1969

A Preliminary Study to Determine the Feasability of the Use of Tritiated Cortisol for Autoradiographic Examination of A/Jax Mouse Fetuses

Allen D. Marks
Loyola University Chicago

A PRELIMINARY STUDY TO DETERMINE THE FEASABILITY OF THE USE OF TRITIATED CORTISOL FOR AUTORADIOGRAPHIC EXAMINATION OF A/JAX MOUSE FETUSES

BY

ALLEN D. MARKS

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

JUNE
1969

BIOGRAPHY

Allen D. Marks was born in Chicago, Illinois on October 29, 1941. He was graduated from the Nicholas Senn High School in January, 1959 and entered the undergraduate division of the University of Illinois at Navy Pier in Chicago in February, 1959.

Following completion of his pre-dental education in June, 1961, he entered the University of Illinois College of Dentistry from which he received his B.S. and D.D.S. degrees in June, 1965.

In July, 1965, Dr. Marks entered the United States Army and served as a dental officer in the Dependent Dental Clinic at Fort Knox, Kentucky from August, 1965 to July 1966. From August, 1966 to June, 1967 he served as a division dentist for the 25th Infantry Division in the Republic of Viet Nam.

In June, 1967 Dr. Marks enrolled as a graduate student in the Department of Oral Biology at the Loyola University School of Dentistry in Chicago and received his Master of Science degree from the University in June, 1969.

Dr. Marks is married to the former Tamara Susan Harris of Chicago.

ACKNOWLEDGEMENTS

I would like to thank the following people who made the preparation of this thesis possible:

Dr. Norman K. Wood, my thesis advisor, for his untiring help and guidance with all stages of preparation of this thesis.

Dr. Patrick D. Toto, who so generously took time from his very busy schedule to give me assistance the many times I required it.

Dr. Donald C. Hilgers, who first gave me the idea for this research.

Dr. Harry Sicher, for his help with the dissection and preparation of the embryoes for histologic and autoradiographic examination and with the final preparation of the thesis itself.

Dr. Douglas C. Bowman for his help with the statistical analysis and interpretation of the data collected during the course of this research.

Dr. Gustavus W. Rapp, for his kindness to me throughout my stay here at the Graduate School.

To my wife Tamara, whose inspiration during the course of my graduate education has been invaluable.

Last, but not least, my parents Harry and Sylvia, who taught me to value education and seek as much of it as I could, and who helped me to weather the difficult periods throughout my education.

LIST OF ILLUSTRATIONS

LIST OF TABLES

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION AND STATEMENT OF PROBLEM

Cleft palate is a congenital anomaly ocurring once every 700 to 800 births in humans. Within the last twenty years, there has been increased investigation of cleft palate embryology. As a result of this research, cleft formation is generally attributed to a disturbance of the normal synchronous growth of the naso-oral complex which leads to a failure of the normal union of the palatine shelves.

One of the most common methods of studying cleft palate embryology involves the use of mice embryoes in which cleft palates are induced by the injection of cortisone into pregnant female mice during the critical period of embryonic palate formation. There exists, however, a great deal of controversy as to the manner in which the cortisone acts to induce the malformation, and whether it acts directly on the palatal shelves, at a site in the fetus other than the palate, or on the maternal physiology, and thus indirectly on the palate.

The purpose of this investigation is (1) to determine if a radioactive label can be detected autoradiographically in the placenta and fetal maxillary process--palatal shelf tissues after injection of tritiated cortisol into the pregnant female Ajax mouse and (2) to attempt to establish parameters for optimum labelling.

This investigation is the preliminary phase of a larger study the ultimate aim of which is to determine whether cortisone injected into a pregnant female mouse acts directly on the palatal shelf tissue of the fetal mouse during the critical period of palate formation.

CHAPTER II

REVIEW OF THE LITERATURE

I. Normal Development of the Palate

Polzl (1904) theorized that the palatal shelves achieve horizontalization by regression of their ventral surfaces with simultaneous new growth from their medial surfaces at approximately the level of the tongue. In 1937, this concept was reaffirmed by Pons-Tortella.

Peter (1924) put forth the idea that horizontalization occurred as a result of medial rotation of the ventrally directed palatal processes, while Lazarro (1940) advanced the notion that such rotation was due to a rapid increase of intercellular substance within the palatine processes with a concomitant removal of the tongue from the plane of horizontalization due to local differential growth changes or actual muscular movement of the tongue.

Reed (1933) felt that the palatal processes horizontalize by differential growth or muscular contraction and grow medially until they contact each other and fuse.

In 1954, Walker stated that the literature then available dealing with the formation of the mammalian secondary palate was reliable except for the stage of closure from the vertical to the horizontal position and then went on to describe seven stages of closure, beginning with the tongue lying between the

vertically positioned palatine shelves and ending with the antero-posterior fusion of the medial portions of the shelves. Walker further proposed that the tongue played no active part in palate formation, since histologic sections had been viewed in which one shelf was horizontal and one shelf was vertical, even though the tongue had not dropped to allow horizontalization.

Walker found that the time required for shelf movement is three hours and the time for fusion six hours in an undisturbed embryo. By experimentally inducing shelf movement, Walker showed that the position of the shelves can change within a minute if the tongue is manually displaced, and hence he feels that the resistance of the tongue accounts for the amount of time needed for shelf movement.

In 1958, Walker and Fraser found that the palatal shelf connective tissue exhibited both metachromasia and an affinity for aldehyde-fuchsin and formulated the idea that shelf movement could be due either to the presence of hyaluronic acid acting as a water binder, and thus regulating tissue turgor, or to the tensions caused by a developing network of elastic fibers.

By 1960-61, however, Walker had discarded both these theories in favor of the idea that an increase in the synthesis of sulfated acid mucopolysaccharides causes a buildup of palatal shelf force.

Larsson (1961) proposed that chondroitin sulfuric acid produced by the mesenchymal cells in the shelf connective tissue was responsible for the internal force due to its ability to change its state of aggregation and water-binding capacity. Larsson further agreed with Walker that the internal force is strong enough to cause the palatine shelves to bulge over the tongue, rather than wait until the tongue moves out of their way.

II. Cortisone-induced Cleft Palate

Isolated cleft palate (cleft palate in which there is no involvement of the alveolar process of the maxilla) was first induced experimentally by Baxter and Fraser (1950) by the injection of cortisone into two strains of mice. Spontaneous clefts were not found to occur in either strain.

Fraser and Fainstat (1951) showed that the A/jax strain was the most susceptible to cleft induction, and that the highest incidence of clefts was noted when the cortisone injections were initiated on the tenth or eleventh day of gestation.

Fraser et al (1954) and Isaacson (1962) were able to obtain a one hundred per cent incidence of cleft palate in A/jax fetuses when pregnant females were administered 2.5 mg of cortisone for four consecutive days beginning on the tenth or eleventh day of pregnancy. Ingalls and Curley (1957), however, used other doses of hydrocortisone injected at 11½ days of gestation to produce cleft palates in mice, but found that more fetal resorptions occurred when a dose of 10 mg was injected

as a single dose.

