1968

Labeling Index and Cellular Density in Palatine Shelves of Cleft Palate Mice

William J. Mott
Loyola University Chicago

LABELING INDEX AND CELLULAR DENSITY IN PALATINE SHELVES OF CLEFT PALATE MICE

BY

WILLIAM J. MOTT

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

1968

AUTOBIOGRAPHY

William J. Mott was born in Chicago, Illinois on October 5, 1937.

In September, 1956 he entered the University of California at Los Angeles. He received the degree of Bachelor of Arts in Zoology in June, 1960.

He entered Loyola University School of Dentistry, Chicago, Illinois in September, 1962. In June, 1966, he received the degree of Doctor of Dental Surgery.

In June, 1966, he began graduate studies in the Department of Oral Biology at Loyola University, Chicago, Illinois.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to all those who have aided in making this investigation possible, particularly to the following:

To Joseph R. Jarabak, D.D.S., Ph.D., my appreciation to his devotion to the orthodontic profession and for giving me the opportunity to study orthodontics.

To Patrick D. Toto, D.D.S., M.S., Professor of Oral Pathology, my thesis director, for his advice, guidance and support in preparing this thesis.

To my wife, Linda, for her never ending encouragement and understanding throughout my professional education.

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION AND STATEMENT OF PROBLEM

Cleft palate is a congenital anomaly occuring once every 700-800 births. Despite this frequency, literature describing research on the embryology of cleft palate as well as normal palate development has been inadequately documented. Furthermore, the majority of this literature has been written within the last fifteen years.

As a result of this renewed interest, it is no longer sufficient to pass off the cause of cleft palate merely as a lack of growth of the palatine processes, or as a mechanical obstruction resulting from a failure of the tongue to drop at the proper time. Recent investigations have seriously questioned these theories, and now the role of acid mucopolysaccharides in the connective tissue of the palatine shelves is of primary interest.

Thru histochemical and autoradiographic studies, various authors suggest a decrease in the acid mucopolysaccharides of a cleft palate mouse compared to a mouse with normal palatal development. Although some evidence has been advanced supporting this theory, the evidence is "semi"-quantitative and has

been based on subjective interpretation of the various investigators.

It is commonly accepted that the cells produce the acid mucopolysaccharides. The purpose of this investigation is therefore two-fold: to determine if there is either a significant difference in the mitotic activity labeling index in the palatine shelves of the control and the treated mice; or if there is a corresponding increase in cellular density relating to the decrease of the acid mucopolysaccharides in the intercellular material.

CHAPTER II

REVIEW OF THE LITERATURE

I. Normal Palatal Development

Polzl (1904) postulated that the palatine processes undergo horizontalization by regression of their ventral extremities and concomitant new shelf growth from their medial surfaces at approximately the level of the tongue. Pons-Tortella in 1937 substantiated this theory of palatal closure.

Peter (1924) proposed the concept that the lateral palatine processes assumed a horizontal position from a ventrally directed position by undergoing a medial rotation. Lazarro (1940) reaffirmed this theory and postulated the following ideas to explain the mechanism of palatal shelf movement: 1. external pressure such as muscular pressure from the tongue; 2. growth changes involving regression of the ventral portion and an outgrowth in the horizontal plane; 3. rapid rotation of the shelves due to some intrinsic forces.

Lazarro favored the idea of rapid movement, and his studies indicated that rotation resulted from an increase of intercellular substance within the lateral palatine processes. Thus the increased intercellular material (swelling of the

shelves) caused the lateral palatine processes to rise once the obstruction of the tongue was removed. Lazarro thought that the release of the tongue had to take place before anything else, even if the mechanism of release was "slightly obscure".

He suggested the following mechanisms for the release of the tongue:

1. lowering of the mandible and tongue
2. forward displacement of the tongue
3. lifting of the roof of the oral cavity
4. changes in form of the tongue due to muscular development
5. muscular movements of the tongue

According to Reed (1933), the vertical palatine processes are rotated into a horizontal plane either by unequal growth or by muscular contraction and growth in the horizontal plane continues until the palatal plates come in contact and fuse.

Walker (1954) stated that the literature on the embryonic development of the secondary mammalian palate appears reliable except for the stage of closure from the vertical to the horizontal position.

Walker described seven (7) stages of palatal closure.

Stage 1: The primary palate and alveolus have already

been formed. The medial portion of each palatine shelf lies in a vertical plane while laterally the shelf lies in a horizontal plane. At first, the tongue lies completely between the shelves, but later as the tongue increases in width it spreads out across the shelves. From a ventral position, the posterior ends of the shelves cannot be seen.

The tongue, therefore, is ventral to the shelves posteriorly and medial to the shelves anteriorly. At the point where the tongue goes from ventral to medial, it makes a grooved impression on the sides of the shelves.

Posterior to the groove, the shelves slope at an obtuse angle away from the roof of the nasal cavity so that the medial surfaces are visible when viewed from the ventral. Anterior to the groove the shelves slope at an acute angle and thus tend to cup the tongue between them, and when the tongue is removed, the medial sides of the shelves are not visible from the ventral.

Stage II: The groove has moved to a point midway along the shelves.

Stage III: Shelf activity is not necessarily synchronized bilaterally and at times a condition is seen where one shelf is completely dorsal to the tongue (horizontal) and the other is not.

Stage IV: Both shelves have now assumed a dorsal position to the tongue. The shelves are at first separated by a small space which is bridged by a further flattening of the shelves.

Stage V: Fusion of the shelf epithelium begins.

Stages VI and VII: Epithelial fusion spreads anterior and posterior until the shelves are fused throughout their length.

Thus Walker says that at the point of transition, where the tongue goes from a medial to a ventral position relative to the shelves, the shelves curve around the tongue in a twisting fashion. This twisting of the shelves over the tongue proceeds in a wave-like manner anteriorly until the whole shelf lies dorsal to the tongue. Walker further states that this horizontalization of the shelves occurs without any active participation of the tongue. This is emphasized in histologic sections in which one shelf is horizontal while the other shelf is vertical. He says there is no sign of the tongue having dropped to allow the shelves to become horizontal as suggested by Peter (1924) and Lazarro (1940).

Walker also says that the idea of rotation of the shelves (Peter, 1924; Reed, 1933; Lazarro, 1940) is unsatisfactory because the relationship of the tongue to the shelves does not suggest such a movement since an intermediate shelf condition

not involving rotation has been shown (bulging of the shelves along their dorso-medial surface, and a regression of the ventral protrusion).

Walker indicated that the time required for shelf movement (stages 2, 3, and 4) is 3 hours and the time required for fusion (stages 5 and 6) is 6 hours. By experimentally inducing shelf movement, Walker showed that the shelves can change position within a minute when the tongue is manually displaced. Resistance of the tongue accounts for the lengthy period required for shelf movement. This discounts the proponents of a growth mechanism (Reed, 1933 and Pons-Tortella, 1937) although they were close to being correct in their theory of how the shelves by-passed the tongue (resorption of the vertically aligned ventral portion and an outward "growth" of the medial wall).

Walker and Fraser (1956) stated that the metachromasia and affinity for aldehyde-fuchsin of the shelf connective tissue suggests two possible mechanisms for shelf mobility:

1. Lazarro's theory of tissue turgor could implicate hyaluronic acid as a water barrier and the acid could account for the metachromasia. (Walker ruled this out however because the dehydrating effect of 70% alcohol on shelf movement was ineffective.) 2. Also, he suggested that shelf movement is

due to the tensions of a developing network of elastic fibers. (They thought that the metachromasia was consistent with an interpretation of elastic fibers.)

