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EXPERIMENTAL PRODUCTION OF CONGENITAL MALFORMATIONS IN AN
INBRED RESISTANT STRAIN OF MOUSE UTILIZING A SINGLE DOSE OF
CORTISONE DURING A CRITICAL GESTATION PERIOD

By

DONALD DEWAYNE KARICH

A Thesis Submitted to the Faculty of the
Graduate School of Loyola University in Partial
Fulfillment of the Requirements for the Degree of
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AUTOBIOGRAPHY

Donald DeWayne Karich was born in Denver, Colorado on December 19, 1944, the son of Leonard and Lorraine Karich. He resided in Colorado for the first twenty years of his life. In 1962 he graduated from Longmont High School and entered the University of Colorado at Boulder where he did his pre dental studies. After spending two years there, he entered the University of Nebraska College of Dentistry in 1964 and received his Doctor of Dental Surgery in 1968.

Following graduation, Dr. Karich worked as a Dental Associate in Denver, Colorado for one year before commencing his own general practice in 1969 in Menlo Park, California. In June of 1972, after three years in Menlo Park, he entered the Graduate School of Loyola University in Chicago, Illinois. Dr. Karich was married to the former Dianne Bumgarner, daughter of Homer and Gloria Bumgarner of Bruning, Nebraska on December 14, 1968.

Dr. and Mrs. Karich are now the proud parents of Eric Kent and Andrew Curran.

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CHAPTER I

INTRODUCTION

Congenital malformations such as cleft palate have been produced in laboratory animals by various modalities. Cleft palate in humans is a congenital defect that occurs approximately one in every eight hundred live births (Grace, 1943). This defect is the result of the failure of the palatine processes to meet and fuse during a specific time in embryological development. In untreated cases severe handicaps appear. Speech, mastication, deglutition and respiration are affected and as a result psychologic problems develop. In 1940, Warkany and Nelson showed that nutritional disturbances were contributing factors to cleft palate production. Fogh-Andersen (1942) demonstrated that genetic factors were also important. Streaan and Peer (1956) thought that physiologic, emotional or traumatic stress played a role in human clefts by stimulating the adrenal cortex to secrete hydrocortisone. This appeared to correlate with the animal studies of Baxter and Fraser (1950), Fraser and Fainstat (1951), and Kalter (1957) who were able to produce clefts in mice before birth by giving cortisone to pregnant A/Jax mice. Experimental production of cleft palate with cortisone in inbred mice has provided a convenient and a reliable tool for investigation into the mechanism of congenital malformations. For over 20 years investigators have reported induction of cleft palates in the offspring of mice after maternal injection of corticoids.

Baxter et al (1950), and Fraser et al (1951) established that the offspring of A/Jax mice were 100% susceptible to cleft palate when maternally injected with 2.5 mg. of cortisone acetate on day 11, 12, 13, and 14 of gestation. Chaudhry and associates (1967) reported that a single intramuscular injection of 10 mg. of cortisone acetate would induce 100% cleft palate if injected on the 11, 12, 13 or 14th day of gestation in the same strain of mouse. Ingalls and Curley (1957) reported the same observation when hydrocortisone (cortisol) was injected instead of cortisone.

It has been established that a strain difference appears to exist between the A/Jax and C57b1 in their sensitivity toward cortisone acetate or hydrocortisone. Fraser reports this difference to be variable according to the genetic constitution of the treated mice, the dosage of cortisone used and the gestational stage at which treatment is begun. The C57b1 strain has been shown to be constitutionally more resistant to the cleft palate inducing effects of the cortisone: Walker and Fraser reported 17-21% of fetuses had cleft palates. All previous work, which has been done on the C57b1 strain utilized a total teratogenic dose of 10 mg. administered over a four day period at the rate of 2.5 mg./day.

In as much as most of the cleft palate studies now being done utilizing the A/Jax strain have adopted the single dose injection technique and in order to facilitate any future comparative study with the C57b1 strain, the purpose of this study is as follows:

1. To determine if a single dose injection technique is feasible

for this C57b1 strain of mouse.

2. To compare the multi-injection technique with the single injection technique with particular emphasis on the number of non-viable fetuses and resorptions as well as the number and percentage of clefts.

3. To determine if fetal weights of the cleft animals differs significantly from the weights of the normal fetuses.

4. To determine if the maternal weights during gestation are related to the cleft formation.

5. To determine what percentage of clefts per animal per viable fetus can be obtained utilizing the single injection technique as opposed to the multi-injection techniques that have been utilized in the past.

6. To correlate uterine position with incidence of imperfect fetuses.

7. To determine what other teratogenic effects are observed in the fetuses which could be related to the drug.

8. Finally, an A/Jax control group is included which will receive a single injection of the drug on the optimal day for cleft production as reported by Chaudhry (1967) to determine that cleft palate percentage in our laboratory is similar to that reported by other investigators.

CHAPTER 2

REVIEW OF LITERATURE

A. Metabolism of the Corticosteroids.

The secretory products of the adrenal cortex are hormones which resemble the lipids in their solubility properties but differ in their chemical structure. They are synthesized from cholesterol or from other intermediate products formed from acetate; these precursors are then transformed into active hormones by a series of enzymatic reactions occurring within the adrenal cortex itself. The basic structure of the adrenocortical hormones is the perhydrocyclopentanophenanthrene nucleus with two additional angular methyl groups attached at carbon atoms 10 and 13, and an α -ketol side chain (carbon atoms 20 and 21) attached at position 17. The principle steroids found in the adrenal cortex include compound E (cortisone), compound F (hydrocortisone or cortisol), 17 hydroxycorticosterone and aldosterone (see Fig. 1). Cortisone (compound F) is the major free circulating adrenocortical hormone in human plasma. Cortisone has a ketone group at C-11, a hydroxyl group at C-17 and only a minute quantity is normally secreted each day. Hydrocortisone contains hydroxyl groups at C-11 and C-17. Hydrocortisone is the predominate adrenal corticosteroid and 15 to 30 mg. are secreted per day. Aldosterone contains an aldehyde group attached at C-13 in place of the methyl group and under average conditions only 150 to 300 micrograms are secreted per day.