It was proposed in 1954 by Fraser et al that two different mechanisms may be operational in the causation of induced clefts: one which causes a delay in the rotation of the palatal shelves before palatal closure, and one which causes a breakdown in palatal tissues after they have fused.

Walker (1954) felt that the primary cause of induced clefts was a delay in the average time and rate at which shelf movement occurred, and cites as evidence the fact that he observed numerous specimens of embryoes in which one palatal shelf had horizontalized while the other remained vertical, a finding which he stated was not often encountered in untreated embryoes. He further 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, although he felt that fusion of the shelves is necessary to promote growth of the shelves beyond the size prior to closure.

Walker (1954) suggested that if cortisone acted upon the shelves directly, there would be a decrease in the force available to cause shelf movement and that 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. fibers, mucopolysaccharides

Larsson (1961-62) demonstrated that cortisone injection lowers the amount of sulfate incorporated into the mouse embryo acid mucopolysaccharides and upheld the idea of Walker and Fraser (1956) that shelves in cleft animals are retarded in horizontalization and that cortisone inhibits the formation of substances necessary to produce the force for horizontalization - i.e. acid mucopolysaccharides.

In 1957, Kalter enumerated some of the factors which determine the frequency of appearance of animals with clefts in a cortisone-treated litter. Among these were: dose of cortisone, gestation time, strain of mouse, maternal genotype, fetal genotype, maternal weight, and fetal weight. Kalter also showed that fetuses from cortisone-treated litters tended to be completely resorbed much more often than untreated 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.

Loevy (1962) felt that it was unlikely that the tongue plays an active role in cleft formation, and that horizontalization was not a major consideration. She further felt that the role of the connective tissue in cleft development has been overemphasized. Her findings were based upon an investigation in which she injected 1.25 mg. of cortisone daily from the 11th to the 14th day in Strong 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 contiguous and ready to fuse.

III. Cortisone Action

Although some investigation has been done, the exact manner in which cortisone acts to induce the formation of cleft palate has yet to be elucidated. It is not known whether cortisone acts directly on the palatine shelves or causes cleft formation through some intermediate action elsewhere in the fetus or mother.

According to Krantz and Carr (1961), cortisone is a glucocorticoid which 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. They state that it also elicits a mild hyperglycemia and an improvement in overall capillary tone, resulting in diminished exudation of plasma into the tissues.

Cortisone per se is not found in adrenal venous blood or peripheral blood and is probably a precursor for hydrocortisone (cortisol) which is found in the peripheral circulation. It is metabolized mainly in the liver.

Hydrocortisone exhibits an anti-inflammatory action possibly owing to its ability to suppress the cell's activity in producing the chemical agents responsible for inflammation; Krantz and Carr (1961).

Goodman and Gilman (1965) state that in addition to causing a lymphocytopenia, cortisone also is responsible for a decrease

in lymphoid tissue mass and an increase in the number of red blood cells (polycytemia). Furthermore, cortisone aids in the regulation of fat, protein, and carbohydrate metabolism.

Dougherty et al (1961) have stated that the anti-inflammatory action of corticosteroids is a focal one and does not depend upon metabolites created at distant sites. They feel further that the influences which cortisol exerts on connective tissue are primarily due to modifications of fibroblast function, since the fibroblast (sic) is the cell responsible for the majority of structures present in connective tissue - i.e. ground substance matrix and fibers. Cortisone inhibits fibroblastic proliferation, decreases collagen deposition, and decreases the secretion of polysaccharides.

Dougherty further states that in tissue culture cortisol causes a pulling in of the processes of the fibroblasts resulting in an epithelioid-appearing cell. Such cells also develop numerous vacuoles filled with reducing substances as shown by various biochemical reactions. These epithelioid fibroblasts have been shown cinemicrographically to be very quiet in their movement. 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.

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.

Bullough (1932) and Sayeed et al (1962) suggested that cortisone can suppress mitosis. In another investigation it was found that the effect of cortisone on mitosis varies from one type of tissue to another, and that it does not seem likely that one of cortisone's fundamental actions is to suppress mitosis. Roberts et al (1952) and Mott (1968), however, concluded that cortisone affected the palatine shelves of embryonic A/jax mice by a suppression of mitosis, thereby causing a deficiency in the number of cells with a subsequent decrease in the amount of intercellular substance.

Layton (1951) felt that high doses of cortisone inhibit the synthesis of chondroitin sulfate in connective tissue. Cavallero and Braccini (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 feels 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

embryoes and that this edema is most striking during the critical period of palate closure. Jacobs attributes this edema to a change in the sulfated mucopolysaccharides.

Paff and Stewart (1953) noted a reduction in the number of mast cells with cortisone treatment, while Birke (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 (oligohydramnios) of the mouse embryo at the time of palatal closure, but the amount of the decrease is exactly the same in normal embryos as in embryoes with a cleft. He therefore concluded that the amniotic fluid reduction cannot be the factor that determines which embryoes develop cleft palate after cortisone treatment. Fraser and his cohorts 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.

Karppala 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 labelled 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-labelled 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 counterstained with hematoxylin and eosin were prepared from the embryoes 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 embryoes revealed that labelling 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 was not able to find labelling in the embryonic palate or maxillary tissue; (private communication)

IV. Autoradiography and Tritium-labelling

Radioisotopes may be localized to a given area in a histologic section, or even to individual cells. The tissue to be studied is placed subjacent to a photographic emulsion for an adequate time to allow exposure of the emulsion which then is developed as in ordinary photography. The processed emulsion is referred to as an autoradiogram and consists of accumulations of black granules which overlie the areas in the tissue which contain the radioisotope. The rapidly moving charged particles emitted from the radioactive material cause ionization of the silver bromide crystals contained in the photographic emulsion, and it is this ionization which enables us to record the presence of the radioisotope; Fitzgerald (1963), Schoenheider (1960).

Both the chemical and biological behavior of a substance labelled with a radioisotope are identical to that of its stable counterpart provided that the amount of radioactivity contained in it is small enough not to have a significant radiochemical effect and the amount of material injected is small enough not to produce a significant increase in the amount of substance in circulation. The labelled substance can then be considered a true "tracer" of normal in vivo metabolism; Schoenheider (1960).

Radioactive tritium is an excellent substance to use as a label because its beta radiation has a very weak energy and, therefore, a very short range. A beta ray will travel a maximum distance of six microns in tissue and half of the particles will travel less than one micron. Consequently, the majority of the activated silver grains in an autoradiogram should lie within one micron of their source within the tissue. Hughes et al (1958)

It can readily be seen from the foregoing that the use of tritium as a tracer substabce to make autoradiograms is a useful adjunct in attempting to determine the presence of a given chemical substance or its metabolites in histologic tissue sections.

CHAPTER III

MATERIALS AND METHODS

Mice:

Thirty A/jax mice (20 female, 10 male) were obtained from the Jackson Laboratory in Bar Harbor, Maine when they were fifteen weeks old. This strain was chosen because of their previous use in studies of cortisone-induced cleft palate.