By 1960, Walker had discarded his "elastic fiber" theory of shelf movement and suggested the force for palatine shelf movement is contained in the sulfated acid mucopolysaccharides of the mesenchymatous ground substance. By using autoradiographs derived from embryos receiving S^{35}, Walker (1961) showed a greater intensity of palatine tissue radioactivity in embryos receiving S^{35} from day 14/8 to 14/18 than from embryos receiving S^{35} from day 12/10 to 14/8. This can be considered as evidence indicating a sulfated acid mucopolysaccharide synthesis causing a build up of palatine shelf force since palatal closure is known to take place from day 14/8 to 14/20. It should also be noted that sulfation increases the viscosity and gelation of acid mucopolysaccharides (Toto).

In 1961, Larsson proposed the theory that : 1) the elasticity of the ground substance permits a change in the fibroblast zones, and that it can be presumed that, 2) changes in the polymerization and/or aggregation of the high molecular weight mucopolysaccharides, and/or binding of water to them gives rise to tensions or changes in pressure in the tissue

resulting in development of a force which causes changes in shape. Larsson therefore considers the presence of chondroitin sulfuric acid, a sulfated acid mucopolysaccharide with powerful synthesis in the ground substance of the fibroblast zones, as responsible for the internal force.

Larsson also agrees with Walker's theory of palatal closure and says the internal force is sufficiently strong to cause the palatine shelves to bulge over the tongue.

Asling et al (1960) said that the lateral processes of normal rat fetuses rotated from a ventromedial to a horizontal plane in the rostral region of developing palate; however palatal closure caudal to the choanae was not described. They thought that the elevated position of the tongue in the floor of the mouth in fetuses with cleft palate acted as a barrier to the palatal processes.

As late as 1965, Coleman said that the rostral segment of rat palate closes in accordance with the rotational theory of Peter (1924) and Lazarro (1940) and the caudal segment in accordance with the mechanism of transformation described by Polzl (1904) and Pons-Tortella (1937).

Cortisone Induced Cleft Palate

In 1950, Baxter and Fraser discovered that cortisone

could induce cleft palate in developing mice. Two strains of mice were used in their investigation, and in neither strain was cleft palate found to occur spontaneously, except in conjunction with cleft lip. The alveolar ridge was not disturbed in the cortisone induced cleft palate.

By giving varying doses of cortisone for varying lengths of time to pregnant female mice, Fraser and Fainstat (1951) showed that the A/Jax strain was the most susceptible to cleft palate formation. Cortisone injections started on the 10th or 11th day showed the highest incidence of cleft palate in the offspring. Fraser et al (1954) and Isaccson (1962) showed a 100% incidence of cleft palate in A/Jax mice treated with 2.5 mg. cortisone for four days beginning on the 10th or 11th day. The incidence of cleft palate declines markedly when treatment is started after 11 days (i.e. 29% on the 12th and 13th days). Fraser and Fainstat (1951).

Ingalls and Curley (1957) reported that 5 and 10 mg. doses of hydrocortisone injected at 11½ days of gestation produced 47% and 100% cleft palates respectively in albino mice. However, there were more fetal resorptions when 10 mg. was injected in a single dose.

Fraser et al (1954) suggested that congenital palatine

clefts may be caused by two different mechanisms: one acting before the palate closed, causing delay in rotation of the palatine shelves, and one causing a regressive change in the palate after it closed.

Walker (1954) stated that when A/Jax mice received a particular schedule of cortisone injections, there was a delay in the average time and rate at which palate shelf movement occurred. He considers this without doubt, the primary cause of cleft palate in newborn mice from a cortisone treated mother. He said that stage three (3) of palatal closure (one shelf vertical, one horizontal), which is normally infrequent, is found repeatedly throughout a wide range of morphological and chronological ages in treated embryos.

The measurements of the palatal region did not show any sign of growth retardation at time of palatal closure due to cortisone treatment (Walker 1954). He also said cleft palate develops in treated embryos with retarded shelf movement because fusion of the shelves is apparently necessary to stimulate shelf growth beyond the size seen during normal palate closure. The degree of delay of horizontalization of the shelves is variable and the shelves that changed shape late in development (day 15/8) were only as wide as normal shelves starting to fuse. Head

growth is not noticeably affected by cortisone during this period, so that the late moving shelves are drawn apart by increasing head width and when the shelves finally become horizontal, they are too far apart to touch and fuse.

Walker (1954) suggested that if cortisone treatment were affecting the shelves directly, there would be an inhibition in the build up or the release of the force causing shelf movement. Walker thought that cortisone was inhibiting the growth of fibers, or the plasticity (acid mucopolysaccharides) of the tissue, or even the spatial relation (pattern) of fibers and tissue masses that lead to a change in the type of configuration, giving maximum stability.

Larsson (1961-62) shows that cortisone administration lowers the sulfate incorporation into the sulfated acid mucopolysaccharides of mouse embryos. He agreed with Walker and Fraser (1956), that the shelves are retarded in horizontalization and that cortisone in the palatine shelves can interfere with substances presumed to be responsible for the internal force (acid mucopolysaccharides). He also presumes there are changes in polymerization and/or aggregation of the high molecular weight mucopolysaccharides and that there is a change in pressure in the tissues. In 1961, Walker also attributed

the decrease in acid mucopolysaccharides as the cause for retardation of the build up of the shelf force.

Kalter (1957) stated the elements so far discovered that interact to determine a particular frequency of cortisone induced cleft palate are: dose of hormone, gestation time, mouse strain, maternal genotype, fetal genotype, maternal weight and fetal weight. He showed that the frequency for control litters to be completely resorbed was 2.7% compared to 28.6% in treated litters. Cortisone decreased litter size by approximately 30%, and the cleft palate newborn weighed less than their normal litter mates. The heavier the mother or the more advanced the parity, the lower the cleft palate frequency.

Leovy (1962) injected 1.25 mg. cortisone daily from the 11th to the 14th day and achieved 100% cleft palate formation with low fetal resorption. She said it is unlikely that the tongue plays an active role in the process of cleft palate formation and unlikely that horizontalization is a significant factor, and furthermore stated that it was unlikely that connective tissue plays an important role in the development of the cleft. She says that if there was an inhibition of connective tissue growth at the moment when the palatine shelves were in close proximity, the delay could be fatal to normal

development because further development of the head would preclude a later fusion of the shelves. She states she has observed a breakdown in basement membrane in early stages of fusion, while in later stages the basement membrane has become reorganized. In two (2) of the experimental animals, an intact basement membrane was seen at 15 and 16 days, when shelves were contiguous. Loevy says these shelves would not have fused and that they would have pulled apart. This theory is not well documented however.

Cortisone Action

The exact mechanism of cortisone action on the palatine shelves is at present unexplained. It is not known whether cortisone acts directly on the shelves or whether there is an intermediate step before the action is manifest. For the above reasons, a review of the effects of cortisone on connective tissue is indicated.

Layton (1951) said that high doses of cortisone appeared to inhibit the synthesis of chondroitin sulfate from the tissues. He said this effect may be secondary to the alteration in protein metabolism indicated by a large negative sulfur balance. A failure to form hexosamines could be partly

responsible for the disappearance of chondroitin sulfate from the tissue; a simultaneous decrease in all mucopolysaccharides would then be expected.

Bullough (1932) suggested that cortisone had a general antimitotic action. Bullough did state, however, that the disturbance in carbohydrate metabolism may be a factor in depressing cell division. In another investigation it was found that the effect of cortisone on mitosis varies from one type tissue to another and that it does not seem likely that one of the fundamental, universal actions of cortisone is to inhibit mitosis. Roberts et al (1952).

Cavallero and Braccini (1951) found that metachromatically stainable material had practically disappeared from the connective interfibrillar tissue. Paff and Stewart (1953) found that the mast cells were reduced in number. It is known that cortisone inhibits new formation of connective tissue. Asboe Hansen (1952) found fibroblasts in healing wounds of treated (cortisone) individuals are smaller and more pynknotic than in wounds of untreated individuals. He also found a decrease in the number of mast cells.

