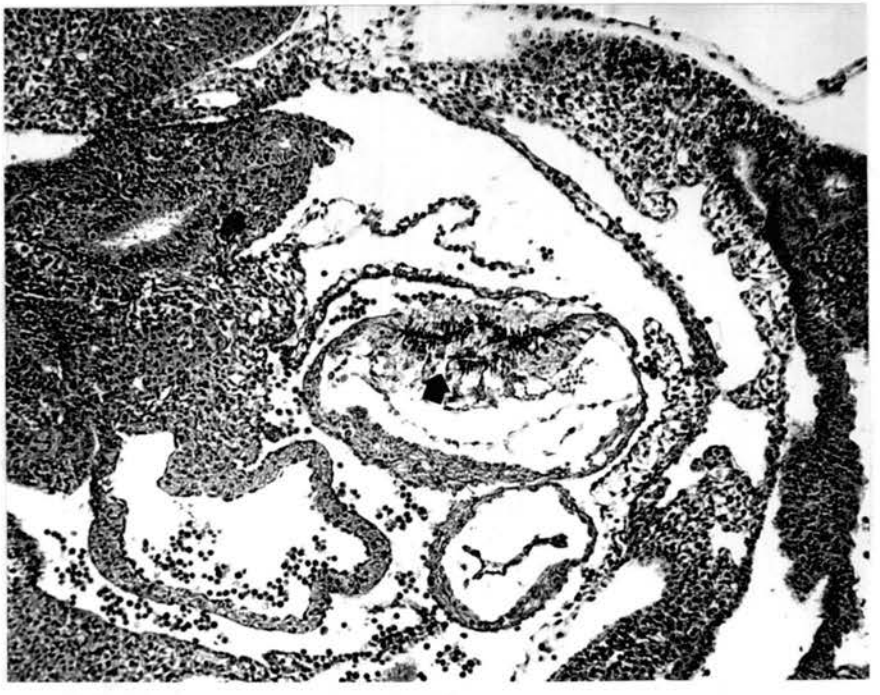

Figure 39. Cardiac Necrosis in a Portion of Developing Ventricle in a 9.5 Day Fetus (arrow) H&E x 30)

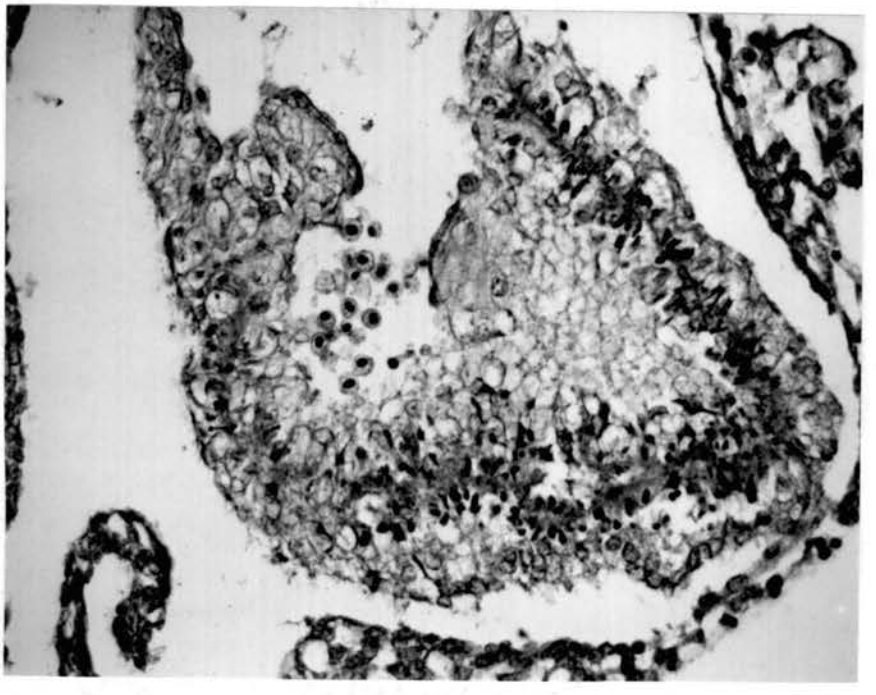

Figure 40. Higher Magnification of Cardiac Necrosis in a 9.5 Day Fetus. Note the Dark Necrospherules Aligned Adjacent to the Zone of Necrosis (arrow) (H&E x 200)

Gestation Day 10. Fetuses in various stages of resorption were similar to those described previously. The cardiac necrosis seen in some fetuses on days 9 and 9.5 was also present in fetuses from 4 animals inoculated on day 9 and killed on day 10. This lesion was seen in live fetuses with and without gross lesions and seemed quite severe in dead fetuses (Fig. 41).

Lesions involving the head and neural tube varied from minimal underdevelopment with transverse ridging of the skull (Fig. 42), through multiple facial and cranial ridging (Fig. 43), to multiple openings in the developing brain (Fig. 44). On day 10 of gestation there were also a number of fetuses with open olfactory lobes (Fig. 45). A few fetuses had variable sized areas of myeloschisis.

By day 10 of gestation some overall growth retardation was noted. Morphologically, this was expressed by slightly smaller size and a lack of complete straightening of the line from the apex of the cranium to the shoulder.

Gestation Day 11. Fetuses in various stages of resorption were similar in appearance to those described previously. Cardiac necrosis similar to that previously described was seen histologically in fetuses from 3 females inoculated on day 9.

Noticeable growth retardation was seen by day 11. Some retarded fetuses had an almost normal appearance but were only 0.5-0.75 as large as normal (Fig. 46). The average size of normal fetuses at this stage of gestation was 0.7 to 0.8 cm. Other fetuses were not only generally small, but had degrees of microcephaly characterized by either compression of the head in a cranial-caudal direction (Fig. 47) or by doming

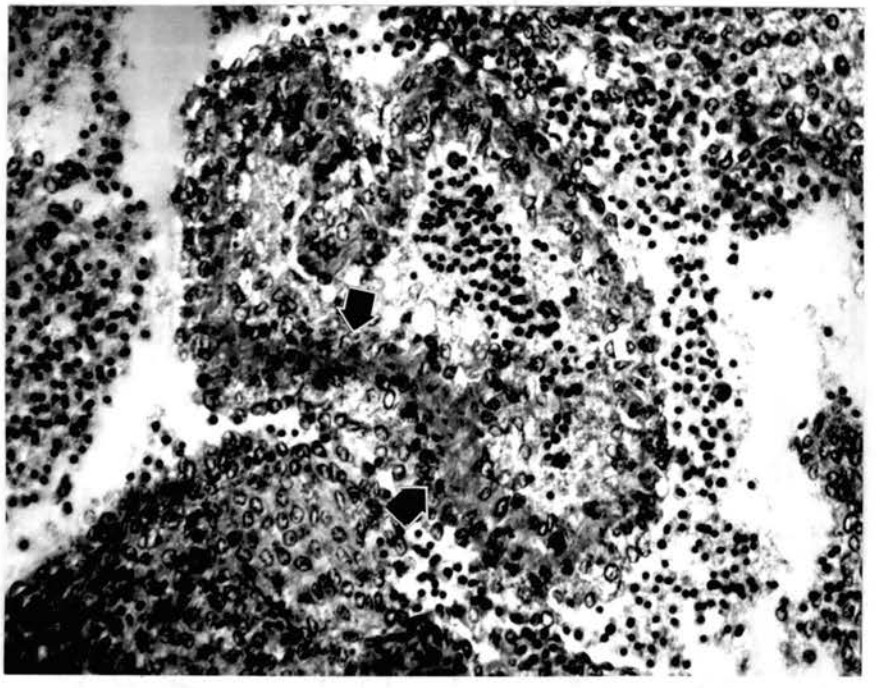

Figure 41. Extensive Cardiac Necrosis in Dead Fetus (arrows). Day 10 of Gestation (H&E x 200)