Mating:

During the period that the mice were being mated, males and females were placed together in cages overnight and the females checked for vaginal copulation plugs the following morning between 8:00 and 11:00 a.m. Females demonstrating copulation plugs were isolated. The date was marked on the isolation cage and designated as day zero of the pregnancy. The animal's weight on day zero was also recorded. The remaining animals were then segregated according to sex until the evening of the same day at which time they were again integrated and allowed to mate. In the beginning the breeding ratio was 1 male for every 3 females, but this ratio became larger as females suspected of being pregnant were removed and placed in isolation.

Cortisone Injections:

On the twelfth day of pregnancy as estimated from the vaginal plug, each female suspected of being pregnant was again weighed. If a weight gain of two or more grams was noted, the assumption was made that the animal was pregnant and suitable

for further use in the experiment. Although this weight gain method was not found to be infallible for determining pregnancy, it did turn out to be quite reliable. Unfortunately, no figures were kept on its degree of accuracy as this was not the primary purpose of the study.

Cortisone acetate S.A.S. (sterile aqueous solution) was obtained from the Upjohn Company in 20 cc. vials containing 25 mg. of cortisone acetate per cc. of solution. All pregnant animals were given a 2.5 mg. (0.1 cc.) loading dose of this solution intramuscularly in the hamstring muscles of the left hind leg on the twelfth day of pregnancy followed 30 minutes later by an injection of a labelling dose of cortisol (hydrocortisone) - 1, 2-tritium. The tritiated cortisol was purchased from the Nuclear Chicago Corporation in 1 ml. vials containing 1 millicurie of radioactivity per milliliter of 50:50 benzene: ethanol solution. A total of nine animals were used, one of them receiving 500 microcuries of radiocortisol in the right hamstring muscles, one receiving 500 microcuries in the subcutaneous tissue of the back, the remaining seven each receiving 50 microcuries in the right hamstring muscles.

All radiocortisol injections were preceded 30 minutes by the 2.5 mg. injections of cortisone acetate because it was assumed necessary to overload the pregnant female mouse's normal cortisone metabolism lest the animal metabolize the minute amount of cortisol which would be present in the tritiated

dose, rendering it useless as a tracer material for fetal cortisone distribution. Nasjleti (1967) reported that the maternal plasma radioactivity level reaches a peak 40 minutes after tracer injection. It was therefore felt that thirty minutes would be enough time for sufficient absorption of cortisone to overload the maternal system of cortisone catabolism. Thus the subsequent injection of tritiated cortisone would not be catabolized and could pass into the fetal circulation in adequate levels for labelling, if, indeed, cortisone is able to cross the placental barrier.

One animal receiving 500 microcuries of radiocortisol was sacrificed by decapitation 2 hours after the tracer material was injected. All others were sacrificed five hours after tracer injection. Nasjleti (1967) has reported that tritiated cortisone injected into a pregnant female mouse was visible autoradiographically 5½ hours later in the blood vessels of the embryonic liver and heart.

Following the sacrifice of the mothers, the uteri were removed with the embryonic sacs intact and allowed to fix for 48 hours in a solution of ten percent neutral formalin. Each embryo and its placenta were then dissected from the uterus. Records were kept of whether the embryo was removed from the right or left uterine horn and what position it had in relation to the cervix for possible use later in determining whether position in the uterus had any effect on the labelling of the embryo with tritiated cortisol.

Histologic Preparation and Autoradiographic Technique:

The embryo heads were detached from the bodies under a binocular dissecting microscope, embedded in paraffin, and sectioned at 5 microns as were the placentas. Each tenth slide was stained with hematoxylin and eosin for cellular and area identification. The staining procedures used were those described by McManus and Mowry (1960).

The autoradiogrpahic technique used was modified from that described by Fitzgerald (1961).

1. Under darkroom conditions, a Kratten #1, Red Safelite 10 watt bulb was used. A humidity of 70% and below was maintained.

2. The slides were dipped in Kodak NTB3 liquid emulsion.

3. The slides were air-dried for 10 minutes.

4. Ten slides were placed in a black lightproof exposure box section sides up. Lithium chloride was placed in the box for maintaining a low humidity, and black masking tape was used to seal the box.

5. The box was exposed for 5 weeks at low humidity and temperature. During the exposure time the box must be maintained in a position which keeps the sections upright.

6. After 5 weeks, the slides were placed in a staining rack and developed for 5 minutes at 60°F. (18°C.) in Kodak D196 developer.

7. The slides were rinsed in distilled water for 30 seconds and then placed in acid fixer for 10 minutes.

8. The slides were rinsed in running tap water for 30 minutes.

9. The slides and staining dish were covered with tissue paper to prevent dust from settling on them and allowed to dry in a stream of air.

The processed autoradiograms were then stained with nuclear fast red and counterstained with indigo carmine.

Grain Counts:

The autoradiograms were examined microscopically and grain counts made of areas on the slide overlying tissue sections and areas with no underlying tissue sections (to determine background radiation effects). If the area with subjacent tissue sections had significantly more grains than the area containing no tissue section, the difference in the number of grains was attributed to the presence of tritium-labelled cortisol or a labelled metabolite of the labelled cortisol.

CHAPTER IV

FINDINGS

Figure 1A demonstrates a low power autoradiographic section of placenta for area identification, while figure 1B illustrates an autoradiogram of the same area with exposed black silver grains in the emulsion clearly visible.

Figure 2A shows a low power section of the fetal head with the maxillary processes well defined. Because of the extremely small size of the 12½ day old A/jax mouse fetal head, it was not always possible to obtain a perfect coronal section with readily defined palatal shelves. In sections where the palatal shelves were not available, grain counts were made in the maxillary processes rather than in the palatal shelves proper. Figure 2B is an autoradiogram of such an area, showing exposed black silver grains overlying the tissue.

Figure 3 is a diagrammatic illustration showing the manner in which fetal positions in the uteri were recorded. For fetuses in succeeding uteri, the next numbers were given in numerical order. All fetuses were numbered from left to right. For example, if the last fetus in the right uterine horn of a given uterus was designated number nine, the first fetus in the left uterine horn of the next uterus would be labelled number ten.