Prolonged treatment with cortisone inhibits both hyaluronidase and streptococci. Birke (1953).

Lattes et al (1953), found that in injured mesenchymal tissues treated with cortisone, the metachromasia of the ground substance is reduced, suggesting that the mucopolysaccharides of the connective tissue do not undergo the same chemical changes as in untreated uninjured animals. Ashton and Cook (1952) found generalized vascular constriction and depression of capillary permeability in rabbit ear chambers after cortisone administration.

In skin wounds, development of granulation tissue was markedly delayed in all cases where cortisone was administered. Ragan et al (1949). There was no significant influence on the composition of the existing granulation tissue, or evidence of lysis of the granulation tissue. Spain et al (1950).

Autoradiography:

With the use of autoradiography, radioisotopes may be localized to an organism, an organ, an area in a histologic section, or even to individual cells. The object (tissue) to be studied is placed against photographic emulsion allowing sufficient time for exposure and then developed as in ordinary photography. The processed photographic emulsion is called an autoradiogram and consists of accumulations of black granules

overlying those areas in the tissue section which contain the radioactive material. The photographic emulsion records in its crystals of silver bromide the ionization produced in the emulsion by rapidly moving charged particles or radiation. (Fitzgerald, 1953; Schoenheider, 1960).

"The chemical behavior of a labeled substance is identical with that of its stable counterpart. This is also true of its biological behavior provided (1) the amount of radioactivity administered is small enough not to have a significant radiochemical effect, and (2) the amount or weight of material injected be sufficiently small not to produce a significant increase in the amount of the substance in the circulation. The labeled substance will then truly act as a "tracer" of normal metabolism." Schoenheider (1960).

In 1959, Leblond, et al investigated the possibility of using tritiated thymidine for studying renewal of cell populations utilizing the mouse tongue. Also in 1959, Cronkite, et al utilized autoradiograms in the study of DNA synthesis, and cell turnover in hemopoietic tissues. They stated that "although thymidine is apparently not a normal precursor of DNA, it can enter the synthetic chain and label DNA at the time of DNA doubling prior to mitosis. Thus if the label is

sufficiently intense and permanent, one can, by autoradiography, follow cells from the time of DNA synthesis to ultimate death."

Hughes et al (1958) stated that: "In order to distinguish cell renewal from the renewal of cellular constituents, the label must be incorporated into a fixed component of the cell which is not lost during the cells life time; and present knowledge suggests that a label incorporated into DNA should be most useful for this purpose. The specific advantage of labeled tritium lies in very high resolution which can be obtained because of the very weak energy and consequently short range of its beta radiation. The maximum range in tissue of a beta ray from tritium is only six microns and half of the betas will travel less than one micron. Consequently, the activated silver grains to an autoradiogram should largely lie within one micron of their source. Tritiated thymidine is apparently rapidly absorbed by all cells when injected into mice. The concentration of tritium hydroxide reaches a maximum within one hour following intraperitoneal injection. Therefore, the precursor pool of DNA synthesis must simultaneously become exhausted suggesting that all labeling of nuclei occurs during the 1st hour after injection. This also means a similar rate of exhaustion of thymidine from DNA synthesizing cells as from

tissue as a whole."

Lajtha et al (1959) also showed thymidine was a specific component of DNA and that it labels only DNA. They noted an eight micron maximum and an average of 1.5 microns. Tritium has a half life of 12.26 years and a disintegration rate of 0.016% a day.

Leblond et al (1959) stated that it is known that mitosis is preceded by a synthesis of DNA. If a radioactive DNA precursor is administered at the time of DNA synthesis, the nucleus becomes radioactive and may be recognized by autoradiography prior to mitosis. The labeled nuclei pass the label on to the daughter cells. The danger of radiation damage seems to be reduced with tritium, and it is possible to use doses which allow cells to be traced over periods of months in the body without apparent signs of damage to these or other cells. Furthermore, the sections need not be subjected to chemical extractions since DNA is the only labeled substance present in significant amounts in the sections following H^3 administration. DNAase treatment eliminates all autoradiographic reactions.

The photographic grains produced by tritium containing structures are found within two or three microns, due to the low beta-ray energy of tritium, and most of them within one

micron from the source as measured within NTB-3 emulsion. "It may be concluded that thymidine H^3 is an adequate tool for the autoradiographic direction of newly formed DNA and therefore, may be used to locate the sites of cell formation." Schoenheider (1960).

Acid Mucopolysaccharides

The term polysaccharide is generally limited to those polymers which contain ten or more monosaccharide units. The size of polysaccharides is variable, but most natural polysaccharides are high molecular weight, containing some 100 to 1000 monosaccharide units. Mucopolysaccharides are most often defined as compounds having a low, but significant protein content but whose reactions are predominently polysaccharide.

The ground substance of connective tissue contains a large amount of mucopolysaccharides and mucoproteins. The acid mucopolysaccharides present in the connective tissue are: hyaluronic acid, chondroitin sulfate A, B and C, heparin and keratosulfate. All of these are sulfated with the exception of hyaluronic acid. Most mucopolysaccharides do not exist as free compounds, but are linked to protein. Dorfman (1961).

There are two theories regarding the source of the mucopolysaccharides of the ground substance. Most investigators

consider the origin to be from the fibroblasts. The presence in fibroblasts of granules and vacuoles having the histochemical reactions of mucopolysaccharides and the occurence of large amounts of mucopolysaccharides in certain types of fibrosarcoma favors the fibroblast. (Gersh and Catchpole, 1949). In scorbutus, where the fibroblasts remain immature and the formation of collagen from reticulum is interfered with, the metachromatic substance persists long after its almost complete disappearance from the completely healed wounds in the normal animals. (Bunting, 1949). The other theory is that the mast cell is the source of mucopolysaccharides. (Asboe-Hansen, 1951). The mast cell is known to store, if not synthesize, heparin.

Chondroitin sulfate A is of primary interest since it is the main mucopolysaccharide of cartilage. These and other sulfated mucopolysaccharides occur in tissues which are normally in a state of dehydration. They have been connected with fibrogenesis and with the binding of cations. Karl Meyer (1954).

Dorfman (1954), states that the acid mucopolysaccharides are flexible chain polymers of high negative charge with a high affinity for cations and water molecules and suggests they may play a critical role in regulating the metabolism of inorganic ions and water. A change in concentration or molecular size of

such a substance would then be of great importance in modifying the capacity of connective tissue to bind water and salts.

CHAPTER III

MATERIALS AND METHODS

I. Animals

Mice:

Thirty A/Jax strain mice (20 female, 10 male) were obtained from the Jackson Laboratory when they were 41 days old. These mice have been inbred for over 114 generations and were chosen because of their susceptibility to cortisone induced cleft palate. Fraser and Fainstat (1951).

Mating:

During the period that the mice were being mated, males and females were grouped in cages on Monday evenings, and were removed on Friday mornings. The females were examined for vaginal plugs each morning between 9:00 and 11:00 A.M. If for some reason the females could not be checked on a given day, the males were removed from the cages the day before. Due to a limited amount of room, a specified method of mating was not used. In the beginning, 2 males were placed in cages with 4 females. Later, as the number of virgin females was reduced, the ratio of male to female varied from 2:3, 2:2, 2:1, and 1:1.

When a vaginal plug was identified or suspected, the

female was isolated. The date the plug was found was written on the isolation cage and identified as day zero of pregnancy.

2:00 A.M. was selected as zero hour in assigning ages to embryos. Snell et al (1940). In order to insure proper timing of the embryos, the morphological criteria of Gruenberg (1943) were used to grade the embryos.

Cortisone Injections:

2.5 mg. (.1 cc.) of cortisone was injected into the flank of the pregnant females at 12:00 P.M. on the 11th, 12th, 13th and 14th days of gestation as estimated by the vaginal plug. The cortisone used was the Merck Cortone (R) preparation, a saline suspension which contains cortisone acetate (25 mg./cc.) with added suspending agents and 1.5% benzyl alcohol. The control females were injected with .1 cc. of sterile isotonic saline solution at the same time as the cortisone treated females.

