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# A Preliminary Growth Study of Embryonic a/Jax Mouse Palatal Shelves in Vitro

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A PRELIMINARY GROWTH STUDY  
OF EMBRYONIC A/JAX MOUSE PALATAL SHELVES IN VITRO

BY  
GERALD A. LAMBERTI

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

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1970

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## AUTOBIOGRAPHY

Gerald A. Lamberti was born in Revere, Massachusetts, on December 13, 1941.

In September, 1959, he enrolled at the University of California at Los Angeles in the College of Arts and Science.

He entered Creighton University School of Dentistry, Omaha, Nebraska, in September, 1962, where upon graduation in June, 1966, he received a degree of Doctor of Dental Surgery.

In July of 1966 he enlisted in the United States Air Force and was relieved of active duty in June, 1968. At this time he began his graduate studies in the Department of Oral Biology at Loyola University, Chicago, Illinois.

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## CHAPTER I

### INTRODUCTION AND STATEMENT OF PROBLEM

The last two decades have produced an increased interest regarding the normal embryological development of the secondary palate with special emphasis directed towards the causes of cleft palate. Only recently has a technique been developed to study the normal growth of the embryonic palatal shelves in vitro. This technique utilized embryonic rat and mouse palatal shelves because the mechanism of palatal development is similar to that of humans. Antimetabolites, drugs and various chemical agents which are known to cause cleft palate in vivo have been added to the organ culture media to observe their effect on the development and fusion of the palatal shelves. However, even after extensive research, little agreement exists as to the mechanism of cleft palate production.

It is the purpose of this investigation to conduct a preliminary growth study of embryonic A/Jax mouse palatal shelves in vitro. Furthermore, as growth and fusion are demonstrated, one may then use this model to record quantitatively and qualitatively the effects of metabolites on the palatal shelf tissue before, during and after fusion.

## CHAPTER II

### REVIEW OF THE LITERATURE

#### A. Normal Palatal Development In Vivo

The development of the secondary palate has been studied using human abortus embryos and various laboratory animals. It is generally agreed to that in the early embryonic stage the palatal shelves are vertical with the tongue placed between them. However, various theories have been postulated concerning the sequence and mechanism involved in the movement of the palatal processes to their final horizontal position.

Polzl (1904) assumed that the palatal shelves attain horizontalization by regression of their ventral surfaces and concomitant new growth from their medial surfaces at the level of the tongue. This concept of palatal closure was substantiated by Pons-Tortella (1937).

Peter (1924), in agreement with Schorr (1908), postulated that the palatal shelves achieved horizontalization by rotating medially from their original vertical position.

Reed (1933) believed that horizontalization occurred in the palatal shelves by one or two methods. He agreed with Schaeffer (1920) that differential growth or muscular contraction was responsible for this growth in the horizontal plane and that growth continued in this direction until the shelves approximated one another and eventually fused.

Lazarro (1940) substantiated this idea and further theorized that such a mechanism occurred as a result of a rapid increase of inter-cellular material within the palatal shelves coupled with a simultaneous withdrawal of the tongue from the horizontal plane because of changes in growth and movements of the tongue.

Walker and Fraser (1956) presented seven stages to represent the morphological states leading to palatal fusion:

Stage I: The primary palate and alveolus have already formed. The medial portion of each palatal shelf lies in the vertical plane while laterally the shelf lies in the horizontal plane. The tongue at first lies completely between the shelves, but after a time, the tongue lies ventral to the shelves posteriorly and medial to the shelves anteriorly. At the place where the tongue goes from ventral to medial it makes a grooved impression on the sides of the shelves.

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

Stage III: Shelf activity is not necessarily synchronized bilaterally and a condition can be seen where one shelf lies completely dorsal to the tongue while the other shelf does not. Lazarro (1940) reported the same phenomenon. The tongue has been depressed sufficiently on one side to make space for the horizontal shelf, but it seems unlikely that any actual rotation of the shelf from a vertical to a horizontal position could have taken place.



Stage IV: When both shelves have assumed a horizontal position and lie dorsal to the tongue, they are at first separated by a small space which is soon bridged by further flattening of the shelves.

Stage V: It is at this stage that fusion of shelf epithelium begins.

Stage VI: Epithelial fusion spreads anteriorly and posteriorly.

Stage VII: The shelves are fused throughout their length at this point.

Walker and Fraser indicated that three hours were required for the palatal shelves to approximate one another and another six hours for them to fuse. They suggest that if the tongue were to be displaced, the time for shelf movement could be drastically reduced to one minute; thus the resistance of the tongue accounts for the lengthy period required for shelf movement.