The secretion of adrenal cortical steroids is largely controlled by

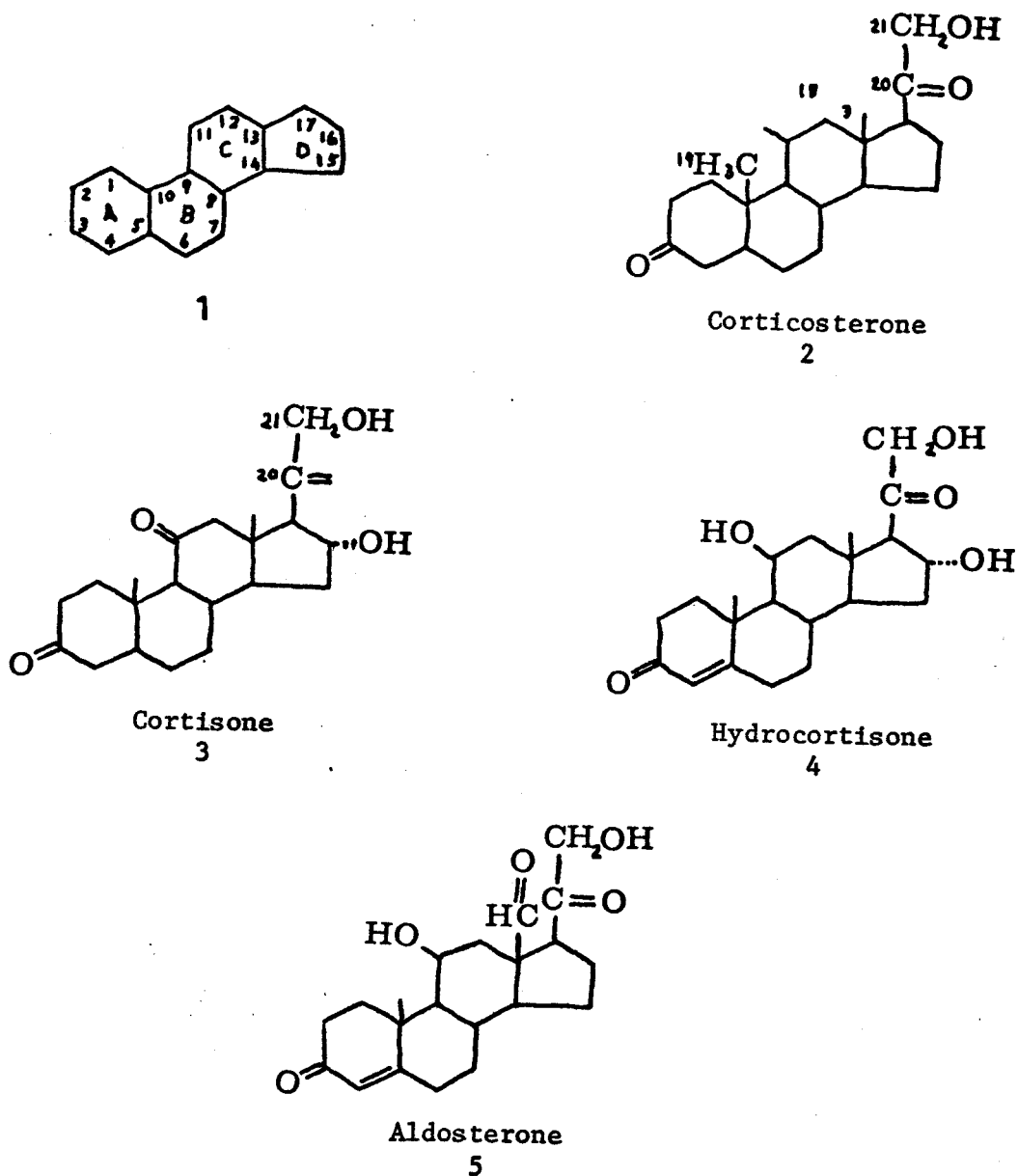


Figure 1. Adrenal corticosteroids. Chemical structures of major steroids secreted by the human adrenal cortex. (1) Perhydrocyclopentanophenanthrene nucleus. (2) Corticosterone. Note that there are angular methyl groups at C-18 and C-19, an α -ketol side chain (C-20 and C-21), a ketone group at C-3, a double bond between C-4 and C-5, an hydroxyl group at C-11; 0.5 to 2.5 mg. of this hormone secreted per day. (3) Cortisone. Ketone group at C-11, hydroxyl group at C-17; minute quantity secreted per day. (4) Hydrocortisone. Hydroxyl groups at C-11 and C-17; 15 to 30 mg. secreted per day. (5) Aldosterone. Aldehyde group attached at C-13 in place of the methyl group; under average conditions, only 150 to 300 μ g. secreted per day.

adrenocorticotrophic hormone (ACTH), a straight-chain polypeptide containing 39 amino acids with a molecular weight of 4,500, synthesized and stored in the anterior pituitary gland, and released from that organ in response to various stimuli.

The adrenocortical hormones exist in plasma in association with a specific binding globulin. Presumably, only the small fraction not bound to this protein is free to diffuse and act physiologically. After a few hours existence in plasma and tissue, these hormones are reduced by hepatic enzymes to a variety of C-21 metabolites and conjugated mainly with glucuronic acid. Both free and conjugated corticosteroid are excreted with the bile into the intestine and may then be reabsorbed by the enterohepatic circulation. These processes render the steroids more water-soluble. Elimination is primarily by the kidney. Measurement of these urinary metabolites is a convenient way in which to assess adrenal function, but determinations of plasma concentration and secretory rate are possible.

ACTH has only a fleeting existence in plasma; the exact pathways of degradation are unknown.

Under normal circumstances, the adrenal cortical steroids are thought to contribute to the preservation of homeostasis by regulating (but not initiating) the metabolism of carbohydrate, protein, fat, and electrolyte. The regulation of biochemical processes by the cortical hormones is undoubtedly accomplished through the inhibition or stimulation of specific enzyme systems. It cannot be stated at this time which

enzymes are primary targets, but the most important measurable effects of hormone action are acceleration of the conversion of glycogenic amino acids to glycogen in the liver (gluconeogenesis), the inhibition of the rate of peripheral utilization of glucose, the conservation of body sodium, the regulation of the rates of protein anabolism and catabolism, and a regulatory influence upon fat mobilization and its site of deposition. The chief hormones which influence carbohydrate, fat, and protein metabolism are hydrocortisone and corticosterone both are known as glucocorticoids. The adrenocortical hormone which is chiefly responsible for the maintenance of sodium and other electrolyte balance in man is aldosterone, a so-called mineralocorticoid. Aldosterone is unique among the biologically important adrenal steroids in that its secretion is affected only slightly by ACTH, but greatly by such stimuli as blood volume, sodium intake and the level of serum potassium. The glucocorticoids have some permissive influence upon electrolyte metabolism under physiologic circumstances, but the mineralocorticoids, natural and synthetic, have insignificant glucocorticoid activity.

When glucocorticoids are circulating in excessive quantities, hyperglycemia or frank diabetes, increased protein catabolism, muscle wasting, osteoporosis, loss of integrity of skin and blood vasculature, increase in serum sodium, renal loss of potassium, edema, hypertension, redistribution of fat to the face and trunk, psychic disturbances, decreased threshold to seizures, acne, polycythemia, eosinopenia, granulocytosis, lymphopenia, increased susceptibility to infection, delayed

wound healing, activation of peptic ulceration, and hirsutism may result. All these undesirable effects are regularly reproduced by the administration of high doses of glucocorticoid, although individual sensitivity plays an important role in determining the rapidity and degree to which they develop.

Cortisol is biologically transported by the protein transcortin (Burton and Jeyes, 1968). The principle reactions in the metabolism of cortisol are oxidation and reduction changes. Conversion of cortisol to cortisone is readily accomplished by a reversible reaction catalyzed by the enzyme 11^B-hydroxydehydrogenase. This biotransformation can occur in the liver and in the lymphocytes.