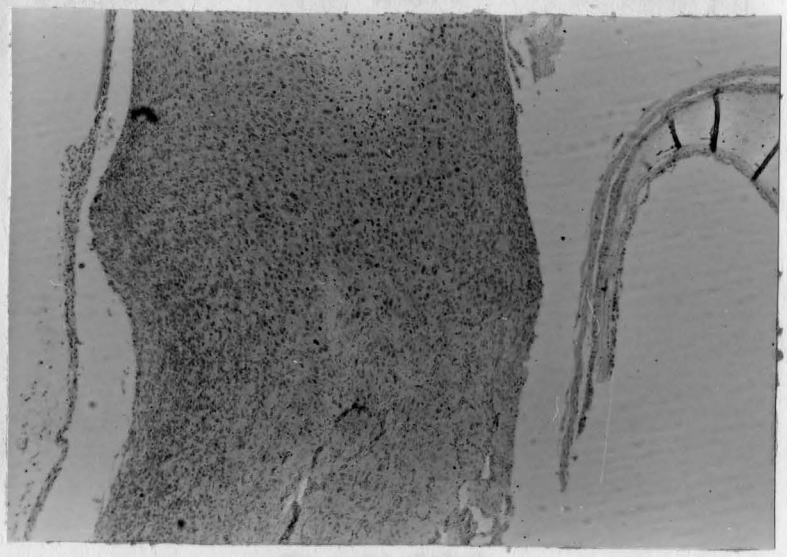


Fig. 1A (40X)

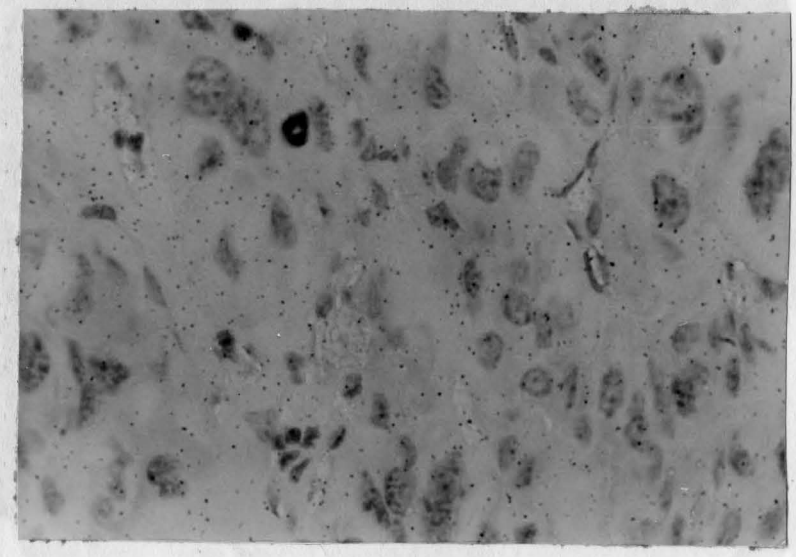


Fig 1B (400X)

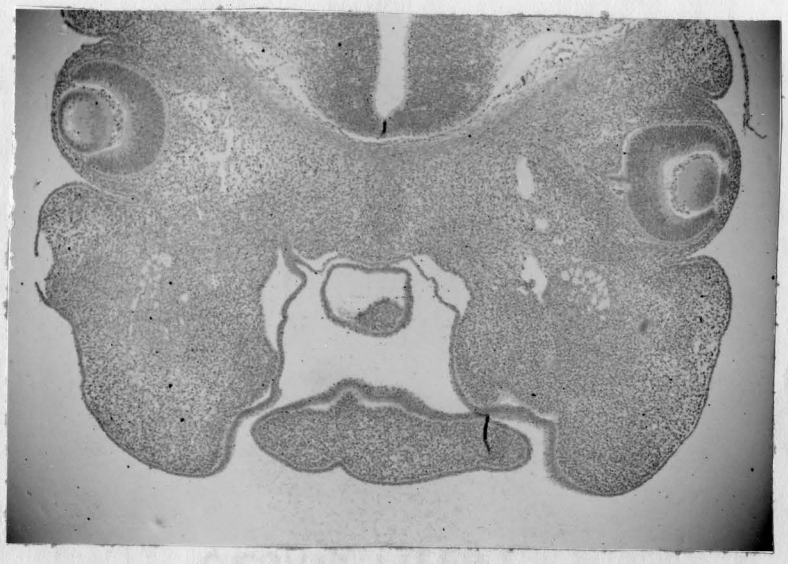


Fig. 2A (40X)

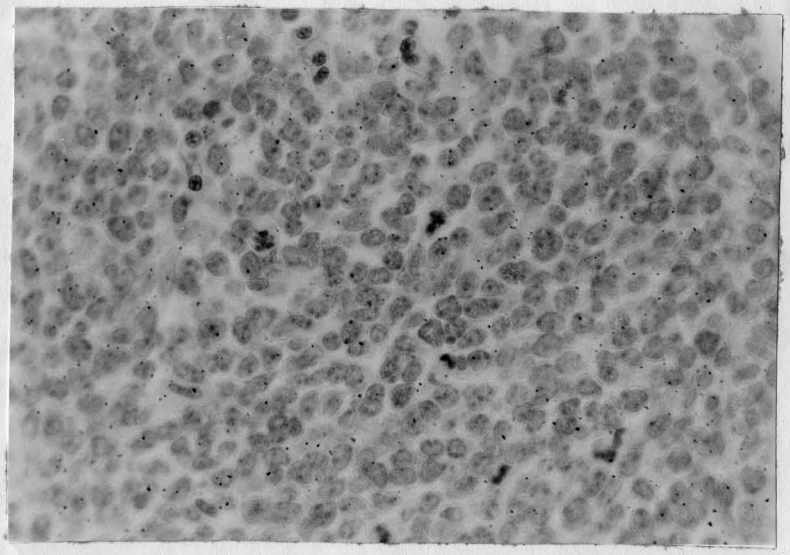
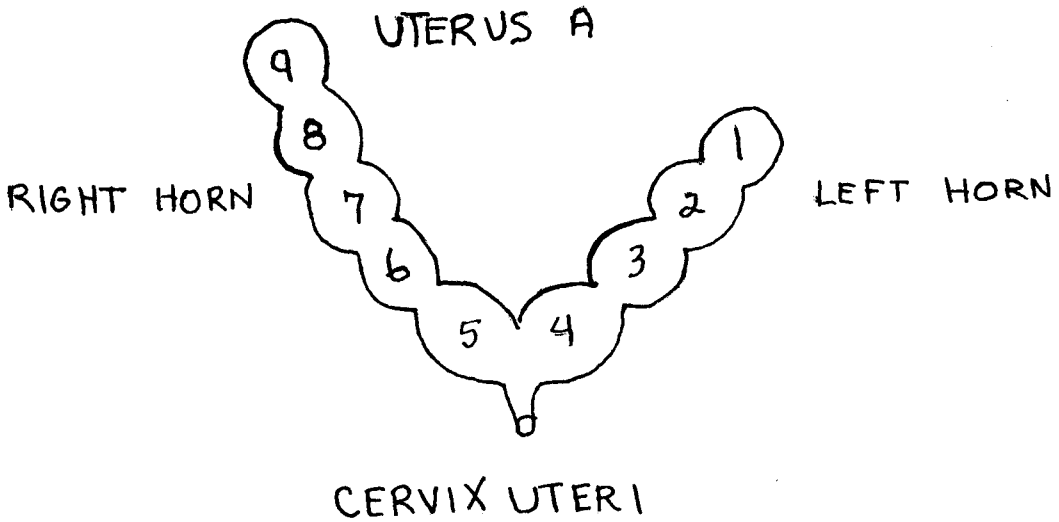


Fig. 2B (400X)