Gross Anatomy:

All pregnant females were injected with .5 cc. labeled thymidine (sp ac. 1.9 c/mmole) peritoneally 1 hour prior to sacrifice. The mice were sacrificed by ether overdose at 15½ and 16½ days in utero. The uterus was removed and placed in Bouin's fixative from 2 to 4 days. The embryos were then

dissected out under a binocular dissecting microscope and the heads were detached from their bodies. The heads were then stored in 75% alcohol. The lower jaw and tongue were removed from several of the embryos in order to examine the palate under the dissecting microscope.

Microscopic Anatomy:

Embryo heads with and without the lower jaw and tongue were used for histologic study. The embryo heads were embedded in paraffin and sectioned at 5 microns. Each 10th slide was stained with hematoxylin and eosin for cellular and area identification. The staining procedures are those described in McManus.

Staining materials used:

1. Hematoxylin and eosin
2. PAS (Periodic Acid Schiff)
3. Alcian Blue-Aldehyde fuchsin
4. Alcian Blue-PAS
5. Azure A extinction pH 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0.

The above histochemistry procedures were used to compare differences in staining capacities during various stages of palatal development. Larsson (1961) and Loevy (1962) could not

show any differences in staining intensities between cortisone induced cleft palate mice and normal mice. Jacobs (1964) showed striking differences in staining reactions in both experimental groups during the period of palatal closure.

Autoradiographic Technique:

The technique used for autoradiography was modified from that described by Fitzgerald. (1951)

1. Under dark room conditions, A Wratten #1, Red Safelite 10 watt bulb is used. A humidity of 70% and below is recommended.

2. The slide is dipped in NTB3 liquid emulsion (Kodak).

3. The slide is air dried for 10 minutes.

4. Ten slides are placed in a black, light proof exposure box, sections sides up. Lithium chloride is placed in the box for maintaining a low humidity and black masking tape is used to seal the box.

5. The box was exposed for 30 days at low humidity and temperature. During the exposure time the box must be maintained in a position which keeps the sections upright.

Developing:

1. The slides are placed in a staining rack and developed for 5 minutes at 60°F. (18°C. in Kodak D196 developer)

2. The slide is rinsed in distilled water for 30 seconds.

3. The slide is then placed in acid fixer for 10 minutes.

4. The slide is rinsed in running tap water for 30 minutes.

5. The slides and staining dish are covered with kleenex (to prevent dust from settling on the slides) and allowed to dry in a stream of air.

Cell Counts:

In order to be consistent in the selection of sections through the palate, only sections which included the nasal septum were utilized for cell counts. The base of the palatine shelf was the region used for recording cell counts.

1. Mitotic Labeling Index: Under high dry magnification the number of labeled connective tissue cells per 1000 cells at the base of the palatine shelf were counted. Three or four black grains over a cell was the criteria for a labeled cell. Four fetuses from each age group were utilized in the counting. The number of labeled cells/1000 cells were counted for each fetus.

2. Cellular density: The number of connective tissue

cells per .25 mm^2 were counted for each fetus. A micrometer was placed over the connective tissue at the base of the palatine shelf and an average was obtained for each fetus.

CHAPTER IV

FINDINGS

Figure 1A demonstrates an H&E section of a 15½ day normal mouse palate. All sections used in counting included the nasal septum for area identification. The palatine shelves have undergone horizontalization and have fused at this stage of development.

Figure 1B demonstrates an autoradiograph at the base of the palate of a 15½ day normal mouse palate.

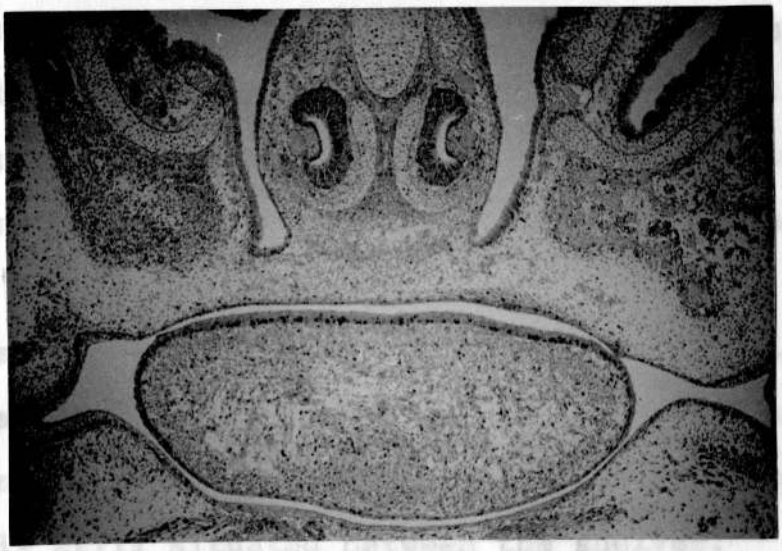

Fig. 1A (40X)

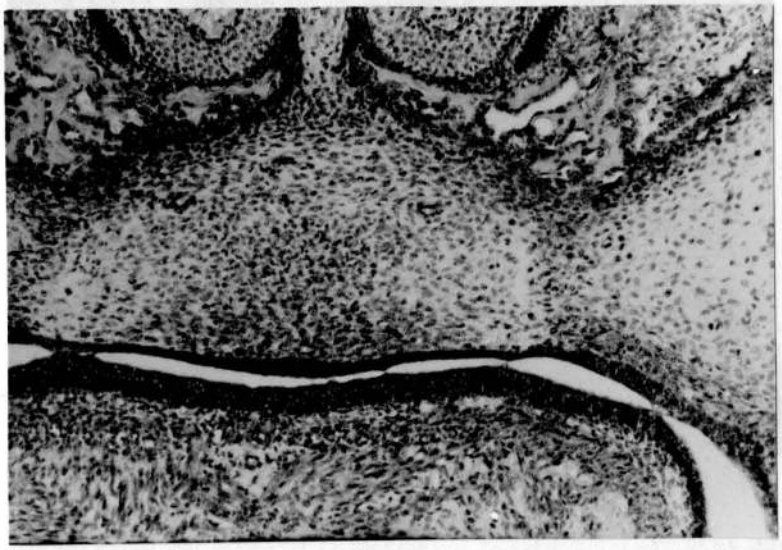

Fig. 1B (100X)

Figure 2A demonstrates an H&E section of a 15½ day mouse with a cleft palate. The palatine shelves are still in a vertical position and the tongue is interposed between the palatine shelves. The tongue appears compressed due to the pressure of the palatine shelves.

Figure 2B demonstrates an autoradiogram of a 15½ day mouse with a cleft palate. The palatine shelf has not undergone horizontalization, but appears at a more oblique angle. The tongue is still situated between the shelves.