Several inactive metabolites of cortisone and cortisol with no known biological activity are known to exist. Some of these conversions include formation of tetrahydrocortisol in the liver, substance E of Reichstein, substance U of Reichstein, dehydrocortisol, pregnane-11^B, 17-dione, and 4-androstene-11^B-ol-3. These biotransformations are the results of combinations of oxidations, reductions, and saturation of the double bonds of hydroxyls, ketones, and double bonds characteristic of cortisol. These metabolites can then be made water-soluble through conjugation mainly in the liver, and secondarily in the kidney. Conjugation with glucuronic acid forms glucuronides while conjugation with sulfate produces sulfate esters. Both water-soluble conjugates can then be excreted through the kidney. The unconjugated metabolites will remain in the blood serum.

B. Cleft Palate Induction in Animals.

In recent years the role of congenital malformation as one of the leading causes of infant mortality and morbidity has been recognized. Whenever a medical problem exists, it is of value to be able to reproduce the pathologic phenomena in animal experiments. Congenital malformations have been produced in lower classes of animals for nearly a century but because mammals have so completely different a prenatal maternal-offspring relationship such experiments did not satisfy those interested in the origins of congenital malformations in children. The first teratologic method practiced in mammals, which dates back to the first decade of this century, was the administration of x-rays to the pregnant mother (Kalter, 1957).

Approximately four decades ago much interest in congenital malformation was aroused when it was shown that these malformations could be induced by dietary deficiencies in pregnant animals. Specifically Hale (1933) showed that malformations could be produced in pigs with vitamin A deficiencies. Since then several investigators have produced malformations in test animals utilizing a variety of agents.

Corticosteroid hormones and ACTH have been administered to pregnant animals of several species by a large number of investigators. Various workers using rats, rabbits and monkeys, usually of unspecified origin, gave different amounts of cortisone acetate, ACTH, or other corticosteroids, intramuscularly or subcutaneously, for selected periods starting at various times before and during gestation. Resorption and/or abortion occurred almost invariably when treatments with large doses of

hormone were begun during the middle third of pregnancy, but the occasional survivors were normal. The effect of lengthy treatment with cortisone on fetal distribution was not minimized or blocked by adrenalectomy of the pregnant female but the influence of ACTH in reducing litter size and fetal weight was prevented by maternal adrenalectomy.

Walker and Fraser (1957) working with mice, produced resorptions by administering intraembryonic injections of about .25 mg. of cortisone between 12 and 14 days after conception. But when injection was made into the embryonic sac, cleft palate resulted. However, intra-amniotic injections of cortisone vehicle also produced cleft palate. This result led the authors to believe that effect was due to the observed leakage of amniotic fluid through the hole made by the hypodermic needle, which caused a reduced hydrostatic pressure in the amnion, allowing the contracting uterus to press the embryo's head onto its chest, lodging the tongue between the palatine processes and thus preventing their movement. This study was repeated by Trasler et al (1956) who, on day 13 of gestation, merely inserted a hypodermic needle through the uterine wall into the amnion, but injected nothing. This procedure also produced cleft palate.

Congenital malformations in offspring of cortisone-treated pregnant animals were first reported by Baxter and Fraser (1950), who produced cleft palate without cleft lip in mice by such treatment. By administration of various doses of the hormone (from 0.625 to 10 mg. per day given for 2-4 successive days beginning different times during the second third

of pregnancy) to several strains and types of mice, Fraser and Fainstat (1951) established that the frequency of cleft palate produced in the offspring varied with the dose given, time treated, and strain used. The highest incidences of the malformation compatible with minimum frequency of resorption were produced by 2.5 mg. cortisone acetate per day given for 4 successive days beginning 11 days after conception. This schedule produced almost 100 per cent affected young in the A/Jax strain, about 20 per cent in the C57b1/6 strain and about 80 per cent in the DBA/I strain.

Fraser et al (1954) suggested two different mechanisms that may be operational in causing induced clefts: one which causes a delay in the rotation of the palatal shelves before palatal closure, and one which causes a breakdown in previously fused palatal tissues.

In 1954, Walker's theory was that a delay in the average time and rate at which shelf movement occurred was the primary cause of induced clefts. He cited as evidence the fact that he observed many embryos in which one palatal shelf had horizontalized while the other remained vertical. This finding was not often encountered in untreated embryos according to Walker. He also found that there was no significant difference in the size of the palatal shelves in treated and untreated animals prior to the time of palatal closure. He did feel that fusion of the shelves is necessary to promote growth of the shelves beyond the size prior to closure.

Walker further suggested in 1954 that there would be a decrease in

the force available to cause shelf movement if cortisone acted upon the shelves directly. Such decrease in available force might be due to:

1. Inhibition of fiber formation.
2. Inhibition of acid mucopolysaccharide synthesis.
3. Change in the pattern of tissue arrangement, i.e., fiber mucopolysaccharides.

Larsson (1961-1962) upheld the idea of Walker and Fraser (1956) that shelves in cleft animals are retarded in horizontalization and that the formation of substances necessary to produce the force for horizontalization is inhibited by cortisone, i.e., acid mucopolysaccharides. He demonstrated that cortisone injection lowers the amount of sulfate incorporated into the mouse embryo acid mucopolysaccharides.

Kalter (1957) enumerated some of the factors which determine the frequency of appearance of animals with clefts in a cortisone-treated litter. Among these were: gestation time, maternal genotype, dose of cortisone, strain of mouse, fetal genotype, maternal weight, and fetal weight. Kalter also showed that there were fewer complete fetal resorptions in untreated litters than in cortisone-treated litters. Treated litters had 30% fewer animals, and the individual cleft palate newborn weighed less than his normal counterpart. A lower frequency of clefts was noted where there were heavier mothers or more advanced parities.

Kalter (1954) made a study of the inheritance of susceptibility to the teratogenic action of cortisone, first, by treating A/Jax and C57b1 females that were crossed to males of the other strain. In this way F1

hybrid fetuses of identical genetic composition (aside from the sex chromosomes) carried by both types of pure strain females were subjected to the influence of cortisone. The frequency of cleft palate in such hybrids of identical genetic constitution was different when they were carried by A/Jax females than if carried by C57b1 females. Hybrids from A/Jax females had an incidence of 43.5 per cent cleft palate and those from C57b1 females had 3.7 per cent, indicating a potent maternal influence operating on the teratogenic efficacy of cortisone. F1 females of reciprocal crosses between the A/Jax and C57b1 strains were then backcrossed to A/Jax males and treated. The frequencies of malformed young from both types of F1 mothers were about the same (25%), eliminating the possibility that the maternal influence mentioned above was due to a cytoplasmic factor. Further analysis showed that the genetic composition of both mother and fetus influenced the cortisone effect.