UTERUS A
9
8
7
6
5
4
3
2
1
RIGHT HORN
LEFT HORN
CERVIX UTERI

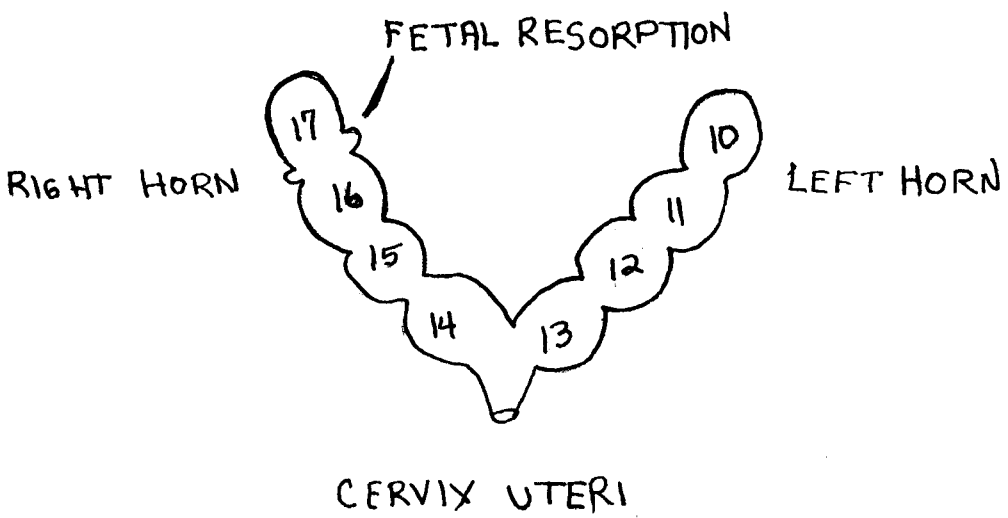

UTERUS B
FETAL RESORPTION
17
16
15
14
13
12
11
10
RIGHT HORN
LEFT HORN
CERVIX UTERI

Fig. 3

TABLE I

Statistical Summary of Tissue vs. Background Grain Counts

for Fetal Maxillary Process - Palatal Shelf Areas

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice Time	Mean Grain Count ± Standard Deviation (N=10) Tissue	Background	T - Value
2	500 uc - IM - 2 Hr.	37.1 ± 9.35	11.1 ± 2.95	31.378 ***
4	500 uc - IM - 2 Hr.	44.0 ± 8.00	7.3 ± 4.22	12.173 ***
5	500 uc - IM - 2 Hr.	46.9 ±14.21	9.3 ± 5.18	16.118 ***
9	500 uc-Subcu.-5 Hr.	44.2 ± 9.77	7.1 ± 2.16	11.116 ***
22	50 uc - IM - 5 Hr.	3.5 ± 2.73	7.0 ± 6.30	1.529 --
27	50 uc - IM - 5 Hr.	2.2 ± 3.71	0.7 ± 0.93	1.177 --
28	50 uc - IM - 5 Hr.	0.5 ± 0.67	0.6 ± 1.02	0.246 --
34	50 uc - IM - 5 Hr.	2.2 ± 2.65	2.2 ± 1.66	0.096 --
38	50 uc - IM - 5 Hr.	4.8 ± 2.37	1.5 ± 2.20	3.060 **
41	50 uc - IM - 5 Hr.	1.8 ± 2.32	2.6 ± 2.49	0.706 --
46	50 uc - IM - 5 Hr.	0.7 ± 1.27	3.7 ± 4.12	2.086 --
52	50 uc - IM - 5 Hr.	9.1 ± 3.08	5.9 ± 4.20	1.841 --
60	50 uc - IM - 5 Hr.	8.1 ± 4.20	7.2 ± 3.37	0.501 --
61	50 uc - IM - 5 Hr.	6.5 ± 3.38	2.5 ± 3.64	2.415 *
67	50 uc - IM - 5 Hr.	13.4 ± 3.70	4.9 ± 2.84	3.221 **

-- = No significant difference between tissue and background count.

* = $.05 > p > .02$

** = $.0 > p > .001$

*** = $p < .001$

TABLE II

Statistical Summary of Tissue vs. Background Grain Counts for Placenta

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice Time	Tissue	Background	T-Value
2	500 uc - IM - 2 Hr.,	41.3 ± 7.37	5.1 ± 4.54	12.532 ***
4	500 uc - IM - 2 Hr.	75.6 ± 18.46	11.4 ± 4.47	10.150 ***
5	500 uc - IM - 2 Hr.	35.4 ± 9.53	5.4 ± 2.58	9.116 ***
9	500 uc - subcu - 5 Hr.	40.8 ± 14.01	10.0 ± 5.21	6.16 ***
22	50 uc - IM - 5 Hr.	0.5 ± 0.92	0.1 ± 0.3	0.3915 --
27	50 uc - IM - 5 Hr.	0.0 ± 0.0	0.1 ± 0.3	1.000 --
34	50 uc - IM - 5 Hr.	4.1 ± 2.93	1.0 ± 1.67	2.720 *
38	50 uc - IM - 5 Hr.	13.9 ± 3.91	1.2 ± 1.54	9.069 ***
41	50 uc - IM - 5 Hr.	8.9 ± 4.25	7.9 ± 4.87	0.464 --
46	50 uc - IM - 5 Hr.	13.0 ± 3.22	3.0 ± 3.66	6.150 ***
52	50 uc - IM - 5 Hr.	10.7 ± 2.19	4.9 ± 3.67	4.068 ***
60	50 uc - IM - 5 Hr.	17.5 ± 3.77	5.2 ± 4.45	6.327 ***
61	50 uc - IM - 5 Hr.	10.8 ± 4.21	4.9 ± 2.70	3.537 **
67	50 uc - IM - 5 Hr.	13.8 ± 4.04	7.2 ± 2.35	4.230 ***

-- = No significant difference between tissue and background count.

* = $.02 > p > .01$

** = $.01 > p > .001$

*** = $p < .001$

Tables I and II are statistical summaries of the tissue vs. background grain counts for the fetal heads and placentas respectively. The "student's t-test" was used to determine if there was a significant difference in numbers of grains counted for the tissue and for the background for a given slide specimen. A p - value greater than 0.05 was not condered to be statistically significant.

All head and placenta specimens studied from mothers which had received doses of 500 microcuries of tritiated cortisol were shown to have highly significant differences in tissue and background counts ($p < .001$) regardless of the route of administration or sacrifice time.

Of the eleven head specimens from 50 uc mothers in which grain counts were made, only three specimens were shown to have statistically significant differences in grain counts ($p < .05$).

Of the ten placenta specimens from 50 uc mothers in which grain counts were made, seven specimens were shown to have statistically significant differences in grain counts ($p < .02$). Of these seven, five had highly significant differences ($p < .001$).

CHAPTER V

DISCUSSION

In 1967, Nasjleti et al found that tritiated cortisol, injected into the pregnant female A/jax mouse in dosages previously reported to cause isolated cleft palate in the fetus, would cause labelling of the placenta and the blood channels of the embryonic liver and heart. Nasjleti and his cohorts, however, were unable to find radioactive labelling in the fetal maxillary process - palatal shelf region; (personal communication).