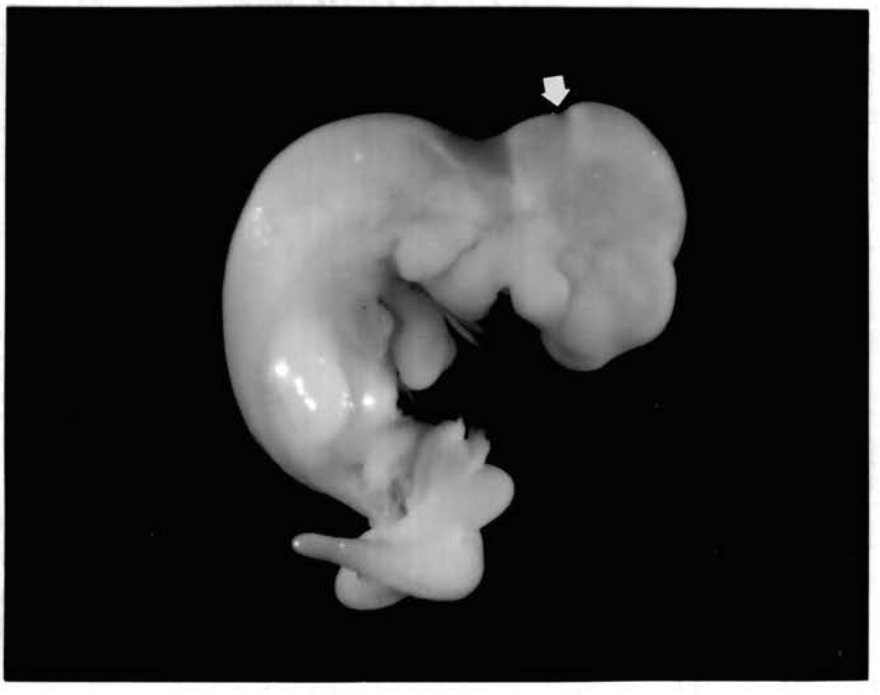

Figure 42. Ten Day Fetus Injected With 4 mg/kg Aflatoxin. Transverse Ridging Is Apparent at the Base of the Skull (Arrow)

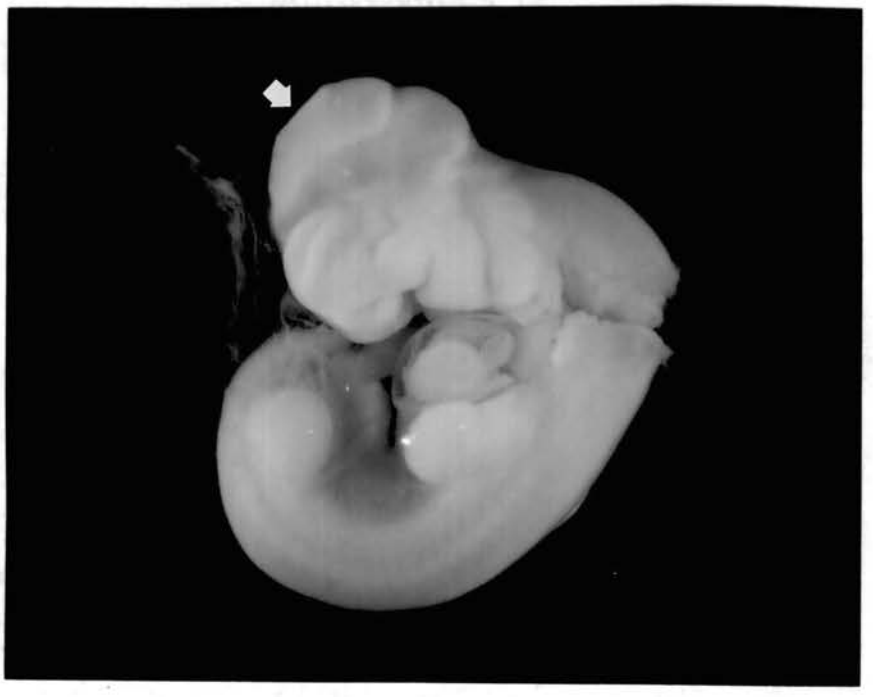

Figure 43. Multiple Cranial Anomalies in a 10 Day Fetus. Note Maxillary and Cranial Ridges and Grooves and a Small Opening in the Top of the Head (arrow). The Thoracic Spinal Defect Is an Artifact

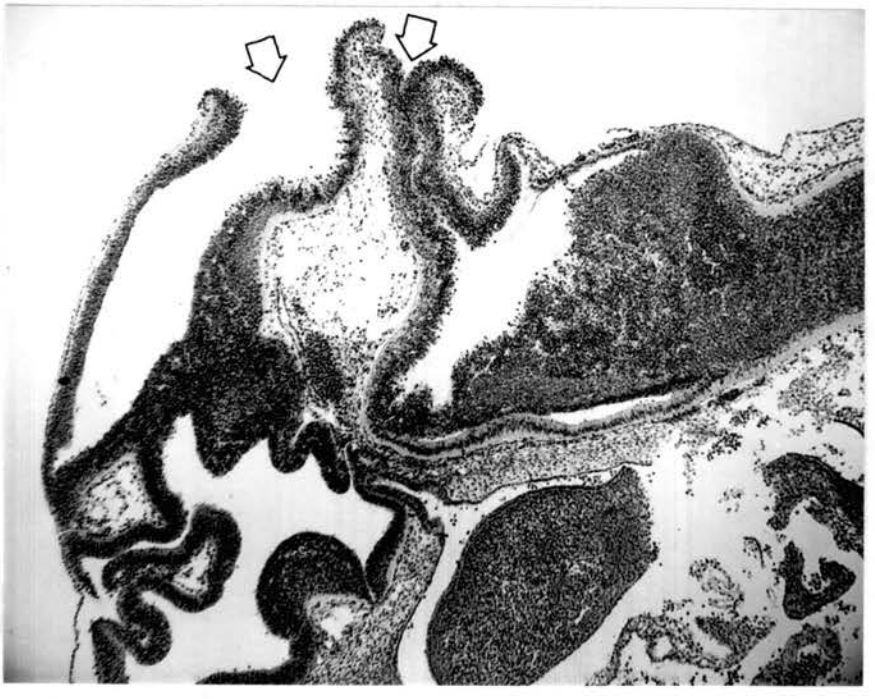

Figure 44. Cranioschisis Involving the Developing Mid and Hind Brain Areas (arrows). 4 mg/kg of Aflatoxin, Day 10 of Gestation. (H&E x 30)

Figure 45. Failure of Closure of Olfactory Lobe of Brain (H&E x 30)

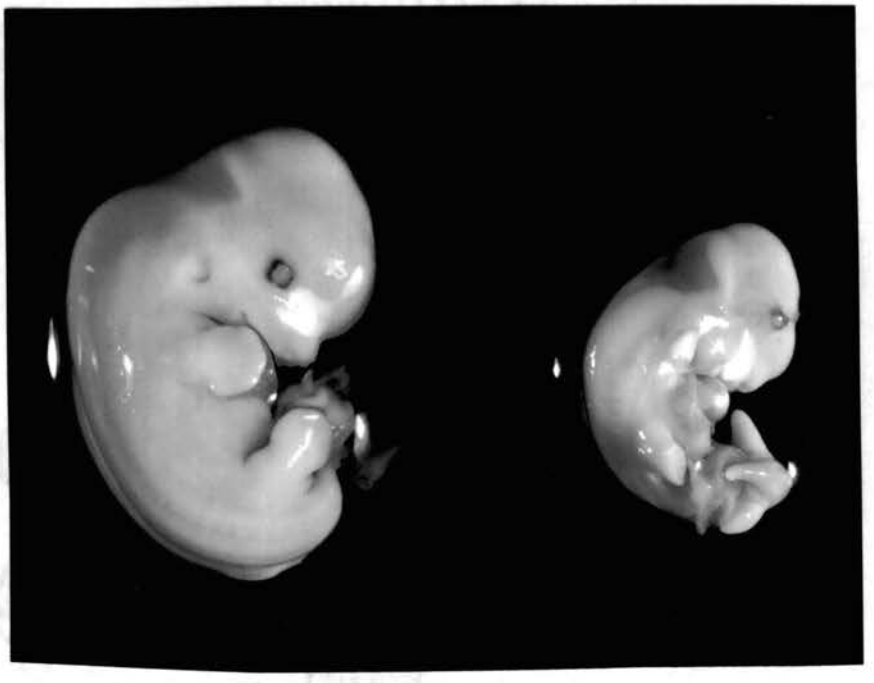

Figure 46. Fetuses on Day 11 of Gestation. The Fetus on the Left Is Normal While Its Littermate on the Right Is Small and Has a Slightly Malformed Head