In an investigation of the food consumption of pregnant cortisone treated mice, Kalter (1955), showed that the hormone caused a significant increase in food eaten by pregnant mice and proved that the teratogenic effect was not a consequence of decreased food intake.

Fainstat (1954) has proved that cortisone is teratogenic in at least one species other than mice. In this study, rabbits of commercial origin were given 25 or 30 mg. of cortisone intramuscularly per day for 4 successive days beginning the 14th or 15th day of gestation; of 35 offspring, 17 had cleft palate.

Several other steroid hormones have been administered to pregnant

mice by Fraser and colleagues. Hydrocortisone was found to be a potent cleft palate-inducing agent (Kalter and Fraser, 1952). This was confirmed by Ingals and Curley (1957). ACTH also produced cleft palate. Strain A females were given 5 mg. ACTH every 6 hours for 2 or 3 days beginning the 13th day of gestation, and cleft palate was induced in some of the young. Injections of 11-desoxycorticosterone were without effect on embryonic development in mice by Burdick, Baird and Rogers (1954) and this substance produced "some embryonic malformations.....in embryos recovered at term from treated ovariectomized pregnant hamsters (Tedford, 1950).

Various studies were undertaken and analyses made to further evaluate some of the residual sources of variability of susceptibility to the cleft palate-inducing property of cortisone. It was first found that a decrease in frequency of cleft palate occurred in the offspring of successive litters of treated pregnant mice (Kalter and Fraser, 1953). By treating primiparous mice, the weights and ages of which varied widely it was found in members of litters of uniform parity that the cleft palate frequency was significantly inversely associated with maternal weight, but not with maternal age (Jost, 1951). In these three studies mice of three different genetic constitutions were used and the average frequencies of cleft palate were all different from one another, but the regression coefficients of maternal weight on cleft palate frequently were approximately the same in all the studies, indicating that the level of protection afforded by increased maternal weight against cortisone-

induced cleft palate may be a constant. Finally, it was found (Kalter, 1957), that affected offspring were significantly lighter than were their siblings with normal palates from cortisone-treated mothers. A decrease in weight of young with cleft palate was also noted in the offspring of cortisone-treated rabbits (Fainstat, 1954). An embryological study was made of palate closure in control and cortisone-treated mice of three inbred strains (Walker and Fraser, 1957). These studies revealed that in control animals the palate closed on the average at an earlier developmental age in C57b1 fetuses than in A/Jax fetuses, with DBA mice being intermediate and claimed that the strain difference between resistant C57b1 and susceptible A/Jax mice in response to cortisone was due to the earlier closing of the palate in the C57b1 strain. Clark (1956) in his histological studies of the embryonic palate observed stainable elements that increase in concentration with advancing stages of palate closure. Control A/Jax fetuses had lower concentrations of these elements than control C57b1's, and both types of treated fetuses showed less stainable material than controls, with the concentration in treated A's less than in treated C57b1's. Since relatively few C57b1 and many A/Jax young show cleft palate after cortisone, Clark proposed that the stainable material was elastin and may be the physical basis for palatal shelf movement, this being normally present in A/Jax mice nearer a critical level, and hence less easily brought below the threshold in the resistant than in the susceptible strain.

In rats, cortisone has repeatedly been found not to be teratogenic,

but a potentiating effect of cortisone on the teratogenic action of hypervitaminosis A was recently reported (Woollam and Millen, 1957).

Loevy (1962) did not feel that the tongue played an active role in cleft formation, nor was it a major consideration in horizontalization. She further felt that the role of the connective tissue in cleft development has been overemphasized. She based her findings upon an investigation in which she injected 1.25 mg. of cortisone daily from the eleventh to the fourteenth day in Strain A mice and achieved 100% cleft formation. Loevy attributed cleft formation to a basement membrane which cannot be penetrated by connective tissue when the shelves are continuous and ready to fuse.

It has not yet been determined exactly how cortisone acts to induce the formation of cleft palate. It is possible that there is an intermediate action elsewhere in the fetus or mother, or it may act directly on the palatine shelves.

Cortisone is not found in adrenal venous blood or peripheral blood. Its function seems to be directed as a precursor for hydrocortisone which is found in the peripheral circulation. Cortisol exhibits an anti-inflammatory action probably due to its ability to suppress the cell's activity in producing the chemical agents responsible for inflammation. It is also known to elicit a mild hyperglycemia and an improvement in overall capillary tone, resulting in diminished exudation of plasma into the tissues. Cortisone also causes a decrease in circulating eosinophils, a decrease in lymphocytes and an increase in the total white cell

count due to an increased number of polymorphonuclear leukocytes (Krantz and Carr, 1961).

Cortisone aids in the regulation of fat, protein, and carbohydrate metabolism. It is also responsible for polycythemia (an increase in the number of red blood cells) and for a decrease in lymphoid tissue mass. Cortisone can produce a lymphocytopenia according to Goodman and Gilman (1965).

Dougherty et al (1961) stated that cortisol in tissue culture causes a "pulling in" of the processes of the fibroblasts resulting in an epithelioid-appearing cell which develops numerous vacuoles filled with reducing substances. Pinocytosis is completely absent, and the epithelioid cells persist intact in spite of enormous damage to neighboring fibroblasts even when the tissue is extensively infiltrated by polymorphonuclear leukocytes. The epithelioid fibroblasts have been cinemicrographically shown to be quite inactive in their movement.

Dougherty stated that the anti-inflammatory action of corticosteroids is a focal one and does not depend upon metabolites created by distant sites. He also felt that the influences which cortisol exerts on connective tissue are primarily due to modifications in fibroblast function. The fibroblast is the cell responsible for the majority of structures present in connective tissue, i.e., ground substance matrix and fibers.

Although the biologically active form of the steroid is cortisol, Dougherty feels that cortisone is reversibly transformed to cortisol and

hence acts as a potential reserve for cortisol when it is needed.

It was found (Bollough 1952, Sayeed 1962) that cortisone can suppress mitosis although it is unlikely that this is one of cortisone's fundamental actions. Roberts (1952) and Mott (1968) concluded that maternal cortisone injections affected the palatine shelves of A/Jax mice by suppression of mitosis, thereby causing a deficiency in the number of cells with a subsequent decrease in the amount of intercellular substance.

Layton (1951) stated that high doses of cortisone inhibit the synthesis of chondroitin sulfate in connective tissue. Cavallero and Braccine (1951) found that metachromatically stainable material had practically disappeared from the interfibrillar ground substance while Lattes et al (1953) also stated that there is a reduction in the metachromasia normally seen suggesting that the mucopolysaccharides of cortisone-treated tissue do not undergo the same chemical changes as in untreated specimens.

Larsson (1962) also felt that cortisone interferes with the synthesis of the usual sulfated mucopolysaccharide content of connective tissue, and Jacobs (1966) concluded that there is more water present in the palates of cortisone-treated embryos and that this edema is most striking during the critical period of palate closure. Jacobs attributed this edema to a decreased level of sulfated mucopolysaccharides in the shelf mesenchyme.