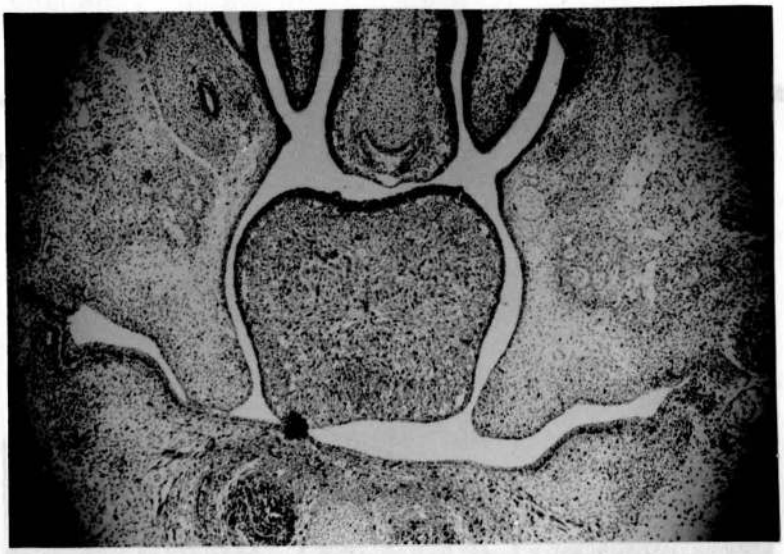

Fig. 2A (40X)

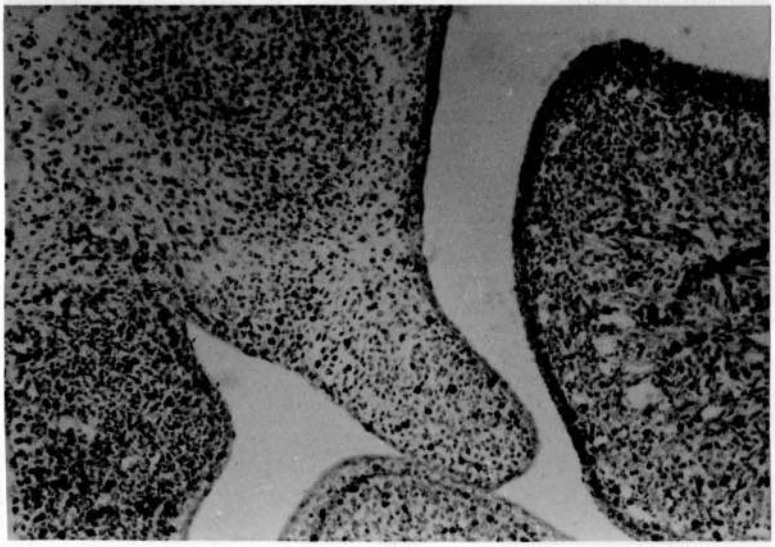

Fig. 2B (100X)

Figure 3A demonstrates an H&E section of a 16½ day normal mouse palate. Figure 3B demonstrates an autoradiograph of a 16½ day normal mouse palate.

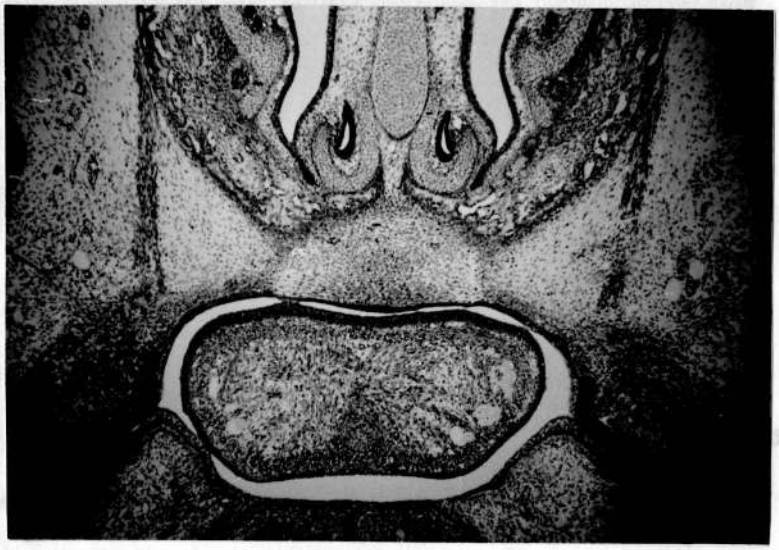

Fig. 3A (40X)

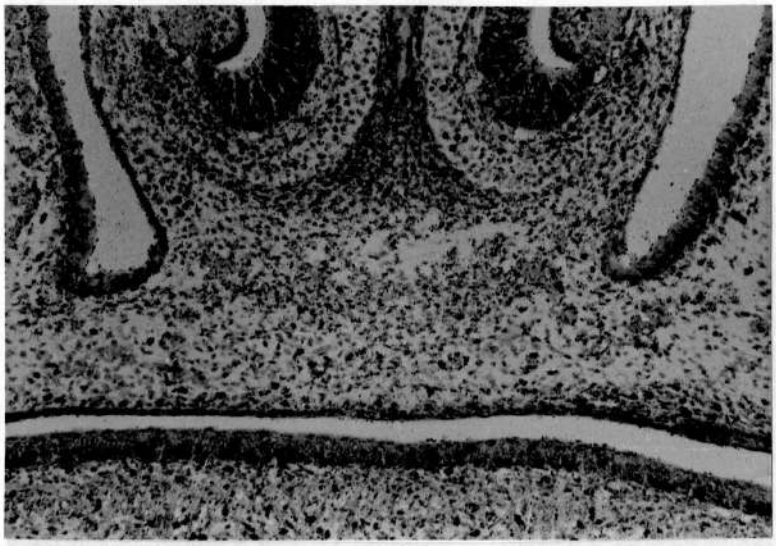

Fig. 3B (100X)

Figure 4A demonstrates an H&E section of a 16½ day mouse cleft palate. The shelves have not yet undergone horizontalization. The tongue is still interposed between the shelves, but is lower and less compressed than in the 15½ day mouse.

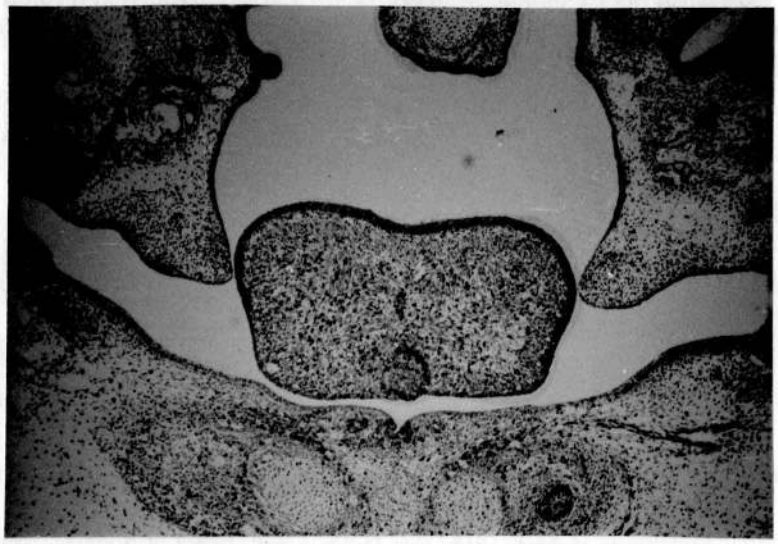

Fig. 4A (40X)

Figure 4B also demonstrates an H&E section of a 16½ day mouse cleft palate in which one shelf is in a horizontal position and the other shelf is still in a vertical position. The tongue has not dropped at this point. The shelves appear to be exerting pressure against the tongue as seen by its shape (i.e. pressed laterally by the vertical shelf and inferiorly by the horizontal shelf).

Figure 4C demonstrates an autoradiogram of a 16½ day mouse with a cleft palate.