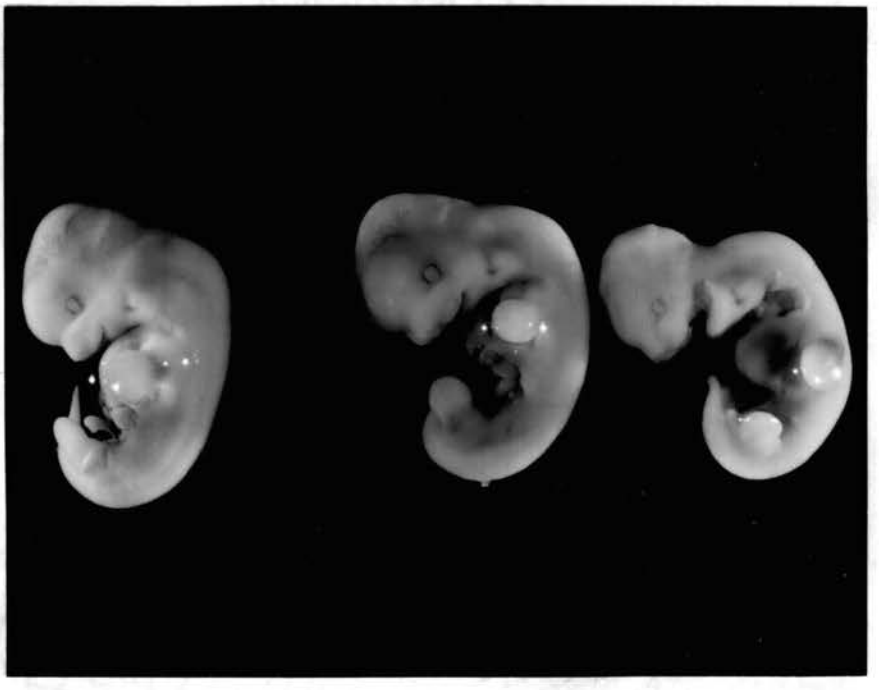

Figure 47. Gestation Day 11. The Fetus on the Left Is Normal, the One in the Center Slightly Undersized, and the One on the Right Undersized and Microcephalic

of the cranium (Fig. 48). Hypoplasia of the maxillary and mandibular areas was also noted in fetuses with growth retardation (Fig. 49, 50).

Well developed cranioschisis was present in a number of fetuses on gestation day 11 (Fig. 51). These fetuses were usually viable. Histologically, a portion of the developing brain neuroepithelium covered the top of the head and much of the external layer was necrotic.

Gestation Day 12. Varying degrees of growth retardation were common especially in fetuses from animals inoculated on day 9 (Fig. 52). Prominent exencephaly was also seen (Fig. 53). Histologically, the exposed neuroepithelium still retained its normal appearance except for a narrow zone of necrosis at the surface (Fig. 54). In one grossly normal fetus there was a small break in the continuity of the neuroepithelium at the base of the developing fourth ventricle. It was the only lesion of its type noted in this group.

Fetuses from severel different females had varying degrees of subcutaneous hemorrhage, edema and ascites. Grossly these animals appeared swollen, with a layer of clear gelatinous material in the subcutis. The hemorrhages were focal and no particular site of predilection was noted. Histologically the subcutaneous edema was characterized by separation of tissue elements, in some cases so severe that the skin was lifted from the underlying connective tissue (Fig. 55). Moderate to severe congestion of internal organs was also noted in these fetuses. All affected fetuses were viable.

Fetuses from 2 females in this group had areas of cardiac muscle necrosis similar to that described previously.

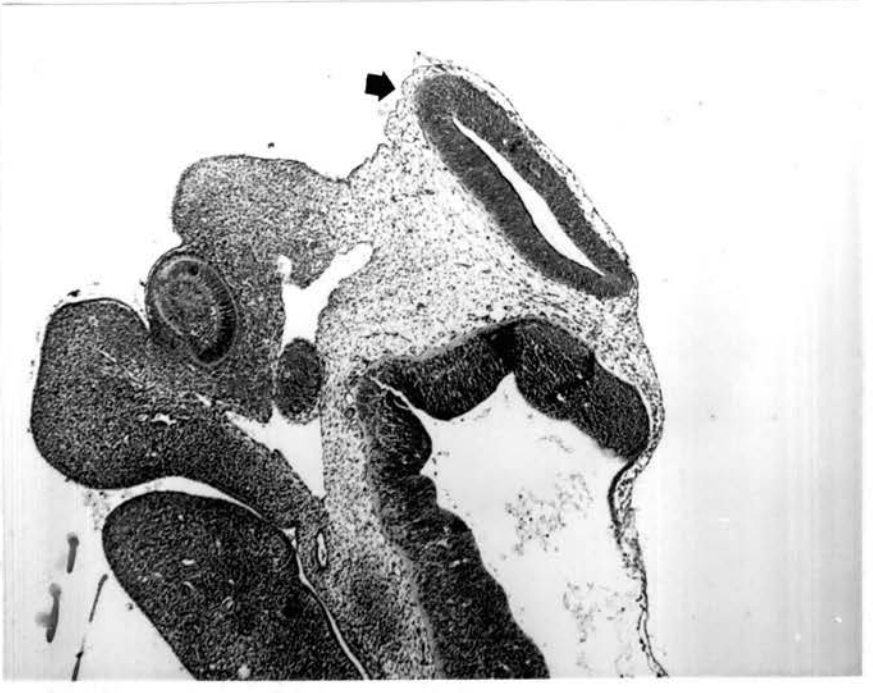

Figure 48. Histologic Section of Day 11 Fetus With Microcephaly. The Cranium (arrow) Has a Domed Appearance (H&E x 30)

Figure 49. Section Through Maxilla (1) and Tongue (2) of Normal 11 Day Fetus (H&E x 30)

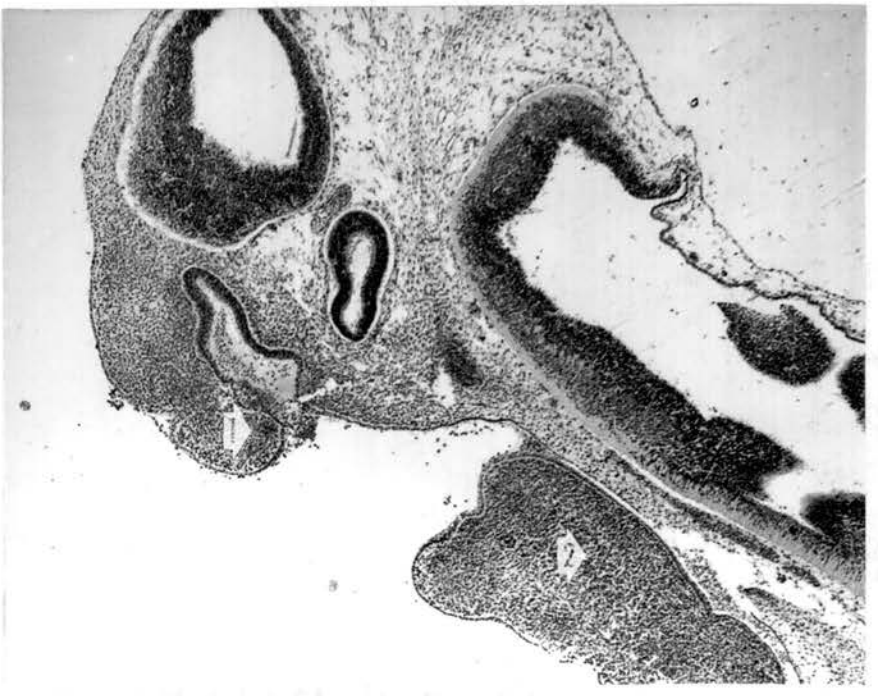

Figure 50. Section of 14 Day Fetus with Hypoplasia of Maxilla (1) and Tongue (2). Compare With Figure 41 (H&E x 30)