The present investigation showed that a dose of 500 microcuries of tritiated cortisol injected into the pregnant female A/jax mouse did cause labelling of the placenta and fetal maxillary process - palatal shelf region ($p < .001$). In pregnant animals injected with 50 uc doses of tritiated cortisol, seven out of ten placentas were shown to have mean grain counts for the tissue which were significantly greater than background grain counts ($p < .02$). Five of these seven placentas showed highly significant differences ($p < .001$). The mean grain counts in placental tissue (Table II) were higher, however, in 500 uc animals than in 50 uc animals, indicating that 500 uc is probably a more reliable labelling dose for the placental tissue.

A 50 uc dose of tritiated cortisol was found to cause significant labelling in only three out of eleven fetal heads examined (in one of the three, $p < .05$, in two $p < .01$). It

would, therefore, seem that 50 microcuries is not as reliable a labelling dose for the fetal head maxillary process - palatal shelf as it is for the placental tissues.

Other observations made during the course of this investigation also favor the use of a 500 uc dose rather than a 50 uc dose in order to get consistently good labelling of the areas studied. Three of the four 500 uc embryoes were sacrificed two hours after the injection of the tritiated cortisol, the fourth, five hours afterward. All these embryoes' mothers showed signs of marked physiologic impairment and became almost comatose shortly after receiving the tritiated cortisol injection. As a result of this observation, a study was undertaken in which it was shown that injection of 0.5 cc. of 50:50 benzene:ethanol solution or 0.25 cc. of ethanol alone would cause a physiologic impairment of the animals which appeared upon gross inspection to be identical to the depression caused by the injection of the 0.5 cc. of 50:50 benzene:ethanol solution containing the 500 uc dose of tritiated cortiso. The depression in the mothers of animals used for labelling studies was therefore attributed to the benzene-ethanol vehicle of the tritiated cortisol.

None of the nine animals receiving 50 uc doses of tritiated cortisol exhibited any marked physiologic impairment, and all nine of them were sacrificed five hours after injection of the labelling material. A 0.05 cc. dose of 50:50 benzene:ethanol solution containing 50 uc of tritiated cortisol

apparently is not large enough to cause marked depression of the test animals by virtue of its toxicity.

Despite the fact that the animals receiving 50 uc doses appeared to be in much better physiologic condition and survived longer (five vs. two hours) than their 500 uc counterparts, labelling was still better in the animals receiving 500 uc doses. 500 microcuries was, therefore, considered to be superior to 50 uc for labelling the placental and fetal tissues.

Further studies would be useful to determine whether some intermediate dose between 50 uc and 500 uc can be used for reliable labelling. Mention must be made here of the fact that the animal receiving its 500 uc dose of tritiated cortisol subcutaneously showed mean grain counts (Table I and II) for placenta and fetal head labelling which were of the same magnitude as those 500 uc animals receiving intramuscular injections. It is possible, therefore, that tritiated cortisol can be administered subcutaneously to attain the same degree of labelling in the fetus and placenta as is achieved with an intramuscular injection. A subcutaneous injection is probably less traumatic to the small A/jax mouse than is an intramuscular one. Further studies in this area would be very useful.

It is possible that some tritiated cortisol did reach the maxillary process - palatal shelf area of all 50 uc fetal heads studied but was not there in sufficient quantities to adequately expose the emulsion in a five week exposure period. Longer exposure times must be attempted in the future to determine if

a significant amount of tritiated cortisol does indeed get to the palate when administered in 50 uc doses. A 50 uc dose, however, probably is not satisfactory for most future studies since an exposure time of greater than five weeks would be rather inconvenient for most investigators.

A final point must be made. Although at the proper dose level, labelling can be seen in the placenta and maxillary process - palatal shelf region of the fetal head, it is not yet certain that the labelled material is indeed tritiated cortisol. Beside the normal cortisone metabolism which occurs in the adult female animal, it has been shown by Villee (1968) that a great deal of steroid metabolism takes place both in the placenta and in the fetus. In view of this, it is not certain whether the labelling seen represents tritiated cortisol, a labelled metabolite of the tritiated cortisol, free tritium, or other unrelated compounds which have received a tritium label from association with the tritiated cortisol or one of its labelled metabolites. Nasjleti et al (1967) called attention to the fact that the tritium label may be removed from the cortisol by biological enolization. Biochemical assay studies, therefore, are necessary to determine the exact chemical composition of the labelled substance represented by the grains in the autoradiograms.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Pregnant female A/jax mice were injected with tritium-labelled cortisol at 12½ days of pregnancy and sacrificed two to five hours after injection of the labelled steroids. Placentas and fetal heads obtained from these animals were examined autoradiographically to determine if any of the labelling material could be found in these tissues subsequent to injecting it into the mother.

Radiation significantly greater than that which could be attributed to background radiation was seen in the autoradiograms of the majority of the placentas and in the maxillary process - palatal shelf region of some of the fetal head sections, indicating that the labelled cortisone or some labelled derivative of the labelled cortisone was actually present in these tissues.

A dose of 500 microcuries administered either intramuscularly or subcutaneously was found to be a reliable labelling dose when the animals were sacrificed two or five hours after injection. A less toxic vehicle for the tritiated cortisol must be found. The present injection vehicle (50:50 benzene:ethanol) exhibits marked toxicity when injected in 0.5 cc. doses.

Further study is needed to determine the optimum dose, vehicle route of administration and sacrifice time for the use of tritiated cortisol in A/jax fetal mouse labelling studies.

Biochemical assay of the labelled material seen in the autoradiograms is necessary for the determination of its exact chemical composition.

BIBLIOGRAPHY

Baxter, H. and Fraser, P.; "The Production of Congenital defects in the Offspring of Female Mice Treated with Cortisone"; McGill Med J; 19:245-49, 1950.

Birke, G.; "The Dermal Spreading of Hyaluronidase and Streptococci Under the Influence of Stress (cold), ACTH, Cortisone, Desoxycorticosterone acetate"; Acta Med Scand; 144:455-466, 1953.

Bullough, W.; "Stress and Epidermal Mitotic Activity. 1. The Effects of the Adrenal Hormones"; J Endoc; 8:265-74, 1932.

Cavallero, C. and Braccini, C.; "Effect of Cortisone on the Mast Cells of the Rat"; Proc Soc Exp Biol and Med; 69: 222-232, 1951.

Chaudhry, A.P., Schwartz, S. and Schmutz, J.A.; "Some Aspects of Experimental Induction of Cleft Palates"; J Oral Ther; 4:98-103, 1967.

Devenuto, F. and Muldoon, T.; "Interaction Between Corticosteroids and fractions of Mitochondria and Nuclei from Normal Rat Liver Cells"; Exp Cell Resp; 50:338-48, 1968.

Dougherty, T.F., Berliner, D.L., Berliner, M.L.; "Corticosteroid-tissue Interaction"; Metab; 50:338-48, 1961.

Fitzgerald, P., Eidenoff, M., Knoll, J. and Simmel, E.; "Tritium in Autoradiographs"; Science; 114:494-98, 1951.