In 1953, Paff and Stewart noted a reduction on the number of mast

cells with cortisone treatment, while Birko (1953) found that it inhibits both hyaluronidase and streptococci.

In skin wounds, Ragan et al (1949) found the development of granulation tissue was markedly delayed by cortisone. Kivirikko (1963) stated that cortisone caused an increase of hydroxyproline and a decrease in the amount of alkali-soluble and neutral-salt soluble collagen in the chicken and attributed to cortisone the ability to alter collagen metabolism.

Fraser et al (1967) determined that the administration of cortisone to a pregnant female mouse caused a reduction in the volume of the amniotic fluid (oligohydrannios) of the mouse embryo at the time of palatal closure, but the amount of the decrease was shown to be exactly the same in normal embryos as in embryos with clefts. He therefore concluded that the amniotic fluid reduction cannot be the factor that determined which co-investigators thought originally that the reduced volume of amniotic fluid might cause a constriction of the embryo with subsequent flexion of the neck pressing the lower jaw against the chest, thus jamming the tongue between the palatal shelves and preventing their closure.

Kerppala and Pitkanen (1960) learned that cortisone inhibits the uptake of oxygen by rat liver mitochondria, reduces the activity of the enzyme cytochrome oxidase, and uncouples the process of oxidative phosphorylation which occurs in the mitochondria.

DeVenuto et al (1968) by the use of cell fractionation and steroids which were labeled with radioactive tritium and Carbon-14, showed that corticosterone and cortisone exhibit a definite interaction with the

nuclear and mitochondrial fractions of rat liver cells.

Nasjleti et al (1967) administered a single intramuscular 10 mg. injection of tritium-labeled cortisone to pregnant female A/Jax mice at 12½ days of gestation. Blood samples were withdrawn from the animals at various time intervals while they were still alive and these were analyzed by liquid scintillation as were various maternal tissues after the animals were sacrificed. Autoradiograms and routine hematoxylin and eosin slides were prepared from the embryos and placentas which were obtained by Caesarean section. The investigators found that the maternal plasma radioactivity reached a peak 40 minutes after the injection of the tritiated cortisone and that radioactivity could be found in the adrenal gland, liver, kidney, spleen, and thymus as well. Autoradiographic examination of the placentas and embryos revealed that labeling was present on the maternal side of the placenta and in the blood channels of the embryonic liver and heart 5½ hours after the injection was administered. Nasjleti (1969) was not able to find labeling in the embryonic palate or maxillary tissue.

Levine, Yaffe and Black (1968) compared the fetal uptake of radioactive cortisol in A/Jax (cleft palate susceptible) and C57 Black Mice (cleft palate nonsusceptible). Subcutaneous injections were made on day 11 of gestation with 2.5 mg. of cold carrier. The mice were sacrificed at intervals of 30, 60, 120, and 240 minutes.

Levine et al reported a similar rate of uptake of radioactivity in both strains but a significantly higher amount in the A/Jax fetuses. He

was able to show that the two strains had similar disappearance rates of the labeled cortisol and the concluded difference in susceptibility between the two strains was not due to variation in placental transport but due to differences in metabolism and/or binding by the fetuses.

Beitins, Kowarski, Shermeta, Delemos, and Migeon (1970) infused three pregnant ewes with C-cortisol while simultaneously infusing their fetuses with ^3H -cortisol in utero. The fetuses were infused by catheters through the common carotid artery, and a catheter was placed into the external jugular vein for withdrawing blood samples. The ewes had catheters for infusion placed in the external jugular vein while blood samples were drawn from the opposite jugular vein by venipunctures. After surgical placement of these catheters and the animal recovery, the double constant infusion of the radioactive cortisol began. Blood samples (maternal and fetal) were withdrawn every 15 minutes for three hours. The uptake of the fetal cortisol in the maternal blood was measured and corrected relative to the blood flow. A similar treatment of data from the fetal blood samples was made. Beitins et al reported a net transfer rate of the hormones across the placenta in both directions but of different magnitudes. There appeared to be a significant barrier for the movement of maternal cortisol to the fetus, thus allowing the fetus to maintain lower concentrations of cortisol for independent homeostatic regulation.

Marks (1969) reported a significant amount of label on autoradiographs in the fetal palatine processes on the twelfth day of gestation

in A/Jax mice. He injected 2.5 mg. of unlabeled cortisone acetate intramuscularly thirty minutes prior to a second injection of tritiated cortisol. The animals were sacrificed five hours after the second injection. Marks reasoned that the first injection of cortisone-acetate would overload the receptors for the metabolism of cortisone which would allow the tritiated cortisol to circulate freely and therefore increase its chances in finding its way to the fetus. Dodds (1971) found that this "loading dose" had just the opposite effect.

Marks, Schmitz (1970) also reported a significantly higher grain count in the palatal processes in fetuses taken from the left uterine horn than those from the right. Schmitz also reported a significant variation between placental and fetal tissues obtained from different mothers, explaining this variation by differences in maternal vascularity.

Dodds (1971) compared the uptake of tritiated cortisol when injected in A/Jax mice on the 12th day of gestation with a 2.5 mg. cortisone acetate "loading dose" and without a "loading dose". Dodds reported no significant variation in the different maternal tissues (except higher in the liver) and no variation in the various fetal tissues. The maternal tissues were significantly higher than the fetal tissues. He also found that the loading dose hinders rather than improves the uptake of label. A comparison of placentas from the right and left sides were reported to have no significant difference in labeling.

Waddell (1971), using wholebody autoradiograms, studied the distribution of ^{14}C -cortisone in pregnant A/Jax mice. He found little

variation in the autoradiograms between the different stages of gestation. He reported the highest concentration of radioactivity in the maternal bile, liver, intestinal contents, and the kidney. At all time intervals, the concentrations of radioactivity in the fetuses were lower than the pregnant female. The fetal tissues reportedly showed a rather even distribution with no greater amounts in the palatal buds. He did show accumulation of radioactivity surrounding the embryo in all gestation periods past twelve days. With thin-layer chromatography of the uterine fluid superimposed on x-ray film, he concluded that the yolk sac is the cause of the difference either from a metabolic or an absorptive standpoint.

In a comparative study between A/Jax mice and C57b1 mice (Reminga and Avery, 1972) the differential binding of ^{14}C -cortisone was examined. On day 12.5 of gestation, each pregnant mouse was administered 2.5 mg. of cortisone acetate intraperitoneally plus 5uCi of ^{14}C -cortisone. Thirty minutes after the injection, the maternal liver, fetuses, and placentas were removed for the study.