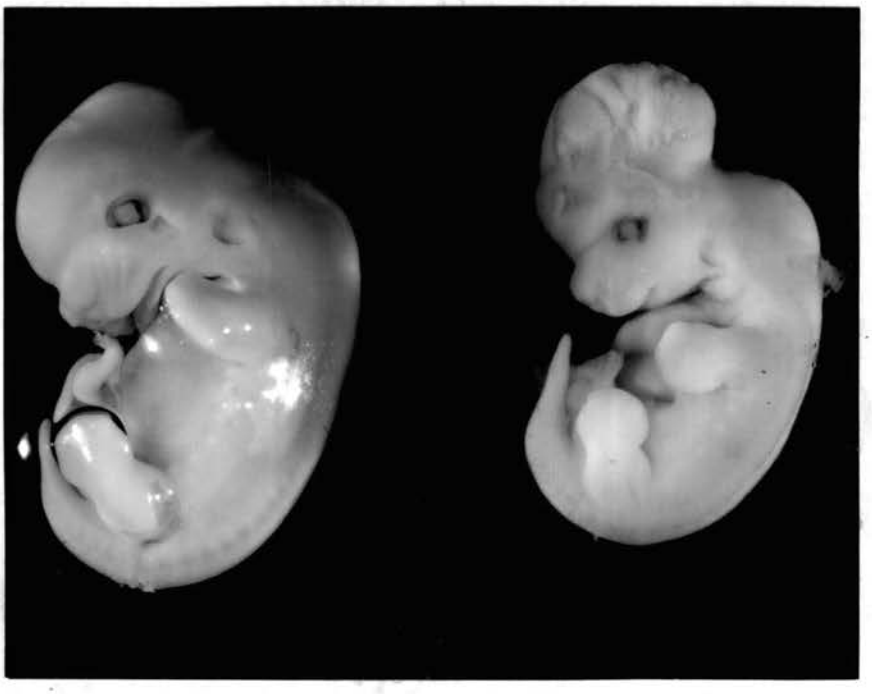

Figure 51. Gestation Day 11 Fetus With Cranioschisis (right). Compare With Normal Day 11 Fetus

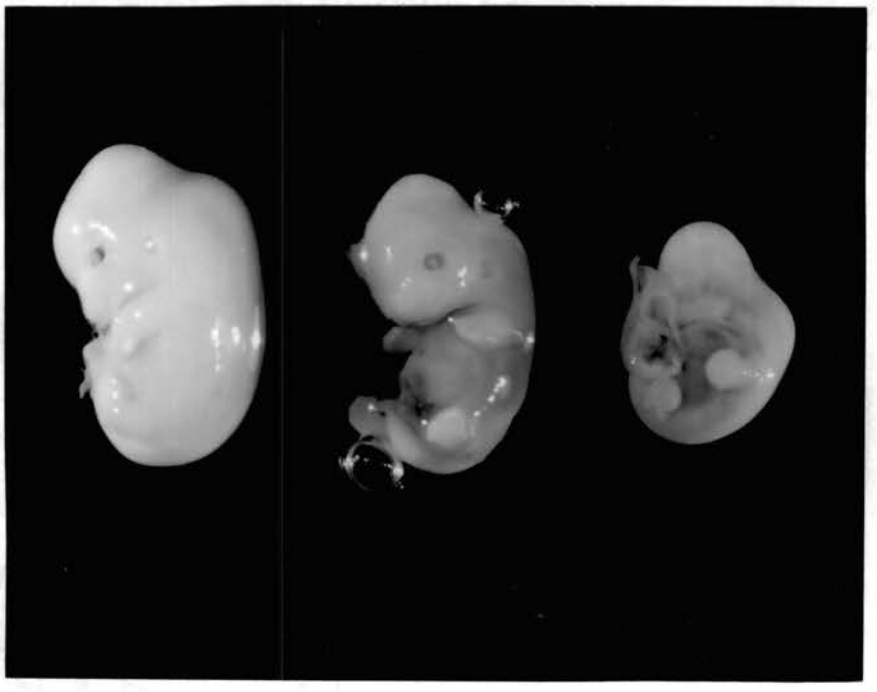

Figure 52. Growth Retardation in Live Fetuses at Gestation Day 12. Normal Fetus at Left for Comparison

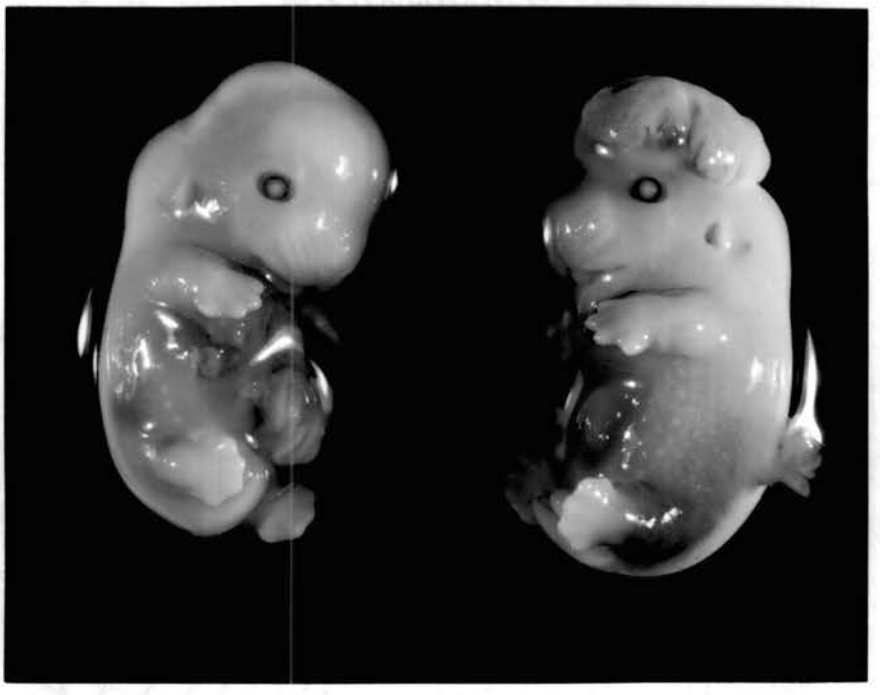

Figure 53. Exencephaly at 12 Days of Gestation.
Normal Fetus on Left for Comparison

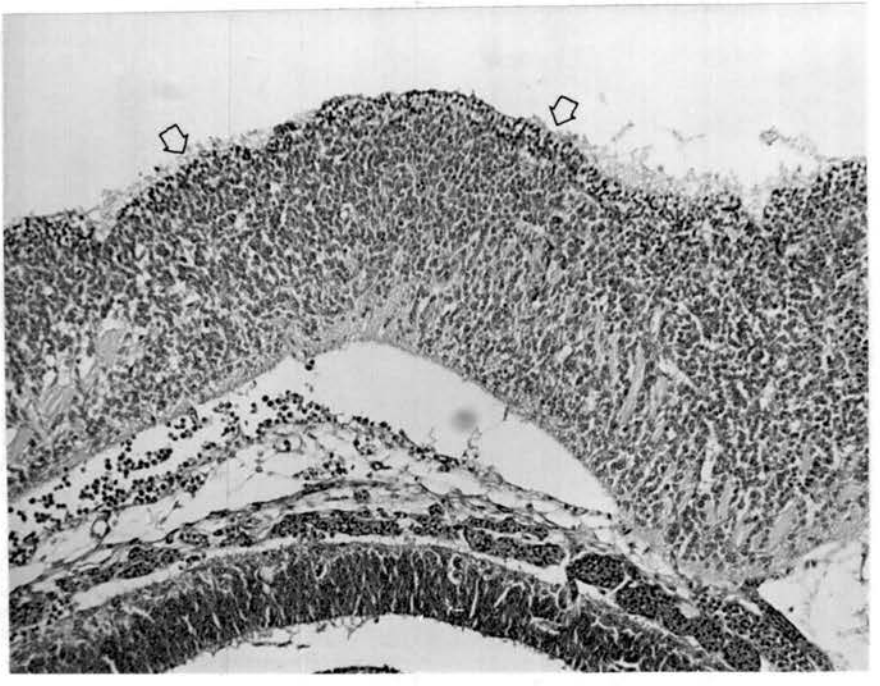

Figure 54. Histologic Section of Brain of Exencephalic Fetus. Narrow Zone of Necrosis at Surface Seen Here as Pinpoint Black Foci Representing Necrospherules (arrows) (H&E x 70)