Fitzgerald, P., Simmel, E., Weinstein, J. and Martin, C.; "Radioautography: Theory, Technique and Applications"; Lab Invest; 2:181-182, 1953.

Fraser, F. and Fainstat, T.; "Production of Congenital Defects in Offspring of pregnant Mice with Cortisone and Other Hormones"; Pediatrics; 8:527-33, 1951.

Fraser, F., Kalter, H., Walker, B. and Fainstat, T.;"The Experimental Production of Cleft palate with Cortisone and Other Hormones"; J Cell and Comp Physio; 43:237-59, 1954.

Fraser, F.C., Chew, D., Verrusio, A.C.; "Oligohydramnios and Cortisone-induced Cleft Palate in the Mouse"; Nature; 214:417-18, 1967.

Goodman, L.S. and Gilman, A.; The Pharmacologic Basis of Therapeutics; (MacMillan Co., New York) 3rd ed., 1965.

Hughes, W., Bond, V., Brecker, G., Cronkite, E., Painter, R., Quaster, H. and Sherman, F.; "Cellular Proliferation in Mouse as Revealed by Autoradiographs with Tritiated Thymidine"; Proc Nat Acad Sci; 44:476-483, 1958.

Ingalls, R., "Relation of Hydrocortisone Injection to Cleft Palate in Mice"; New Eng J Med; 256:1035-39, 1957.

Ingalls, R. and Curley; "Factors Influencing the Frequency of Cortisone Induced Cleft Palate in Mice"; J Exp Zool,; 134:449-67, 1957.

Isaacson, R and Chaudhry, A.; "Cleft Palate Induction in Strain A Mice with Cortisone"; Anat Rec; 134:449-67, 1962.

Jacobs, R.M.; "Effects of Cortisone Acetate Upon Hydration of Embryonic Palate in two Inbred Strains of Mice"; Anat Rec; 156:1-4, 1966.

Kalter, H. and Fraser, F.; "The Modification of the Teratogenic Action of Cortisone by Parity"; Science; 118:625-26, 1953.

Kalter, H.; "Inheritance of Susceptibility to the Teratogenic Action of Cortisone in Mice"; Genetics; 39:185-96, 1954.

Kalter, H.; "Factors Influencing the Frequency of Cortisone Induced Cleft Palate in Mice"; J Exp Zool; 134:449-68, 1957.

Kalter, H. and Warkany; "Experimental Production of Congenital Malformations in Mammals by Metabolic Procedure"; Physio Rev; 39:69-115, 1959.

Kerppala, W., Pitkanen, E.; "The Action of Cortisone on Oxidation and Glycolytic Liver Enzyme Activities in Rats of Different Age and Sex";Endoc; 67:162-65, 1960.

Kivirriko, K.I.; "Effects of Cortisone on Collagen Formation in the Chick Embryo"; Nature; 214:417-18, 1963.

Krantz, J.C., Jr. and Carr, C.J.; The Pharmacologic Principles of Medical Practice; (Williams and Wilkins Co., Baltimore) 1961.

Lajtha, L., Phil, D. and Oliver, R.; "The Application of Autoradiography in the Study of Nucleic Acid Metabolism"; Lab Invest; Vol 8, No 1, 1959.

Larsson, K., Bostrom, H., and Carsloo, R.; "Studies on the Closure of the Secondary Palate"; J Exp Cell Res; 16: 379-383, 1959.

Larsson, K.; "Studies of the Closure of the Secondary Palate, III. Autoradiographic and Histochemical Studies in the Normal Mouse Embryos"; Acta Morph Neerl Scand; 4:349-367, 1961-62.

Larsson, K.; "Studies on the Closure of the Secondary Palate, IV. Autoradiographic and Histochemical Studies of Mouse Embryos from Cortisone-treated Mothers"; Acta Morph Neerl Scand; 4:369-86, 1961-62.

Larsson, K.; "Studies on the Closure of the Secondary Palate; Acta Odont Scand; 20:1-13, 1962.

Lattes, R., Blunt, J., Rose, H., Jessar, R., Vaillancourt, G., Ragan, C.; "Lack of Cortisone Effect in the Early Stages of Inflammation and Repair"; Am J Path; 29:1-19, 1953.

Layton, L.; "Cortisone Inhibition of Mucopolysaccharide Synthesis in the Intact Rat"; Arch Biochem; 32:224, 1951.

Layton, L.; "Effect of Cortisone Upon Chondroitin Sulfate Synthesis by Animal Tissues"; Proc Soc Exp Biol; 76:596-98, 1951.

Lazzaro, C.; "Sul Meccanismo di chiasura del Palato Secondario"; Monit Zool Ital; 51: 249-265, 1940

Loevy, H.; "Normal and Abnormal Palate Development in the Mouse Fetus: Morphological and Histological Factors"; Ph.D. Dissertation, University of Illinois, 1961.

Loevy, H.; "Developmental Changes in the Palate of Normal and Cortisone Treated Strong A Mice"; Anat Rec; 142:375-90, 1962.

McManus, J. and Maury, R.; "Staining Methods: Histologic and Histochemical; (Hoeber, Inc., New York), 1960.

Mott, W.J.; "Labelling Index and Cellular Density in Palatine Shelves of Cleft Palate Mice"; M.S. Thesis, Loyola University, Chicago, Illinois; 1968.

Nasjleti, C.E., Avery, J.K., Spencer, H.H. and Walden, J.M.; "Tritiated Cortisone Distribution and Induced Cleft Palate in Mice"; J Oral Ther; 4:71-82, 1967.

Paff, G. and Stewart, R.; "Free Wandering Cells and Cortisone"; Proc Soc Exp Biol and Med; 83: 591-92, 1953.

Peter, K.; "Die Entwicklung des saugetiergaumens"; Ergebn Anat Entwicklungsgesch; 25:448-564, 1924.

Pölzl, A.; "Sur Entwickelungsgeschiete des Manschilchen"; Gaumens Anat Hefte; 27:244-301, 1904.

Pons-Tortella, E.; "Uber die bildungsweise des Sekundaren"; Gaumens Anat Ans; 84:13-47, 1937.

Ragan, C., Howes, E., Plotz, C., Meyer, K. and Blunt, J.; "Effect of Cortisone on Production of Granulation Tissue in Rabbit"; Proc Soc Biol and Med; 72:718-21, 1949.

Ragan, C., Howes, E., Plotz, C., Meyer, K. and Blunt, J. and Lattes, R.; "The Effect of ACTH and Cortisone on Connective Tissue"; Bull N.Y. Acad Med; 26:251-254; 1950.

Reed, S.C.; "An Embryological Study of Harelip in Mice"; Anat Rec; 56: 101-110, 1933.

Roberts, K., Florey, H. and Joklik, W.; "The Influence of Cortisone on Cell Division; Quart J Exp Physiol; 37:239-57, 1952.

Sayeed, M.M., Blumenthal, D.S., Blumenthal, H.I.; "Effect of Cortisone and Growth Hormones on Cellular Proliferation During Early Embryogenesis"; Proc Soc Exp Biol and Med; 109:261-264, 1962.