Spain (1973) using twenty-one pregnant A/Jax mice injected a solution of 50 uCi of ^3H -cortisol and 10 mg. of cortisone acetate on day 12 of gestation. Four animals were sacrificed at each of the following time intervals after injections: 0.5 hour, 1.0 hour, 1.5 hour, 2.0 hours, 3.0 hours and one animal at 6.0 hours. The maternal liver, adrenal, brain, placenta, yolk sac and the fetuses in three segments, torso, jaws, and the remainder of the head were removed for analysis. Each tissue was

analyzed for total tritium content and the percents of labeled cortisol and cortisol metabolites present. Oxidative combustion and liquid scintillation combined with thin-layer chromatography techniques were utilized. He was able to show that injected cortisol did reach the fetal tissues, and significant percents of cortisone and other cortisol metabolites could be identified from the 0.5 hour interval and remained significant through the 3.0 hour interval. His comparative results between fetuses, yolk sac, and the placenta indicate that both the yolk sac and placenta act as a quantitative "barrier" for the passage of certain corticoids to the embryo. He further found that fetal tissues do not significantly differ in the uptake of tritium label through 3.0 hours after injection and the fetal tissues do incorporate a significantly lower amount of total labeled corticoids than the maternal liver, adrenal, placenta, and yolk sac.

CHAPTER III

METHODS AND MATERIALS

A. Mice.

Segregated quantities of two strains of mice were obtained from Jackson Laboratory in Bar Harbor, Maine. A total of 60 female strain C57b1 and 10 female strain A/Jax were used in this study. These mice were obtained at the age of 12-15 weeks and used for approximately 7 months. Comparably aged males of the same strains were provided for mating.

B. Feeding and Environment.

An environmental control room in the animal care facility was used, where constant temperature and humidity was maintained. A day/night cycle was maintained by a clock mechanism which automatically turned the lights on at 6:00 a.m. and off at 6:00 p.m. Standard Purina Rat Chow and tap water were given ad libitum.

C. Mating.

Prior to mating of the mice, all females were placed in separate cages for conditioning to the environment, for one month. At the end of this period, mating commenced. One male was placed in a cage with a single female at 6:00 p.m. The female was then checked for the presence of a vaginal plug at 8:00 a.m. on the following morning. At this time the males were removed from the cage of the female and segregated until 6:00 p.m. when they would again be placed with the female for mating.

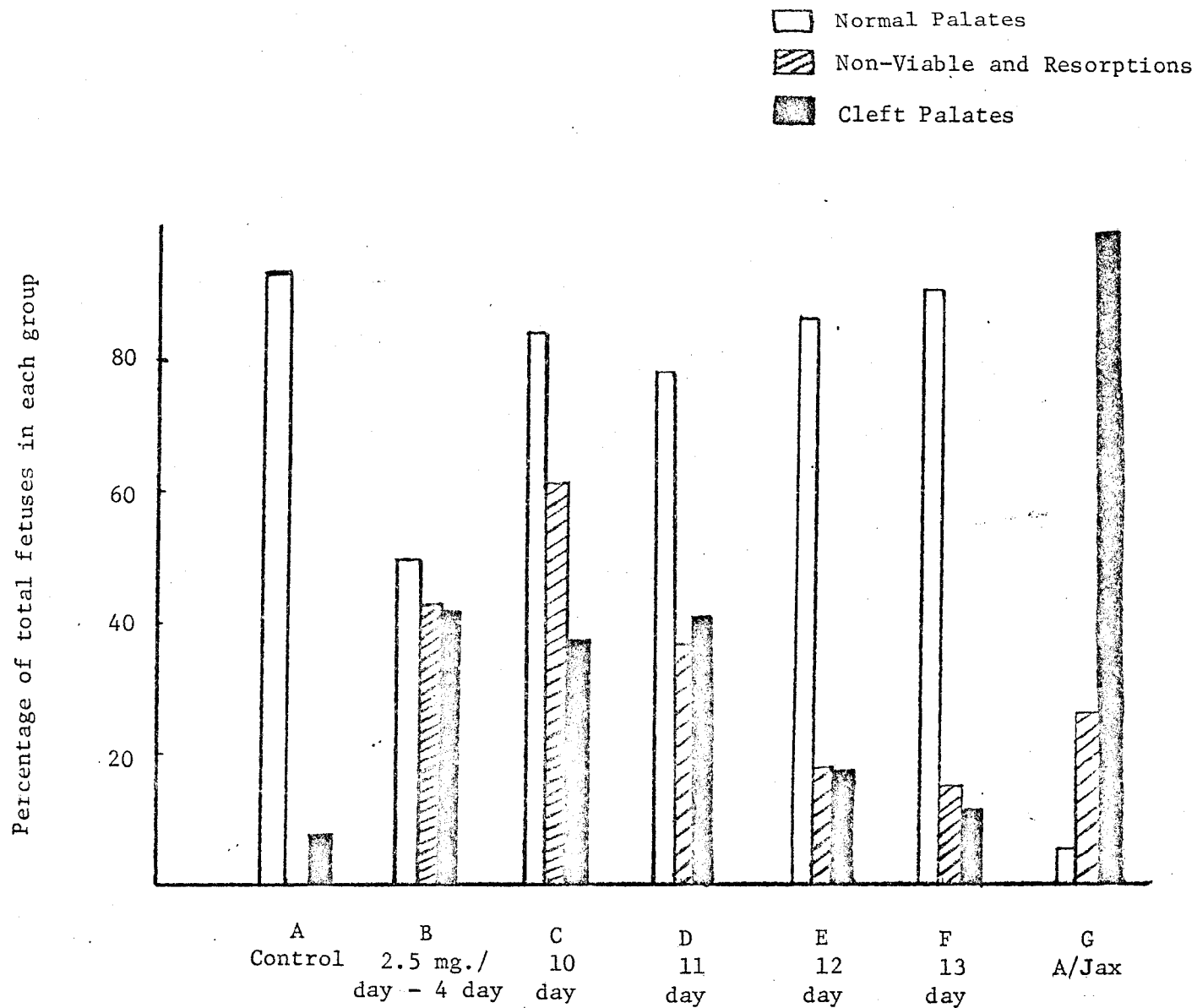


FIGURE 2

When a vaginal plug was found, the mouse was weighed and that day recorded as day zero. Since ovulation usually takes place shortly after midnight (Grunebery, 1952) and copulation any time afterwards, an unavoidable error of up to 8 hours must be realized. The mouse was again weighed at 8:00 a.m. on day 10. If a weight gain of two grams or more was measured, the mouse was considered pregnant and placed in one of seven groups which will be described.

D. Group Assignment.

Pregnant mice were assigned at random to each of the seven experimental groups. Group A consisted of two C57b1 mice which were weighed and given a 10 mg. injection of normal saline on the 12th day of gestation. This control group of pregnant mice was used to compare the effect of cortisone on fetal and maternal weights.

Group B consisted of ten C57b1 mice which were weighed and given 2.5 mg. of cortisone at 12:00 noon for a four day period commencing on day ten.

Group C through F consisted of ten C57b1 each which received a single 10 mg. injection of cortisone on days 10, 11, 12 or 13 of gestation respectively.

Group G consisted of ten A/Jax mice which were given a single dose of 10 mg. of cortisone on the 12th day of gestation.

E. Dissection and Inspection.

On the 18th day postconception, the animals were weighed and sacrificed by a sharp blow to the occiput followed by immediate decapitation.