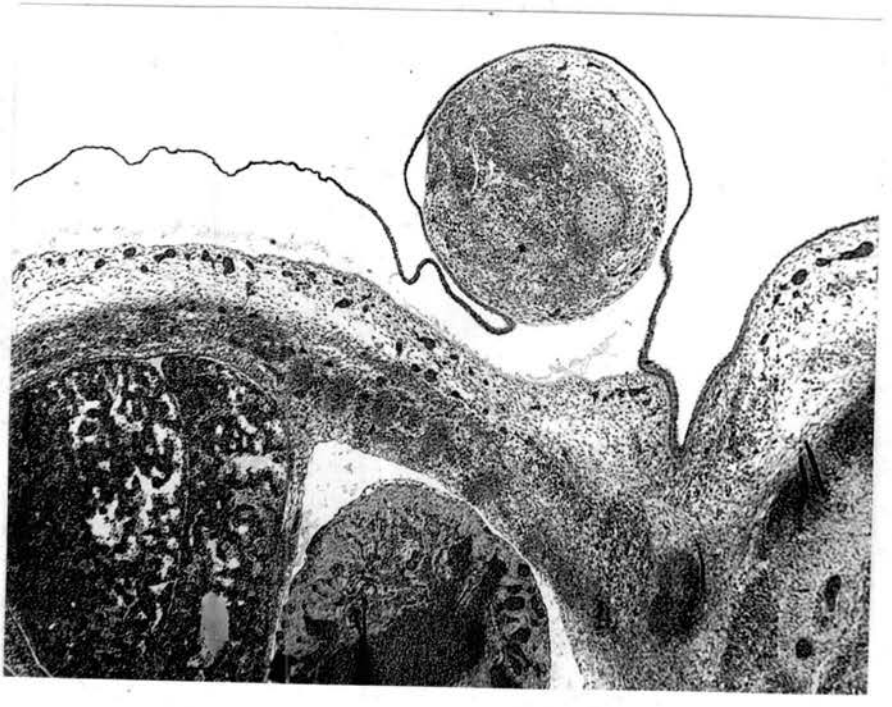

Figure 55. Day 12 Fetus With Severe Subcutaneous Edema. The Skin Was Lifted From the Connective Tissue by the Edema Fluid (H&E x 30)

that individual gestation sacs were not easily visualized. Very few dead or resorbing fetuses were seen.

The most common abnormality noted was growth retardation. This was seen in fetuses from females that became sick and were killed before day 15, and was very prominent in affected females on day 15. In fetuses that were the full 15 days, there was a symmetrical reduction in size, the animal appearing morphologically normal except for size (Fig. 56).

Gross malformations were limited to 2 fetuses with anencephaly (Fig. 57). Histologically there was a relatively sharp demarcation between normally developing connective tissues and brain, and the affected portion of the brain (Fig. 58). The exposed brain was necrotic and no vestige of intramembranous bone formation was seen over the top of the brain.

Histologically in several day 15 fetuses there were focal liver lesions characterized by dense foci which contained eosinophilic debris and what appeared to be pyknotic nuclei (Fig. 59). The normal sinusoidal architecture was lost and these foci appeared to be areas of parenchymal degeneration and early necrosis.

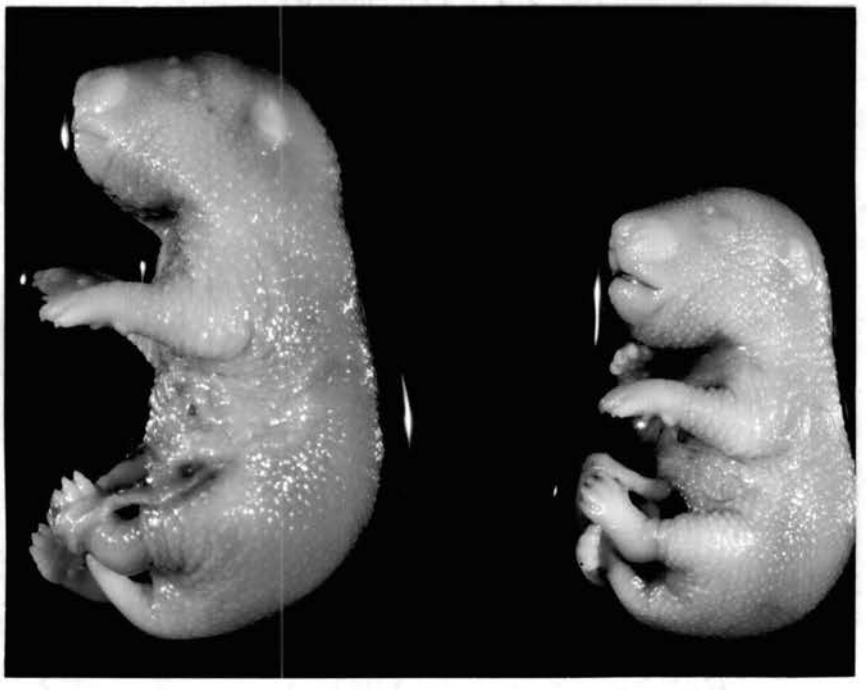

Figure 56. Fetuses on Day 15 of Gestation. The Undersized but Normal Appearing Fetus Is on the Right, While a Control 15 Day Fetus Is on the Left