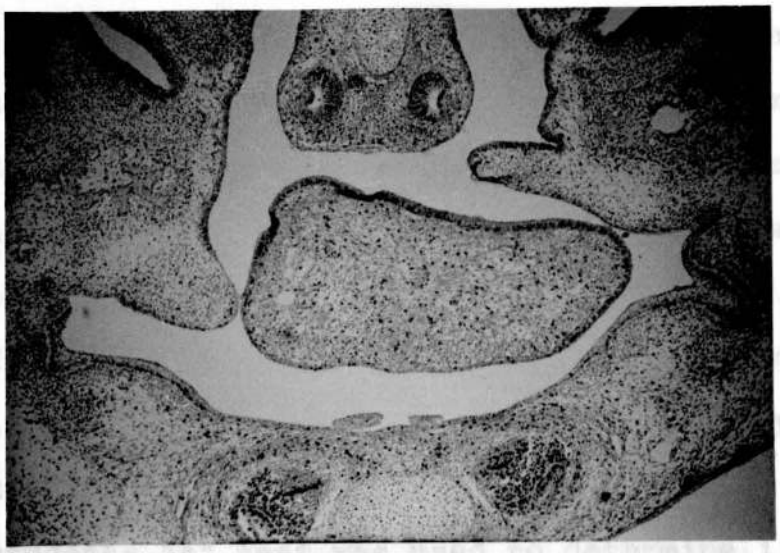

Fig. 4B (40X)

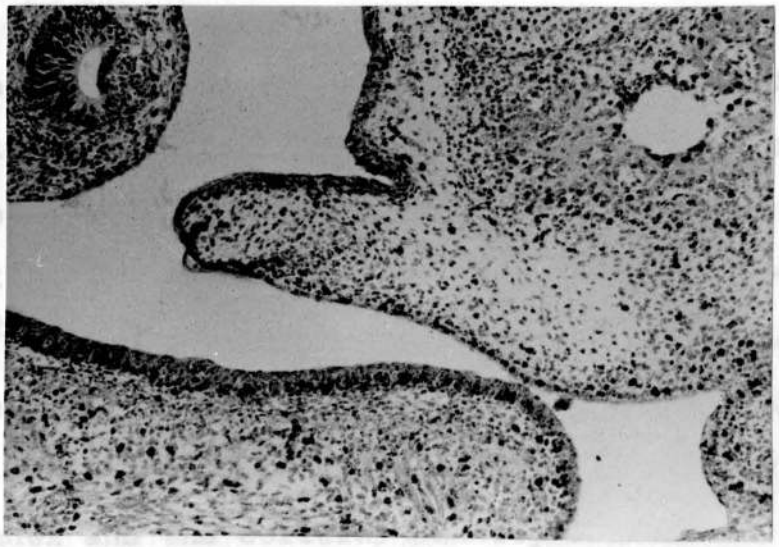

Fig. 4C (100X)

Four fetuses from each age group were used for the mitotic labeling index and cellular density counts. The number of labeled cells per 1000 cells in the 15½ day normal mouse palate was 229, 218, 210, and 223. In the 15½ day cleft palate mouse, the number of labeled cells per 1000 cells was 164, 155, 146, and 149. In the cellular density counts, the average number of cells per .25 mm^2 was 10.13 for the 15½ day normal palate and 12.96 for the 15½ day cleft palate mouse.

The students "t" test was used to determine if there was a significant difference in the number of labeled cells and in the number of cells per .25 mm^2. In both cases, the difference was highly significant ($p = .001$).

In the 16½ day normal mouse palate, the number of labeled cells per 1000 cells was 212, 218, 235, and 208. In the 16½ day cleft palate mouse, the number of labeled cells per 1000 cells was 136, 126, 148, and 130. The average number of cells per .25 mm^2 was 9.25 in the 16½ day normal mouse palate and 13.15 in the 16½ day cleft palate mouse. The students "t" test was also highly significant ($p = .001$) for both the mitotic labeling index and the cellular density counts.

The increased cell density indicates that the cells are packed closer together in the cleft palate mice compared to the

normal mice. The fact that there are more cells per unit area in the cleft palate mice indicates that conversely there is less intercellular substance (acid mucopolysaccharides) within the same unit area.

The palatine shelves consist of a core of loose connective tissue surrounded by epithelium. There was bone present in the base of the palatine shelves, but not in the shelf portion. In the sections stained with PAS, the connective tissues of the palatine shelves were PAS-positive, indicating the presence of neutral mucopolysaccharides. The intercellular substance stained a light magenta and the cytoplasm of the cells stained a darker magenta. The nuclei appeared to be light blue.

In the alcian blue-PAS sections, the intercellular substance stained a light blue indicating the presence of acid mucopolysaccharides. The cells in the base of the connective tissue were a darker turquoise blue. The cell boundary is deeply stained and the stain intensity decreases toward the center of the cell. The bone being formed in the base of the shelves was a dark purple.

In the alcian blue-aldehyde fuchsin sections, the intercellular substance stained a light blue color. The cytoplasm of the cells stained a darker blue compared to a very light

blue nucleus. Both the cartilage of the nasal septum and Meckel's cartilage exhibited a bluish-violet color.

CHAPTER V

DISCUSSION

This investigation shows there are significantly ($p = .001$) fewer labeled cells per 1000 cells in the palatine shelves of cleft palate mice as compared to normal mice. Since the labeled tritiated thymidine is picked up by the cells that are preparing to undergo mitosis, the decreased amount of labeling in the palatine shelves of cleft palate mice indicates that there are less cells being produced in these mice.

Although gross or microscopic measurements were not done in this investigation, it was observed that the palatine shelves of cleft palate mice appeared smaller than those of normal mice. This is substantiated by the fact that there were less cells in the palatine shelves of the cleft palate mice than in the normal mice. It has been shown however, that the palatine shelves of cleft palate mice are smaller than those of normal mice (Walker, 1954). It was also shown that the palatine shelves of cleft palate mice (which undergo horizontalization at day 15/8) are the same size as the shelves of normal mice which undergo horizontalization at day 14/8.

Due to the retarded growth of the palatine shelves of

cleft palate mice, they are unable to keep up with the growth in width of the head. Therefore, once the shelves become horizontal, they are unable to fuse since they cannot come into contact with each other.

The deficiency in the number of cells is also significant since the cells produce the acid mucopolysaccharides. A decreased number of cells therefore indicates there should also be a corresponding decrease in the amount of intercellular substance (acid mucopolysaccharides) produced in the palatine shelves of cleft palate mice. This was substantiated by the cellular density counts. The decrease in the number of cells per .25 mm^2 in the normal mouse palate compared to the cleft palate mouse proved to be highly significant ($p = .001$).

A deficient amount of intercellular substance (acid mucopolysaccharides) at the time of normal horizontalization can be considered a major factor in the development of cleft palate. The synthesis of sulphated acid mucopolysaccharides is considered responsible for the build up of an internal pressure within the shelves. This agrees with Walker (1960) and Larsson (1962) who have shown there is a greater S^{35} uptake in the palatine shelves from day 14/8 to 14/18 than there is from 12/10 to 14/8. They have also shown that there is a

considerably greater uptake of S^{35} in the normal mouse embryonic palatine shelves than there is in the cleft palate mice. This increased S^{35} uptake indicates a sulphated acid mucopolysaccharide synthesis prior to horizontalization of the palatine shelves in normal mice.

It was demonstrated that the upward movement of the palatine shelves is retarded in mouse embryos who have been given cortisone at a prescribed time during gestation. This correlates with the findings of Walker and Fraser (1956) and Larsson (1962). The exact mechanism of cortisone action on the connective tissues of the palatine shelves is unknown however.

Due to the decreased amount of labeled cells in the cleft palate mice, it can readily be seen that cortisone depresses the mitotic activity of the cells. This has also been shown by Bullough (1952) and Roberts (1952).

The decreased amounts of intercellular substance in cleft palate mice indicates that cortisone interferes with the formation of acid mucopolysaccharides by the cells and/or the decreased number of cells is the reason for less intercellular substance.

The process of sulphation increases the gelation and viscosity of the acid mucopolysaccharides. The adhesive

properties of the acid mucopolysaccharides (Toto and Grandel, 1966) are also responsible for the binding of H_2O and electrolytes. A decreased synthesis of acid mucopolysaccharides and/or a chemical alteration (i.e., interference of sulphation) due to the cortisone action would interfere with the binding properties, elasticity and swelling capacities of the intercellular substance. This interference causes a delay in the build up of the internal shelf pressure and thus a delay in the horizontalization of the palatine shelves.