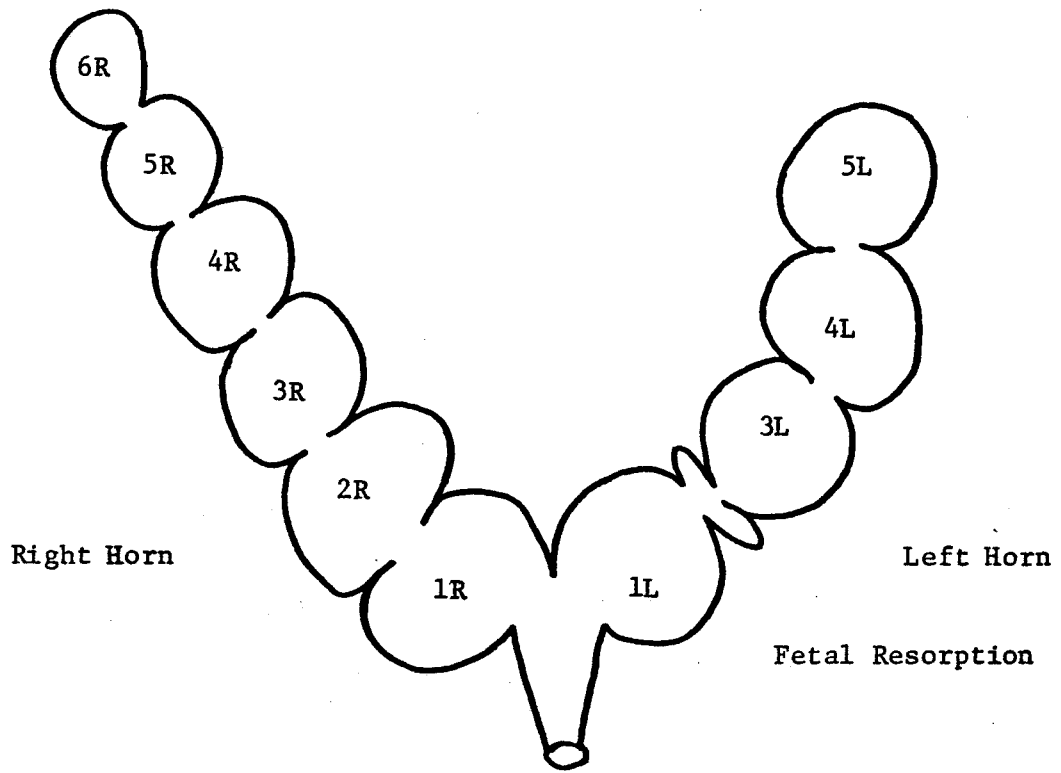
The fetuses were delivered through Caesarean section and the number and position of the fetuses were noted. They were then weighed and the viable, non-viable and resorbed fetuses were recorded. A gross microscopic dissection was then performed on all fetuses and all abnormalities were recorded. The palate was more closely examined and classified as normal or cleft.

The data on the frequency of cleft palate, cleft lip and palate, viable, non-viable fetuses and resorptions were analyzed by chi square tests. Fetal and maternal weights were analyzed by the T-test. In calculating the frequency of cleft palate fetuses the cleft lips were omitted as this latter abnormality occurs spontaneously in both strains of mice used.

F. Drugs and Route of Administration.

The drug used in this study was an aqueous solution of cortisone acetate manufactured by Upjohn and supplied in a multidose vial containing 25 mg./cc. A total of 0.4 cc. or 10 mg. was aspirated into a 1 cc. tuberculin syringe and administered to each test animal in Groups C through G. One half of the solution was injected intramuscularly into the hamstring muscle of each leg being careful to aspirate first and inject slowly. Group B received 0.1 cc. (2.5 mg.) total per day for four consecutive days. Group A was the control group and received on day 12 of gestation a 0.4 cc. (10 mg.) injection of sterile normal saline instead of cortisone acetate.

UTERUS



Fetal Positions in Uterine Cervix

FIGURE 3

CHAPTER IV

FINDINGS

Table 1 shows the test results of 66 mice injected with Cortisone Acetate, arranged by day of gestation and dose of the hormone. Figure 2 represents the graphical distribution of normal and cleft palates among the 425 fetuses.

Group G, consisting of A/Jax mice, shows the highest number of clefts.

In the C57b1 mice, Group B, (2.5 mg./4 days) was obviously more affected by the cortisone than were Groups A (control receiving 0.4 cc. normal saline), C (receiving 10 mg. on the 10th day of gestation), D (receiving 10 mg. on the 11th day of gestation), E (receiving 10 mg. on the 12th day of gestation), F (receiving 10 mg. on the 13th day of gestation). This was shown by the unusually high percentage of cleft palates produced in this resistant strain of mouse and also by the high number of resorptions and non-viable fetuses. Group C (10 day) and Group D (11 day) responded with 17 and 21% cleft production respectively to the effects of the drug although Group C exhibited a high percentage of resorptions.

Other malformations observed were cleft lip associated with cleft palate. Four instances of this combination was observed, one of these was seen in a fetus whose mother was in Group B (2.5 mg./day) and received four injections over a four day period commencing on day 10 of pregnancy.

This was a unilateral cleft palate and cleft lip. Three instances were observed in the A/Jax group (G) that was given a single dose of 10 mg. of cortisone. Two of these fetuses had unilateral cleft lip associated with a midline cleft palate and the other fetus demonstrated a bilateral cleft lip associated with a unilateral cleft palate.

One monster was observed in the day 13 group (Group E). This fetus showed fusion of the lips and lacked one eye and pinnae.

No attempt was made to find additional congenital malformations other than using gross microscopic inspection of the external fetus. It is pertinent to point out that cortisone injections during pregnancy of C57b1 resulted sometimes in maternal death a few days afterwards. More often, however, it produced early and late embryonic deaths within litters and of entire litters.

Maternal weights were recorded on the morning that the vaginal plug was observed and again at the time of drug administration once pregnancy had been established. A final maternal weight was recorded just prior to sacrificing. Final weights of all mothers were compared utilizing the T test to see if any significant differences could be shown between weights of control animals, maternal weights of treated litters with no cleft offspring and maternal weights of treated animals producing fetuses exhibiting cleft palates. Weight differences were determined to be nonsignificant ($P < .05$).

Fetal weights were also recorded as were uterine positions of defective offsprings and evaluated for statistical significance. It was

found that the weights of fetuses obtained from animals in the control group (A) were significantly higher than weights of fetuses obtained from non-cleft animals ($t = 10.76$, $P < .001$) and also significantly higher than the weights of fetuses obtained from mothers which showed cleft fetuses ($t = 11.79$, $P < .001$). No difference, however, could be shown between weights of fetuses obtained from treated animals showing clefts and treated animals producing cleft free litters. Uterine position was evaluated with respect to location of cleft or resorbed fetuses in order to determine if either the distal or proximal position was more susceptible to cleft frequency or that left uterine horn or the right was more vulnerable. Figure 3 illustrates how uterine positions were labeled. The various possible positions were statistically evaluated by chi square and found to be insignificant. Uterine position could not be correlated with either cleft formation or frequency of resorption.