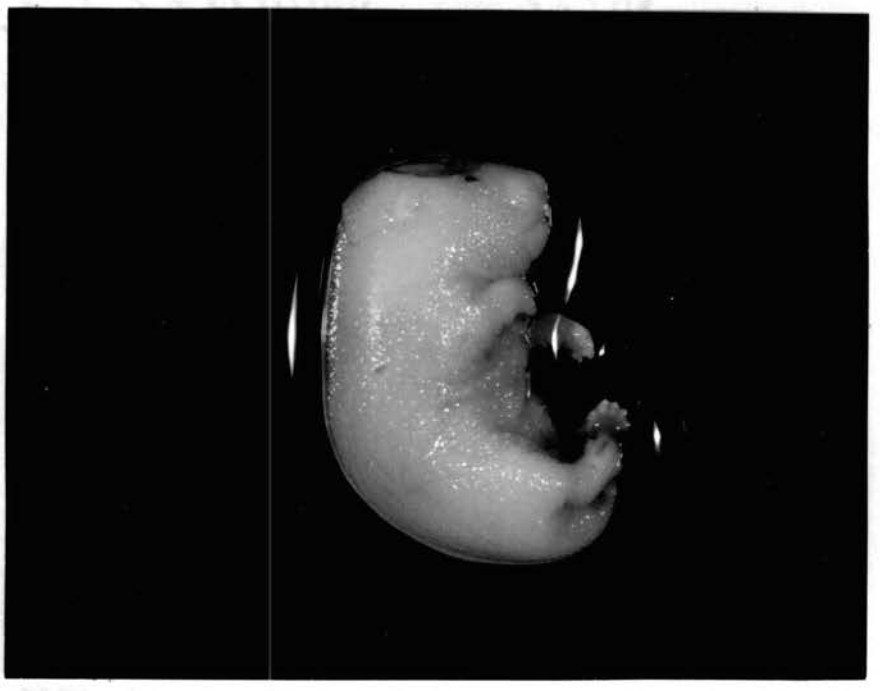

Figure 57. Anencephaly in a 15 Day Fetus

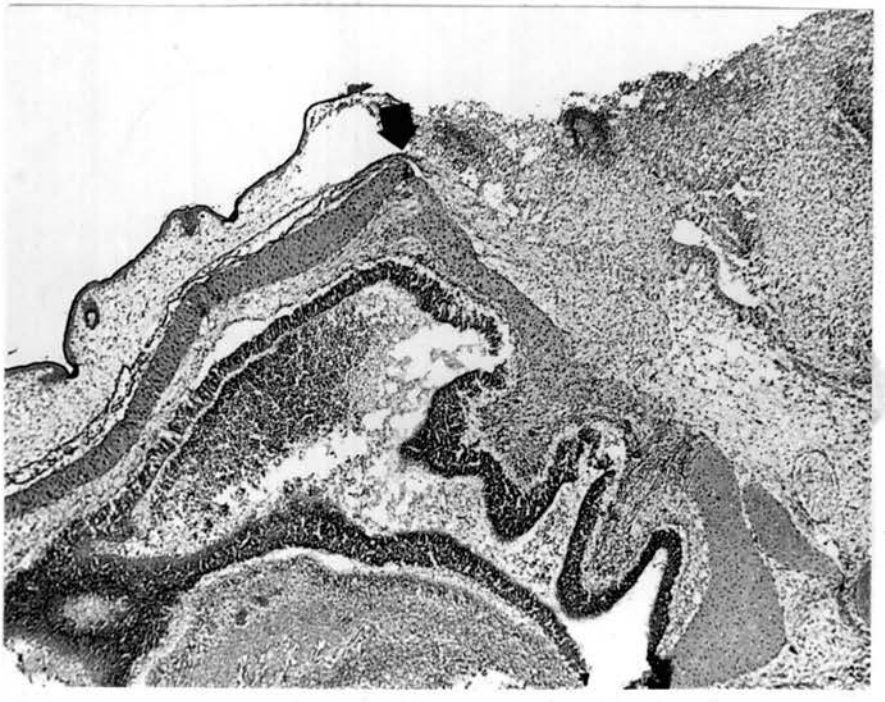

Figure 58. Histologic Section of Anencephalic 15 Day Fetus. Note Area of Demarcation Between Developing Bone and Skin and Exposed Brain (arrow) (H&E x 30)

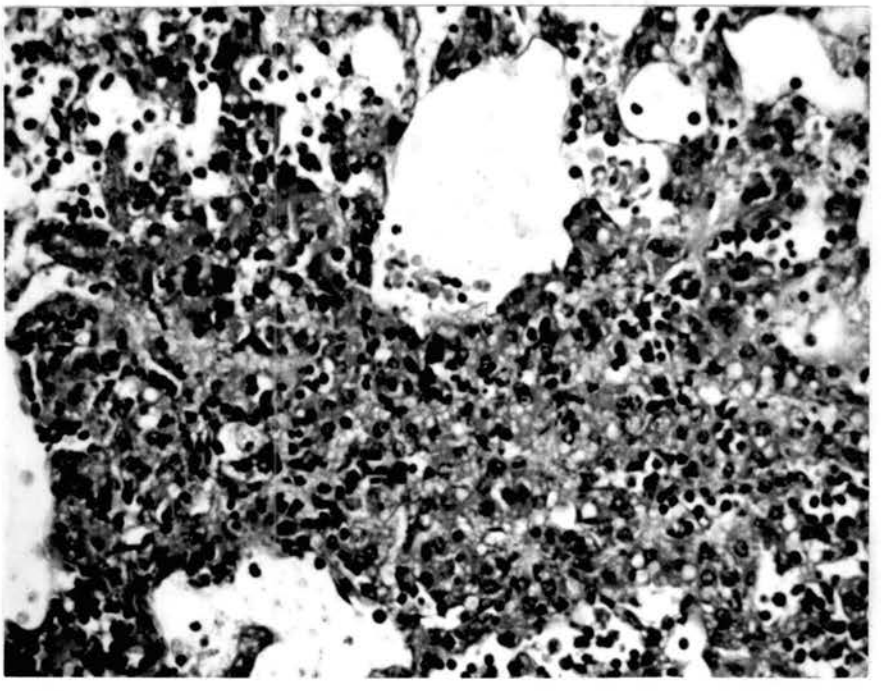

Figure 59. Focus of Degeneration in Liver of a 15 Day Fetus. There Is Swelling and Degeneration of Hepatic Cells and Nuclear Pyknosis (H&E x 200)

CHAPTER V

DISCUSSION AND CONCLUSIONS

Effect of Aflatoxin on Pregnant Female Hamsters

The progressive liver lesions seen in adult female hamsters are similar to those described in rats given aflatoxin (8) and are apparently similar to those seen in female hamsters by Elis and DiPaolo (17), since they mentioned the similarity between the lesions they saw and those described by Butler (8). The only differences noted in animals inoculated with 4 mg/kg and 6 mg/kg was the more rapid development of the liver lesion in the 6 mg/kg group.

Studies on the acute toxicity of aflatoxin in other species have led to reports of both periportal and centrilobular liver damage (16). Considering that the route of excretion of aflatoxin is via the bile (63), the periportal lesion described previously would appear to be a logical consequence. Perhaps those animals in which centrilobular damage was reported have some difference in excretion or in the location within liver lobules of the enzymes involved in aflatoxin metabolism.

Nephrosis following aflatoxin injection has been reported in rats and guinea pigs (8, 9), but not in hamsters. The occurrence of nephrosis in subjects of this experiment is somewhat clouded by the ability of glycerin itself to induce a hemolytic crisis, hemoglobinuria and necrotizing nephrosis in animals (34, 45, 59). In the present experiments,

control animals inoculated with quantities of glycerin equivalent to those used as diluent with aflatoxin did not develop tubular lesions. Of those inoculated with triethylene glycol alone only a few developed degenerative lesions, but these were not as severe as the lesions seen in aflatoxin inoculated animals. The tubular epithelium of triethylene glycol controls was flattened and probably represented a stage of repair similar to that described by Aizawa and Mostofi (1) following glycerin injection. The results of the present experiments indicate that the renal damage was primarily due to aflatoxin although the possibility that the diluents used were contributory should not be overlooked.

Several observations indicate that death following a 6 mg/kg dose might not be due primarily to the action of the toxin on the liver. Liver lesions which were morphologically as severe as those in the 6 mg/kg group occurred with 4 mg/kg, but no deaths resulted. However, the kidney lesions seen in animals given 6 mg/kg appeared to be more severe than those seen in the 4 mg/kg animals and less evidence of attempted regeneration was noted. A limited number (2) of animals given 6 mg/kg had serum glutamic pyruvic transaminase (SGPT) and blood urea nitrogen (BUN) determinations done and in each case 3-4 days postinoculation SGPT values were over 1,000 Sigma-Frankel units, while the BUN was elevated only in those animals that had severe clinical illness. The above points indicate that the degree of kidney change may be the determinant of the LD_{50} in pregnant female hamsters.

Effects of Aflatoxin on Fetuses

A significant difference in mean percent between control and aflatoxin inoculated groups was seen for the variables PVIAB, PDEAD,

PTWL and PGR in Experiment 1, PVIAB, PTWL and PGR in Experiment 2, and PVIAB, PDEAD, PTOT, PVWL, PDWL and PGR in Experiment 3. These results indicate that aflatoxin has definite effects on developing fetuses. Certain similarities exist between the results of these experiments and those of Elis and DiPaolo (17), primarily in the total percent of abnormal fetuses seen in Experiment 1. However, marked differences in the results of the present experiments and those of Elis and DiPaolo are also present.

Elis and DiPaolo (17) reported a decrease in number of malformations as a function of day of examination, ranging from 29.4% on day 9 to 6.2% on day 15. In Experiment 1 the results were comparable (27%-7%) but in Experiments 2 and 3 the tendency was reversed, with a higher mean percent of abnormalities (48% and 43%) seen on day 15. The explanation for the above mentioned differences may lie in the type of diluent used to dissolve the aflatoxin. Elis and DiPaolo (17) used triethylene glycol in their study, and when triethylene glycol was used in the present experiment 1, there was a greater percent of resorptions and less growth retardation of live or dead fetuses (PGR). In addition, triethylene glycol had a more severe effect on the pregnant control females than did glycerin and may well have been a factor in earlier and greater numbers of embryonic or fetal deaths. In glycerin-aflatoxin inoculated animals the effect may be more of aflatoxin alone, rather than a combined aflatoxin-triethylene glycol action leading to earlier deaths and more resorptions.