This agrees with Walker (1960) who stated the only modification necessary to enable the intercellular substance (acid mucopolysaccharides) to bring about movement of the palatine shelves is that it should not be in its conformation of maximum stability prior to the actual movement. He thought that the instability might be due to asymetrical deposition and subsequent growth stresses, to growth pressures from outside sources, or to chemical alteration of the material itself. It is not known at present whether such mechanisms are possible, or whether any of them exist in the embryo. However, an interference in the synthesis of acid mucopolysaccharides due to cortisone would not allow the intercellular substance in the palatine shelves to be in the "state of maximum stability" at

the time of normal horizontalization as declared by Walker.

Many cleft palate microscopic sections were seen in which one palatine shelf was horizontal and the other was vertical. The tongue did not appear to have dropped since it was still medial to the vertical shelf. This was described by Walker (1954) who classified it as stage III of palatal closure. He also said there was no sign of the tongue having dropped, thus allowing the shelves to become horizontal as suggested by Peter (1926) and Lazarro (1942). Because this stage of palatal closure appears so frequently, Walker felt that the tongue played a passive role in the closure of the secondary palate.

It is doubtful that the tongue is entirely passive in the process of palatal closure since it must at least change shape to accomodate the changes in shelf position during palatal closure.

It was not possible to determine any difference in the staining intensities of the palatine shelves in cleft palate and normal mice. A system of assigning plus signs according to the degree of intensity was initiated, but was discarded. However, the special stains were excellent in showing the various staining capacities of the acid mucopolysaccharides.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Pregnant female mice were injected with cortisone to induce cleft palates in their offspring. 2.5 mg. of cortisone acetate was injected in the flank of the pregnant female mice on the 11th, 12th, 13th, and 14th day of the gestation period. The cortisone treated mice and the controls were sacrificed at 15½ and 16½ days in utero (one hour after intraperitoneal injection of .5cc tritiated thymidine).

The number of labeled cells per 1000 cells and the number of cells per .25 mm^2 in the base of the palatine shelves were counted.

There were significantly more labeled cells per 1000 cells in the shelves of normal mice than in cleft palate mice. The decreased number of labeled cells indicates there are less cells preparing to undergo mitosis, and consequently, a decreased cellularity in the shelves of cleft palate mice.

There is a greater cell density in the palatine shelves of cleft palate mice than in normal mice. This results in a deficient amount of intercellular substance (acid mucopoly-saccharides). This deficiency in intercellular substance

causes a delay in the horizontalization of the palatine shelves.

The anti-mitotic effect of cortisone and/or a chemical alteration of the cells results in a deficiency in the number of cells and therefore less intercellular substance.

It was not possible to determine any differences in the staining intensities in the palatine shelves of cleft palate and normal mice.

BIBLIOGRAPHY

Asboe-Hansen, G. 1952. The mast cell. Cortisone action on connective tissue. Proc. Soc. Expt'l. Biol. and Med., 80:677-679.

_____1954. Connective Tissue in Health and Disease. Copenhagen, Ejnar Munksgaard, Publisher.

_____1958. Hormonal effects on connective tissue. Physiol Rev., 38:446-462.

Ashton, N. and Cook, C. 1952. In vivo observations of the effects of cortisone upon the blood vessels in rabbit ear chambers. Brit. J. Expt'l. Biol. and Med.,33:445-450.

Baxter, H. and Fraser, F. 1950. The production of congenital defects in the offspring of female mice treated with cortisone. McGill Med. Journ.,19:245-249.

Birke, G. 1953. The dermal spreading of hyaluronidase and streptococci under the influence of stress (cold), ACTH, cortisone, desoxycorticosterone acetate. Acta. Med. Scand.,144:455-466.

Boyd, G. and Williams, A. 1948. Stripping film techniques for histological autoradiographs. Proc. Soc. Expt'l. Biol. and Med.,69:222-232.

Bullough, W. 1952. Stress and epidermal mitotic activity. 1. The effects of the adrenal hormones. Jour.Endocrinology, 8:265-274.

Bunting, H. 1950. The distribution of acid mucopolysaccharides in mammalian tissues as revealed by histochemical methods. Ann. New York Acad. Sci.,52:977-982.

Bone Structures and Metabolism: CIBA Foundation and Symposium. Little, Brown and Co. Boston, 65-75. 1956.

Callas, G. and Walker, B. 1963. Palate Morphogenesis in mouse embryos after x-irradiation. Anat. Rec. 145:61-72.

Cavallero, C. and Braccini, C. 1951. Effect of cortisone on the mast cells of the rat. Proc. Soc. Expt'l. Biol. and Med., 69:222-232.

Coleman, R. 1965. Development of the rat palate. Anat. Rec., 151:107-112.

Cronkite, E., Bond, V., Fliedman, T., and Rubine, J. 1959. The use of tritiated thymidine in DNA synthesis and cell turnover in hemopoietic tissue. Lab. Invest., Vol. 8, No. 1.

Dorfman, A. 1961. Biochemistry of Connective Tissue: Conference on the Biology of Connective Tissue Cells.

_____ 1956. The metabolism of mucopolysaccharides. Proc. Intern. Conf. on the Peaceful Use of Atomic Energy. 12:286.

Firket, H. and Verly, W. 1957. Autographic visualization of DNA in tissue culture with tritiated thymidine. Nature, 181:274.

Fitzgerald, P., Eidenoff, M., Knoll, J., and Simmel, E. 1951. Tritium in Autoradiographs. Science, 114:494-498.

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

Fraser, F. and Fainstat, T. 1951. Production of congenital defects in offspring of pregnant mice with cortisone and other hormones. Pediatrics, 8:527-533.

Fraser, F., Kalter, H., Walker, B., and Fainstat, T. 1954. The experimental production of cleft palate with cortisone and other hormones. J. Cell. and Comp. Physio. 43:237-259.

Gersh, I., and Catchpole, H. 1949. The organization of ground substance and basement membrane and its significance for tissue injury, disease and growth. Am. J. Anat. 85:457-521.

Heiberg, K., Kalter, H., and Fraser, F. 1959. Production of cleft palate in the offspring of mice treated with ACTH during pregnancy. Biol. Neonatal, 1:33-37.

Huges, W., Bond, V., Brecker, G., Cronkite, E., Painter, R., Quaster, H., Sherman, F. 1958. Cellular proliferation in mouse as revealed by autoradiographs with tritiated thymidine. Proc. Nat. Acad. Scd. 44:476-483.

Ingalls, R. 1957. Relation of hydrocortisone injections to cleft palate in mice. New England Journal Med. 256: 1035-1039.

Ingalls, R., and Curley. 1957. Factors influencing the frequency of cortisone induced cleft palate in mice. J. Exp. Zool. 134:449-467.

Isaccson, R. and Chaudry, A. 1962. Cleft palate induction in strain A mice with cortisone. Anat. Rec. 142:479-484.

Jacobs. R. 1964. Preliminary survey of the effects of cortisone upon palate formation, litter size and fetal weight in CD-1 strain of mice. Jour. Dent. Research. 43,#5:715.

Ibid. 1964. Histochemical study of morphogenesis and teratogenesis of the palate in mouse embryos. Anat. Rec. 149: 691-698.

Kalter, H. and Fraser, F. 1953. The modification of the teratogenic action of cortisone by parity. Science. 118:625-626.

Kalter, H. 1954. Inheritance of susceptibility to teratogenic action of cortisone in mice. Genetics. 39:185-196.

_____1957. Factors influencing the frequency of cortisone induced cleft palate in mice. J. Exp. Zool. 134:449-468.

Kalter, H. and Warkany. 1959. Experimental production of congenital malformations in mammals by metabolic procedure. Physio. Rev. 39:69-115.

Lajtha, L., Phil, D., and Oliver. R. 1959. The application of autoradiography in the study of nucleic acid metabolism. Lab. Invest., Vol. 8, No. 1.