Finally in order to determine if any statistical difference could be shown between the multi-injection technique and the single injection technique, the data was summarized and evaluated as to cleft versus non-cleft and viable versus non-viable and subjected to chi square analysis. The data is shown in Table 1. The results show that if the 10 mg. single injection technique is used on day 10 or 11 of pregnancy in the C57b1 strain of mouse that there is no significant difference when compared to the 2.5 mg./day multi-injection technique.

Mice which were administered cortisone on day twelve (Group E) were considerably different both in fewer clefts (chi square = 7.3) and

fewer resorptions (chi square = 11.4, $P < .001$) when compared to the 2.5 mg./day group. Resultant litters from Group F (day 13 injection) exhibited even greater differences with a chi square = 17.5 for clefts and a chi square = 20.4 for resorptions, ($P < .001$) when compared to the same group B, 2.5 mg./day.

CHAPTER V

DISCUSSION

A ten milligram injection of cortisone acetate had considerable teratogenic effect on the developing embryo of the C57b1 pregnant mouse. The effect of the drug administration was most profound when given approximately 48 hours before palate closure as previously noted by Chaudhry (1967). When a ten milligram dose was given on day eleven of gestation, the results consistently produced around 20% cleft palate in this previously defined resistant strain (Walker, 1957). The findings corroborate those of Fraser and his colleagues, who injected inbred strains of mice with daily doses of cortisone for a period of four days and demonstrated its capacity for inducing cleft palates in the young. Our experiments show that this four day regimen can be narrowed to a single day with a single dose and further that the response is dependent upon the timing and dose of cortisone given. There is no question that genetically determined susceptibility also influences expression of the acquired defect; but genetic susceptibility is shown to be one of multiple factors that contribute to the genesis of cleft palate in unborn mice. The data shown in Table 1 narrow markedly the apparent discrepancy in timing between the cortisone induced disturbances. Palates of embryos in the C57b1 strain of mice normally close about the 14th or 15th day as opposed to the 16th day for the A/Jax strain (Kalter, 1952). Thus it would be expected that this earlier maturing mouse would behave as it has

been shown and exhibit the teratogenic effects one to two days and earlier than its highly susceptible counterpart, the A/Jax mouse. This so called incubation period of 48 hours immediately precedes the critical period of accelerated differentiation and fusion when the embryo appears to be in a highly susceptible state and ready to depart from normal ontogenetic sequences of morphogenesis.

From the observed incidence of cleft palate, it is quite obvious that cortisone in a 10 mg. dose is equally as effective as a teratogenic agent when administered on day 10 or 11 of gestation. However this effect declines sharply when the drug is given on the 12th or 13th day of pregnancy. This study indicates a trend toward a higher percentage of clefts in groups subjected to the four day regimen of injections (2.5 mg./day) although no statistical difference could be shown between drug administration regimes. While animals were randomly selected for the other groups, apparently significantly younger and lighter mothers were used for multi-injection group B. This could probably account for the relatively higher percentage of clefts and resorptions in this group (Kalter, 1955).

The data further suggest that there is a significant reduction in fetal weights of animals borne by cortisone treated mothers. It was shown that not only are cleft fetuses significantly lighter than control but also non cleft fetuses of cortisone treated mothers are significantly lighter than the normal palated fetuses in the control group. From this fact we suggest that the cleft manifestation may partly stem from a more

general stress imposed on the maternal environment and not solely by direct action of cortisone against the affected fetus.

From the foregoing it can be seen that congenital malformations are but one manifestation of pathology inflicted by cortisone injections. Such experimental methods are capable of inducing more severe disturbances such as abortion, fetal resorption, or neonatal death. But while it is relatively easy to interrupt pregnancy by environmental interference, it is more difficult to obtain young with congenital malformations, since this requires an experimental arrangement that permits damaging the embryo without killing it. To achieve such balance, the teratogen, its dose, and timing, must be coordinated.

CHAPTER VI .

SUMMARY AND CONCLUSIONS

Cortisone acetate was administered as a teratogenic agent to 5 groups of C57b1 mice and one group of the A/Jax strain of mice. An additional C57b1 group served as a control. Each test group was injected on a single day of pregnancy except for one group which received a four day regimen of injections, equal in amount to that given the other groups on a single injection. These injections were given on the 10th, 11th, 12th and 13th day of gestation for the purpose of determining on which day the agent was most effective in producing cleft palate.

1. The eleventh day of pregnancy proved to be the most feasible from the standpoint of percentage of clefts produced, per viable fetuses and number of resorptions suffered.

2. It was determined that fetal weights of treated animals were significantly lighter than weights of control litters.

3. Maternal weights were determined to be unrelated to cleft formation.

4. It was shown that in the C57b1 strain, a single injection drug administration can adequately replace a multi-injection technique spread over a 4 day period if the single injection is done on the eleventh day.

5. Occurrences of malformation are unrelated to uterine position of the fetus.

The induced defect was always in the midline and not associated

with the cleft lip. The four observed occurrences of cleft palate with cleft lip was 1%, a finding in agreement with other investigators (Hackman and Brown, 1972). This defect resembled the inherited cleft which to the author's knowledge has never been produced to date by known teratological methods.

TABLE 1. Results of Cortisone Acetate Injection in 66 Pregnant Mice

<u>Group</u>	<u>Injection Day(s) of Gestation</u>	<u>Number of Litters Examined</u>	<u>Dose Administered</u>	<u>Number of Fetuses</u>	<u>Number Resorptions and Non-Viable Fetuses *</u>	<u>Number of Clefts **</u>	<u>Percentage of Viable Fetuses with Cleft Palate</u>
A	Control (12 day)	10	0.4 cc. Sterile Saline	55	4	0	0
B	10, 11, 12, 13	10	10 mg. (2.5 mg./day) (0.1 cc.)	59	27	14	44%
C	10th day	10	10 mg. (0.4 cc.)	65	36	5	17%
D	11th day	10	10 mg. (0.4 cc.)	57	10	10	21%
E	12th day	10	10 mg. (0.4 cc.)	59	10	8	16%
F	13th day	10	10 mg. (0.4 cc.)	77	12	7	11%
G	A/Jax 12th day	6	10 mg. (0.4 cc.)	53	15	36	98%

* Significant chi square comparisons between groups for non-viable fetuses (P 0.05)

A vs. B, A vs. C, A vs. G, B vs. D, B vs. E, B vs. F, C vs. D, C vs. E, C vs. F, C vs. G.

** Significant chi square comparisons between groups for clefts (P .05)

A vs. B, A vs. C, A vs. D, A vs. E, A vs. F, A vs. G, B vs. E, B vs. F, B vs. G, C vs. G, D vs. G, E vs. G, F vs. G.

CHAPTER VII

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APPROVAL SHEET

The thesis submitted by Donald DeWayne Karich has been read and approved by three members of the faculty of the graduate school.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

5-17-74

Date

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