A more disturbing difference between these experiments and those of Elis and DiPaolo (17) is finding malformations in control fetuses in the present experiment. Several possible explanations for this may

be considered. The first would be the presence in the colony environment or in the animals themselves of a teratogenic agent that was capable of inducing a "background" number of malformations. Since no animals except hamsters were present in the room, no chemicals or ionizing radiations were in the vicinity and the feed was negative for contamination with aflatoxin, environmental contamination was not considered highly probable. No attempt was made to rule out the presence of some viral agent in female hamsters, however no clinical illness was seen in any of the females and all hamsters were obtained from a commercial supplier who states that their colony is free from known hamster viruses. The animals were supposed to have originated from a broad genetic pool, reducing the chances of malformations as a function of inbreeding.

Another possibility is that many of the malformations were not really malformations except by the criteria applied to evaluate the experimental results. Welch (60) stated that, "The rapid rate of maturation causes embryos of a litter to vary so that several developmental stages may be in the same litter at a given time." In addition, Welch illustrated a day 9 fetus which, although not specifically noted, appeared to have a small spinal opening. This may well be an example of a fetus that would be normal if seen on gestation days 12 through 15, having been just a little slower than "normal" in neural tube closure. Although the neural tube is supposed to close on day 9 of gestation (22), a consideration of biologic variation indicates that time of closure in each fetus might vary, with some conceivably not closing until early on the 10th day. Thus, the "defect" noted on day 9 may be gone by day 10. Unfortunately, no good method is presently available for observing the same group of hamster fetuses over a period

of time, however some evidence for the above possibility is present in Experiments 1, 2, 3 and 4. In none of the control groups was there any significant difference in the mean percent of the total number of fetuses (PTOT) as a function of the day of examination. PTOT was relatively the same whether examined on day 9, 10, 11 or 12. During this same span of examination days however, there was a marked reduction in the total number of fetuses with abnormalities (PTWL) with progressive days after inoculation. These findings could indicate that some of the "malformations" seen on gestation days 9.0 or 9.5 are no longer present on day 11 or 12, and cannot be accounted for by an increase in resorptions.

A third explanation for malformed control fetuses might be that there is a natural incidence of malformations in fetuses from normal female hamsters. Boyer (7) indicated that in selecting fetuses for use in describing embryologic development he discarded any with obvious malformations. Unfortunately he did not describe any of the malformations seen, nor the percent of fetal malformations. Further evidence of malformations in normal litters was given by Welch (60). In his study of the normal development of the hamster he indicated that embryos or fetuses which appeared malformed or abnormal were not used. He, like Boyer, did not describe the types of abnormalities encountered, however he mentioned that he only used 90 of 214 possible embryos or fetuses. Even though some of these were lost due to faulty technique, it seems that there must have been a fair percentage of malformed fetuses.

If this does indeed occur, the question is why haven't more abnormalities been seen in control fetuses in experiments on hamster teratogenesis. Only 1 litter containing malformations (18) was mentioned in 15 papers (17-21, 23, 24, 26, 27, 31, 33, 35, 41, 53, 56)

concerned with teratogenesis in hamsters.

Ferm (25) stated that most teratogenic stimuli were measured on well developed fetuses near term and that this assessment does not indicate the total range of embryonic responses. Since controls are usually examined at the same time, it seems possible that problems other than fetal death and severe changes and resorption, could be overlooked. The majority of control fetuses mentioned in the above noted 15 papers were examined on days 13-15 of gestation. Either variation in closure time or natural occurrence of malformations would fit with the patterns seen in controls in the present experiments. There was a significant difference in the number of malformations seen in diluent controls (Experiments 1, 2, 3) and non-diluent controls (Exp. 4) according to the day of sampling. The mean percent PTWL was especially high on gestation day 9 as compared with the other days of examination.

If either of the above mentioned mechanisms was responsible for the results noted in controls, then the results of the aflatoxin inoculated groups must be analyzed in more detail than simply the percent of viable versus dead or abnormality versus normal.

The statistically significant effect of aflatoxin was growth retardation, with an indication of elevated mean percent of PM in inoculated animals. However, the possibility that growth retardation was in some degree secondary to liver and kidney damage cannot be ruled out. The miscellaneous lesions noted consisted primarily of gross edema and hemorrhage, and histologic cardiac necrosis, possibly indicating susceptibility of the fetal cardiovascular system to aflatoxin. Since the morphologic lesion seen in the fetal hearts was noted in one control it is considered that this may be an expression of myocardial cell death

death regardless of cause, with aflatoxin acting as a stimulus to either earlier or more extensive lesion development. The possibility of the cardiac lesion being due to triethylene glycol or glycerin was considered, however, in vitro exposure of cultured fetal myocardial cells to glycerol and ethylene glycol up to 5 M concentration was found to be non-toxic (52). The overall impression from the results of the present experiments is that aflatoxin may have an effect on growth and on developing myocardium.

McCutcheon (42) said that practically all teratogenic effects occur during organogenesis and that there was little susceptibility to exogenous agents after organogenesis ends. He also distinguished between teratogenic substances and those that are toxic to the fetus. In considering the effect of aflatoxin on growth, the determination of whether growth retardation compatible with life is a teratogenic or toxic effect may become a matter of semantics or individual interpretation. Wilson (61) indicated that exposure during the fetal period leads to growth disturbances whereas the same exposure during the embryonic period would lead to structural defects.

The biochemical effects of aflatoxin must also be considered in discussing the type of abnormality produced. Different investigators (14, 72, 63) have shown that aflatoxin has effects on DNA replication and RNA and protein synthesis. The various morphologic expressions of teratogenesis can result from altered nucleic acid integrity or function as a result of interaction with an exogenous chemical (61). If the effect of aflatoxin is indeed a slowing of mitotic activity, then it could be postulated that aflatoxin would exaggerate the possible normal variation in closure, however no real indication of this effect was

seen in these experiments. This same biochemical action would also give the results noted for the PGR including a tendency for higher mean PGR in animals inoculated on day 9, which is after the completion of organogenesis for most of the fetuses.

Considering the experimental results as a whole, 3 points should be emphasized: 1) There is need for further investigation of anomalies in litters from "normal" hamsters, especially in regard to the effect of day of examination on the mean percent of malformations. 2) Teratogenic experiments may become more meaningful if they are designed to be analyzed statistically, with the effects of all sources of variation on the results considered. One reason for the lack of statistical significance in the AOV of some of the variables was the size of the error (residual) term. This is due in part to the sources of variation, but also to the considerable natural variability among animals. Disregarding these sources of error could lead to some unsupported assumptions. 3) The present study was an initial step in studying aflatoxin teratogenesis. In order to conclusively prove its effects in the hamster, further studies on dose rate, route of administration, and time of inoculation must be done. The speed with which aflatoxin reaches the fetus will probably be altered by the route of administration, and will therefore affect the developing fetus at differing stages of organogenesis. In addition, the present study does not help to pinpoint the locus of aflatoxin action either in organs or cells. Use of tools such as radioactive labeling, electron microscopy and clinical chemistry would be of value in reaching this goal.

CHAPTER VI

SUMMARY

This study was designed primarily to evaluate possible teratogenic effects of aflatoxin on hamster fetuses and pregnant female hamsters. Female hamsters were randomly assigned to 4 experimental groups, and the effects of aflatoxin on hamster fetuses and pregnant females noted and compared with several types of control groups.

Variables analyzed included the percent of viable and dead fetuses, percent of resorptions, percent of viable and dead fetuses with abnormalities, the total percent of fetuses (viable + dead) and the total percent of fetuses with abnormalities. Other variables included 4 categories of abnormality; malformations of the head, malformations of the spine, growth retardation and miscellaneous malformations which were made up primarily of lesions related to the cardiovascular system. Morphologic lesions in adult females and fetuses were described grossly and histologically.

A number of malformations was noted in fetuses from control females and several possibilities for this occurrence were discussed. The total percent (day 8 and day 9 inoculations) of head and spine malformations was about the same in control and inoculated groups. A definitely higher percent of miscellaneous lesions was noted in aflatoxin inoculated animals and a statistically significant difference was seen in the mean percent of growth retarded fetuses, with a much

higher mean percent in the inoculated groups.

The primary effect of aflatoxin on adult females was progressive necrosis of liver and kidneys.

It was suggested that the present experiments represent only a start on the problem of aflatoxin teratogenesis and that modifications of the experimental design would be of value in investigating this problem.