Schoenheider, W.A., Jr.; DNA Metabolism of the Basal Cell Layer of the Epithelium of the Tongue in the Mouse"; M.S. Thesis, Loyola University, Chicago, Illinois, 1960.

Villee, A.; "The Placenta and Fetal Tissues. A Cooperative Enterprise for the Synthesis of Steroids"; Israel J Med Sci; 4:270-6, 1968.

Walker, B.; "Genetico-embryological Studies on Normal and Cleft Palates in Mice"; Ph.D. Dissertation, McGill University.

Walker, B. and Fraser, F.; "Closure of the Secondary Palate in Three Strains of Mice"; J Embryol and Exp Morph;9:22-31, 1956.

Walker, B.; "A Special Component of Embryonic Mesenchyme"; Anat Rec; 136:298, 1960.

APPENDIX

GRAIN COUNTS

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice time	Head		Placenta	
		Tissue	Background	Tissue	Background
2	500 microcuries IM 2 Hr.	46	6	54	0
		30	9	51	1
		34	10	39	4
		20	13	41	2
		37	10	40	10
		42	14	39	7
		40	16	42	2
		43	11	26	2
		26	8	45	8
		53	14	36	15
4	500 microcuries IM 2 Hr.	38	14	66	19
		51	8	78	16
		35	10	83	5
		38	12	36	4
		43	10	75	11
		35	0	83	10
		49	5	79	10
		39	8	98	16
		53	3	71	11
		59	3	107	12

APPENDIX

GRAIN COUNTS

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice time	Head		Placenta	
		Tissue	Background	Tissue	Background
5	500 microcuries IM 2 Hr.	25	14	23	6
		25	5	27	8
		37	14	33	6
		39	12	27	9
		37	11	40	3
		39	5	48	7
		33	19	53	3
		29	6	43	3
		33	2	29	1
		41	5	31	8
9	500 microcuries subcutaneous 5 Hr.	52	8	16	6
		60	9	70	4
		44	6	39	5
		37	3	42	2
		47	6	55	5
		53	11	42	7
		26	9	29	5
		50	5	37	7
		40	7	47	8
		33	7	31	3

APPENDIX

GRAIN COUNTS

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice Time	Tissue	Background	Tissue	Background
22	500 microcuries IM 5 Hr.	4	3	3	0
		5	5	0	0
		9	5	0	0
		5	12	0	1
		4	2	0	0
		0	7	0	0
		3	15	0	0
		0	0	1	0
		0	8	0	0
		5	13	1	0
27	50 microcuries IM 5 Hr.	0	2	0	0
		0	1	0	0
		0	0	0	0
		4	0	6	1
		12	0	0	0
		0	0	0	0
		0	2	0	0
		1	2	0	0
		5	0	0	0
		0	0	0	0

APPENDIX

GRAIN COUNTS

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice Time	Head		Placenta	
		Tissue	Background	Tissue	Background
28	50 microcuries IM 5 hr.	0	0	-	-
		0	1	-	-
		0	0	-	-
		0	3	-	-
		0	0	-	-
		2	2	-	-
		1	0	-	-
		1	0	-	-
		1	0	-	-
		0	0	-	-
34	50 microcuries IM 5 Hr.	0	2	10	0
		0	2	4	5
		0	3	1	3
		0	5	2	0
		0	2	8	0
		4	1	1	2
		8	2	2	0
		3	0	3	0
		3	0	7	0
		5	5	3	0

APPENDIX

GRAIN COUNTS

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice Time	Head		Placenta	
		Tissue	Background	Tissue	Background
38	50 microcuries IM 5 Hr.	2	1	19	2
		7	5	7	5
		4	3	12	0
		7	0	16	0
		7	0	13	0
		9	0	15	0
		4	0	14	0
		3	0	10	2
		2	0	21	1
		3	6	12	2
41	50 microcuries IM 5 Hr.	2	3	4	6
		8	1	9	1
		2	2	4	0
		3	3	2	7
		2	0	11	7
		0	8	10	6
		1	5	11	14
		0	4	12	10
		0	0	15	13
		0	0	11	15

APPENDIX

GRAIN COUNTS

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice Time	Head		Placenta	
		Tissue	Background	Tissue	Background
46	50 microcuries IM 5 Hr.	0	0	16	5
		2	3	11	7
		0	0	9	6
		0	4	11	4
		0	6	14	4
		0	5	11	4
		1	5	16	0
		4	14	17	0
		0	0	17	0
		0	0	8	0
52	50 microcuries IM 5 Hr.	9	1	12	5
		8	7	11	7
		7	11	11	0
		5	7	11	7
		9	0	6	9
		16	12	10	8
		11	6	14	0
		11	0	11	0
		10	5	8	10

APPENDIX

GRAIN COUNTS

Embryo Number	Dose and Route of Tritiated Cortisol Sacrifice Time	Head		Placenta	
		Tissue	background	Tissue	background
60	50 microcuries IM 5 Hr.	3	10	13	9
		18	5	18	4
		7	11	21	1
		6	0	22	0
		10	9	21	5
		3	7	22	4
		6	7	15	13
		10	4	18	4
		7	9	13	0
		11	10	12	12
61	50 microcuries IM 5 Hr.	8	7	15	3
		9	4	17	1
		4	3	13	9
		5	0	14	9
		3	10	10	5
		12	0	4	7
		8	0	14	5
		11	0	5	5
		3	0	8	4
		2	11	8	1

APPENDIX

GRAIN COUNTS

Embryo Number	Dose and Route of Tritiated Cortisol and Sacrifice Time	Head		Placenta	
		Tissue	Background	Tissue	Background
67	50 microcuries IM 5 Hr.	14	8	16	7
		15	2	11	5
		6	7	22	3
		7	7	15	6
		15	3	19	8
		18	5	9	7
		14	2	13	12
		13	4	11	10
		17	10	13	7
		12	1	9	7

APPENDIX

Mother	Dose, Route of Administration Of Tritiated Cortisol And Sacrifice Time	Numerical Designations Of Embryoes Obtained Upon Sacrifice
A	500 uc - IM - 2 Hr.	1 2 3 4
		5 6 7 8
B	500 uc - Subcu. - 5 Hr.	9 10 11 12
		13 14 15 16
		17 18 19
C	50 uc - IM - 5 Hr.	20 21 22 23
		24 25 26 27
		28 29
D	50 uc - IM - 5 Hr.	30 31 32 33
		34 35 36 37
		38 39
E	50 uc - IM - 5 Hr.	40 41 42 43
		44 45 46 47
		48 49
F	50 uc - IM - 5 Hr.	50 51 52 53
		54 55 56 57
		58 59 60
G	50 uc - IM - 5 Hr.	61 62 63 64
		65 66 67
H	50 uc - IM - 5 Hr.	68 69 70 71
		72 73 74 75
		76
I	50 uc - IM - 5 Hr.	77 78 79 80
		81 82 83 84
		85

APPROVAL SHEET

The thesis submitted by Allen D. Marks 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.

May 8/69
Date

Dr. N.K. Wood
Signature of Advisor