Larsson, K., Bostrom, H., and Carsloo, R. 1959. Studies on the closure of the secondary palate, I. Experimental Cell Research, 16:379-383.

Larsson, K. 1961-62. 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. Studies on the closure of the secondary palate, IV. Autoradiographic and histochemical studies of mouse embryos from cortisone-treated mothers. Acta Morph. Neerl. Scand. 4:369-386.

_____1962. Studies on the closure of the secondary palate. Acta Odont. Scand. 20:1-13.

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.

Layton, L. 1951. Cortisone inhibition of mucopolysaccharide synthesis in the intact rat. Arch. Biochem. 32:224.

_____1951. Effect of Cortisone upon chondroitin sulfate synthesis by animal tissues. Proc. Soc. Exp. Biol. 76: 596-598.

Lazzaro, C. 1940. Sul meccanisimo di chiasura del palato secondario. Monit. Zool. Ital. 51:249-265.

Leblond, C., Messier, B., and Kapriwa, B. 1959. Thymidine H^3 as a tool for the investigation of renewal of cell population. Lab. Invest., Vol. 8, No. 1.

Loevy, H. 1962. Developmental changes in the palate of normal and cortisone treated Strong A mice. Anat Rec., 142: 375-390.

McManus, J., and Mowry, R. Staining Methods: Histologic and Histochemical. Heober Inc. New York, 1960.

Meyer, K. 1947. The biological significance of hyaluronic acid and hyaluronidase..Physio. Rev., 27:335-339.

_____1955-56. The chemistry of the mesodermal ground substances. The Harvey Lectures, 51:Copy 2, 88-111.

_____1961. Biochemistry of connective tissue: conference on the biology of connective tissue cells.

Mowry, R. 1956. Alcian Blue. Techniques for the histochemical study of acidic carbohydrates. J. Histochem. and Cytochem., 4:407.

Paff, G. and Stewart, R. 1953. Free wandering cells and cortisone. Proc. Soc. Exp. Biol. and Med., 83:591-592.

Pearse, A. Histochemistry, Theorhetical and Applied. Little, Brown and Co. Boston. 1953.

Pelc, S. 1947. Autoradiographic technique. Nature, 160:749-750.

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

Plotz, C., Howes, E., Blunt, J., Meyer, K., and Ragan, C. 1950. Action of cortisone on mesenchymal tissues. Arch. Derm. Syph., 61:919-921.

Polzl, A. 1904. Sur Entwickelungsgeschiete des manschlichen. Gaumens. Anat. Hefte, 27:244-301.

Pons-Tortella, E. 1937. Uber die bildungsweise des sekundaren. Gaumens. Anat. Ans., 84:1347.

Ragan, C., Howes, E., Plotz, C., Meyer, K., and Blunt, J. 1949. Effect of cortisone on production of granulation tissue in rabbit. Proc. Soc. Exp. Biol. and Med., 72:718-721.

Ragan, C., Howes, E., Plotz, C., Meyer, K., Blunt, J., and Lattes, R. 1950. The effect of ACTH and cortisone on connective tissue. Bull. N.Y. Acad. Med., 26:251-254.

Reed, S. C. 1933. An embryological study of harelip in mice. Anat. Rec., 56:101-110.

Roberts, K., Florey, H., and Joklik, W. 1952. The influence of cortisone on cell division. Quart. J. Exp. Physiol.,

Snell, C. Biology of the Laboratory Mouse (Jackson Laboratory). Blakiston Co. New York. 1941.

Snell, C., Fekete, E., Hummel, K., Law, L. 1940. The relation of mating, ovulation and the estrus smear in the house mouse to the time of day. Anat. Rec., 76:39-54.

Stark, R. 1954. The pathogenesis of harelip and cleft palate. Plastic and Reconstructive Surgery, 13:20-39.

Toto, P. Personal Communication.

Toto, P., and Grandel, E. 1966. Acid mucopolysaccharides in the oral epithelium. Peridontics, Vol. 4, No. 3:127-130.

Walker, B. 1954. Genetico-embryological studies on normal and cleft palates in mice. Ph.D. Dissertation. McGill Univ.

Walker, B., and Fraser, F. 1956. Closure of the secondary palate in three strains of mice. J. Embryol. and Exp. Morph., 4:176-189.

Walker, B. 1960. A special component of embryonic mesenchyme. Anat. Rec., 136:298.

_____1961. The association of mucopolysaccharides with morphogenesis of the palate and other structures in mouse embryos. J. Embryol. Exp. Morph., 9:22-31.

Whitehouse, P. 1960. Effect of cortisone, hydrocortisone and some of their synthetic analogues upon the biogenesis of cartilage in vitro. Acta. Endocrinol Suppl., 51:957.

APPENDIX

TABLE 1

15½ Day Normal

Fetus No.	No. labeled cells/1000 cells	No. cells/.25mm^2				
1-1	229	12	11	10	9	10
		8	10	9	13	11
		11	9	12	11	10
		10	11	10	10	8
		12	10	9	9	10
1-3	218	9	11	10	10	12
		9	10	8	10	9
		10	11	9	10	10
		10	10	12	9	11
		12	10	11	9	11
1-A	210	11	10	10	13	10
		10	9	12	8	9
		9	10	9	10	10
		8	11	12	11	12
		10	9	10	11	9
1-4	223	10	12	10	11	11
		8	9	9	11	11
		9	10	12	10	10
		9	10	9	12	9
		11	10	9	10	10

TABLE 2

16½ Day Normal

Fetus No.	No. labeled cells/1000 cells	No. cells/.25mm^2				
1V-1	212	8	10	8	9	9
		9	8	8	8	11
		8	10	8	9	10
		10	8	9	9	9
		10	9	6	13	8
1V-2	218	11	8	10	9	11
		10	10	11	9	10
		8	9	9	10	9
		10	10	10	10	9
		10	9	10	9	10
1V-6	235	10	7	9	10	7
		7	10	9	10	9
		10	8	10	9	9
		9	10	10	8	10
		8	10	11	12	8
1V-3	208	11	13	12	10	8
		9	8	13	8	7
		10	7	7	10	10
		9	10	9	11	8
		10	7	7	8	8

TABLE 3

15½ Day Cleft Palate

Fetus No.	No. labeled cells/1000 cells	No. cells/.25mm^2				
111-1	164	9	14	14	15	12
		15	12	14	12	9
		17	12	13	17	11
		10	11	15	13	12
		9	12	10	11	12
111-2	155	16	14	14	16	12
		16	14	12	14	14
		16	14	14	13	11
		16	16	16	16	15
		15	16	15	13	13
111-3	146	13	12	12	13	12
		12	12	15	13	13
		15	12	15	14	14
		14	11	13	12	11
		13	14	14	15	12
111-4	149	13	12	13	9	11
		13	12	13	10	11
		10	11	10	11	14
		14	13	15	14	11
		14	14	13	14	13

TABLE 4

16½ Day Cleft Palate

Fetus No.	No. labeled Cells/1000 cells	No. cells/.25mm^2				
11-1	136	14	12	11	10	15
		15	13	10	15	10
		15	13	14	10	6
		11	13	14	14	9
		13	10	10	14	11
11-2	126	14	11	15	12	13
		13	12	15	10	15
		12	15	16	13	12
		15	13	10	14	14
		13	14	12	15	16
11-3	148	13	13	14	14	15
		12	12	10	14	12
		12	11	10	16	11
		14	12	12	14	12
		14	14	9	15	14
11-4	130	15	16	13	16	14
		13	16	15	16	14
		13	15	13	15	15
		15	10	16	16	15
		14	10	14	17	14

APPROVAL SHEET

The thesis submitted by William J. Mott has been read and approved by members of the department of Oral Biology. The final copies have been examined by the director of the thesis and his 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 requirement for the degree of Master of Science.

May 16, 1968

Date

Patrick D. Tate

Signature of Advisor