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APPENDIXES

TABLE VI

RAW DATA FOR CONTROLS - EXPERIMENT 1

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	9	12	10	2	0
8	9	13	11	1	1
8	11	14	0	0	14
8	11	2	2	0	0
8	15	13	10	0	3
8	15	9	0	0	9
9	10	13	11	0	2
9	10	6	4	0	2
9	12	13	12	1	0
9	12	10	7	0	3
9	15	13	13	0	0
9	15	11	11	0	0

TABLE VII

RAW DATA FOR CONTROLS - EXPERIMENT 1

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	9	5	2	7	0	0	0
8	9	5	0	4	2	0	0
8	11	0	0	0	0	0	0
8	11	0	0	0	0	0	0
8	15	0	0	0	0	0	0
8	15	0	0	0	0	0	0
9	10	0	0	0	0	0	0
9	10	0	0	0	0	0	0
9	12	5	1	6	0	0	0
9	12	2	0	1	0	0	1
9	15	0	0	0	0	0	0
9	15	0	0	0	0	0	0

TABLE VIII

RAW DATA FOR AFLATOXIN INOCULATIONS - EXPERIMENT 1

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	9	10	8	1	1
8	9	11	7	3	1
8	11	13	12	0	1
8	11	13	1	9	3
8	15	6	0	0	6
8	15	12	6	0	6
9	10	11	10	1	0
9	10	12	1	11	0
9	12	14	14	0	0
9	12	14	0	0	14
9	15	14	0	0	14

TABLE IX

RAW DATA FOR AFLATOXIN INOCULATIONS - EXPERIMENT 1

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	9	4	1	4	1	0	0
8	9	3	0	3	0	0	0
8	11	1	0	1	0	0	0
8	11	1	9	1	0	9	0
8	15	0	0	0	0	0	0
8	15	6	0	0	0	6	0
9	10	4	1	0	0	2	2
9	10	0	0	0	0	0	0
9	12	10	0	7	0	0	3
9	12	0	0	0	0	0	0
9	15	0	0	0	0	0	0

TABLE X

RAW DATA FOR CONTROLS - EXPERIMENT 2

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	8.5	7	4	0	3
8	9	14	8	4	2
8	11	14	14	0	0
8	15	12	12	0	0
9	9.5	11	8	1	2
9	10	12	12	0	0
9	12	7	7	0	0
9	15	11	9	2	0

TABLE XI

RAW DATA FOR CONTROLS - EXPERIMENT 2

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	8.5	0	0	0	0	0	0
8	9	1	4	5	0	0	0
8	11	3	0	0	0	3	0
8	15	0	0	0	0	0	0
9	9.5	5	1	4	3	0	0
9	10	5	0	4	1	0	0
9	12	0	0	0	0	0	0
9	15	0	2	2	0	0	0

TABLE XII

RAW DATA FOR AFLATOXIN INOCULATIONS - EXPERIMENT 2

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	8.5	11	8	0	3
8	8.5	13	10	0	3
8	9	12	2	8	2
8	9	10	0	0	10
8	11	14	13	1	0
8	11	8	6	1	1
8	15	13	13	0	0
8	15	8	8	0	0
9	9.5	16	12	4	0
9	9.5	13	10	2	1
9	10	13	12	0	1
9	10	14	10	3	1
9	12	16	9	6	1
9	12	12	11	1	0
9	15	10	9	1	0
9	15	14	7	3	4

TABLE XIII

RAW DATA FOR AFLATOXIN INOCULATIONS - EXPERIMENT 2

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	8.5	0	0	0	0	0	0
8	8.5	0	0	0	0	0	0
8	9	1	6	5	1	1	0
8	9	0	0	0	0	0	0
8	11	2	1	0	0	3	0
8	11	1	1	1	0	0	1
8	15	0	0	0	0	0	0
8	15	8	0	0	0	8	0
9	9.5	4	4	8	1	0	0
9	9.5	5	1	5	1	2	0
9	10	2	0	2	0	0	0
9	10	4	3	6	0	2	0
9	12	6	6	0	0	12	0
9	12	11	1	0	0	5	7
9	15	9	1	0	0	10	2
9	15	7	3	1	0	8	1

TABLE XIV

RAW DATA FOR CONTROLS - EXPERIMENT 3

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	8.5	12	9	0	3
8	9	4	3	0	1
8	11	12	12	0	0
8	15	12	12	0	0
9	9.5	10	5	5	0
9	10	13	13	0	0
9	12	14	13	0	1
9	15	14	14	0	0

TABLE XV

RAW DATA FOR CONTROLS - EXPERIMENT 3

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	8.5	0	0	0	0	0	0
8	9	1	0	0	1	0	0
8	11	0	0	0	0	0	0
8	15	0	0	0	0	0	0
9	9.5	2	2	4	1	0	0
9	10	4	0	4	0	0	0
9	12	1	0	0	0	0	1
9	15	0	0	0	0	0	0

TABLE XVI

RAW DATA FOR AFLATOXIN INOCULATIONS - EXPERIMENT 3

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	8.5	18	15	0	3
8	8.5	12	11	0	1
8	9	13	12	1	0
8	9	15	14	1	0
8	11	10	0	4	6
8	11	11	0	8	3
8	15	12	4	3	5
8	15	9	0	5	4
9	9.5	12	11	0	1
9	9.5	12	10	2	0
9	10	8	3	4	1
9	10	10	9	1	0
9	12	13	0	0	13
9	12	13	11	1	1
9	15	13	11	0	2
9	15	12	0	10	2

TABLE XVII

RAW DATA FOR AFLATOXIN INOCULATIONS - EXPERIMENT 3

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	9.5	0	0	0	0	0	0
8	8.5	0	0	0	0	0	0
8	9	11	1	8	3	3	1
8	9	8	1	4	4	1	0
8	11	0	4	2	0	4	1
8	11	0	8	0	0	8	1
8	15	4	3	0	0	7	0
8	15	0	5	0	0	5	1
9	9.5	3	0	1	0	0	3
9	9.5	5	2	6	2	1	1
9	10	1	4	1	0	4	0
9	10	9	1	0	0	0	0
9	12	0	0	0	0	0	0
9	12	9	1	0	0	1	10
9	15	8	0	1	0	7	0
9	15	0	10	0	0	10	0

TABLE XVIII

RAW DATA FOR NON-MANIPULATED CONTROLS - EXPERIMENT 4

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	9	13	8	4	1
8	9	15	10	5	0
8	11	9	8	1	0
8	11	17	12	0	5
8	15	13	13	0	0
8	15	15	15	0	0

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	9	4	4	8	0	0	0
8	9	2	3	5	0	0	0
8	11	1	0	0	0	1	0
8	11	1	0	1	0	0	0
8	15	0	0	0	0	0	0
8	15	0	0	0	0	0	0

TABLE XIX

RAW DATA FOR NEEDLE PUNCTURE CONTROLS - EXPERIMENT 4

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	9	13	0	0	13
8	9	11	9	2	0
8	11	15	14	0	1
8	11	13	11	0	2
8	15	7	6	0	1
8	15	13	13	0	00

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	9	0	0	0	0	0	0
8	9	5	1	5	1	0	0
8	11	0	0	0	0	0	0
8	11	0	0	0	0	0	0
8	15	0	0	0	0	0	0
8	15	0	0	0	0	0	0

TABLE XX

RAW DATA FOR SALINE INOCULATED CONTROLS - EXPERIMENT 4

Day of Inoculation	Day of Observation	Number of Gestation Sacs	Number of Viable Fetuses	Number of Dead Fetuses	Resorptions
8	9	15	9	5	1
8	9	13	1	9	3
8	11	14	10	2	2
8	11	7	7	0	0
8	15	11	11	0	0
8	15	13	15	0	0

Day of Inoculation	Day of Observation	Number of Viable Fetuses With Lesions	Number of Dead Fetuses With Lesions	Number of Head Lesions	Number of Spine Lesions	Growth Retardation	Miscellaneous
8	9	2	4	5	0	0	1
8	9	0	3	3	0	0	0
8	11	3	0	3	0	0	0
8	11	2	0	2	0	1	0
8	15	0	0	0	0	0	0
8	15	0	0	0	0	0	0

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