Walker and Fraser discarded the idea of a rotational theory (Schorr, 1908; Peter, 1924; Asling et al, 1960) as being unsatisfactory, since histological study shows that the relationship of the tongue to shelves does not suggest such a movement and because an intermediate shelf condition not involving rotation has been found. Also, the proponents of a growth mechanism (Polzl, 1904; Pons Tortella, 1937) were incorrect regarding the force bringing about a change in shape but were close to being correct in their theory of how the shelves bypass the tongue.

Walker and Fraser finally state that the palatal shelves close by a rapid movement. This movement consists of a bulging of the medial wall and a regression of the ventral wall of each shelf, with the transformation proceeding in a wave-like motion from the posterior end of the shelves to the anterior end. The flow of the palatal shelf tissue into the bulge of the dorsal medial wall carries the shelves dorsal to the tongue and forces the latter into a more ventral position. This was substantiated by Larsson et al (1959). The shelves move by means of an internal force, which increases until it is sufficiently strong enough to drive the shelves dorsal to the tongue. Evidence suggested that this force resides in a network of elastic fibers in the connective tissue of the shelves.

Walker (1961) discarded the elastic fiber theory of shelf movement. Stark and Ehrmann (1958), Loevy (1962), Andersen and Matthiessen (1967) and Frommer et al (1969) have failed to verify the presence of elastic fibers in the palatal shelves.

Larsson (1960), Walker (1961) suggested that an internal force is responsible for palatal shelf movement. A sulfated acid mucopolysaccharide called chondroitin sulfuric acid could be responsible due to its forceful synthesis in the ground substance of the fibroblast zone.

Atnip (1963) studied the role of surface tension in the elevation of embryonic palatal shelves. Before placing the shelves in 0.9%

saline, he displaced the tongue and lowered the mandible. He discovered while the shelves were submerged they did not horizontalize, but when removed from the saline and dried, movement did occur. Atnip concluded that elevation of the palatal shelves in nonsubmerged embryos following displacement of the tongue is due to surface tension rather than an intrinsic shelf force. Ross et al (1967) achieved movement when they submerged their embryos.

Coleman (1965) postulated that palatal closure was achieved rostrally by rotation of the palatal shelves from ventromedial to a horizontal position, in accordance with the rotational theory of Schorr (1908), Peter (1924), Ashling et al (1960). While caudally, closure resulted from outgrowths of the medial surface and a regression of the ventral areas, which is in agreement with Polzl (1904), Pons Tortella (1937).

Andersen and Matthiessen (1967) are of the opinion that mitotic activity, the hydration of mucopolysaccharides and the ingrowth of blood vessels into the laterally oriented mesenchymal areas are responsible for the horizontalization of the palatal shelves.

DeAngelis (1969) suggests that a relationship between glycogen, and an internal shelf force is responsible for shelf transposition. He postulates that glycogen could act as a source of potential energy and a reservoir for building blocks for the formation of mucopolysaccharides.

There is a definite difference in opinion in the literature as to which area of the palatal shelf first begins to change from vertical to horizontal. Walker and Fraser (1956) state that the processes change their position from vertical to horizontal in a wave-like motion that begins posteriorly and proceeds anteriorly. Contrastingly, Pons-Tortella (1937), Coleman (1965), Burdi and Faist (1967), Nanda (1969) have found the opposite to occur.

It is generally agreed to by most investigators that the onset of fusion occurs in the anterior region and continues posteriorly. The difference in opinion is in what specific area of the anterior region. Coleman (1965), Nanda (1969) agree that fusion originates in the middle third of the anterior region. While Stark and Ehrmann (1958) suggest that fusion begins in the incisive foramina region.

#### B. Normal Palatal Development In Vitro

Until recently, there has been very little in vitro experimentation concerning the development of the palate. By this method, growth of the palatal shelves can be studied directly in its entirety without the presence of any extrinsic factors such as the tongue, head, mandible, or apparent changes in maternal physiology.

Moriarty et al (1963) using Sprague-Dawley rats attempted to fuse embryonic palatal shelves in a semidefined medium. The medium consisted of nine and one-half day chick embryonic extract and chicken plasma.

The tissue cultivated contained the maxillary ridges, palatal processes, part of the nasal septum, the primary palate and in most cases the tongue. The initial contact of the cultivated processes occurred in the posterior area. Fusion was assumed when no definite demarcation was noticed between the two tissues. Fusion appeared to begin in the middle anterior third and progressed anteriorly and posteriorly. Moriarty noticed that after twenty-four hours closure was nearly complete, demonstrating an opening only in its middle third. After forty-eight hours fusion was nearly complete except for the posterior region and after seventy-two hours fusion was complete in vitro.

Konégni et al (1965) employing procedures similar to those described by Moriarty et al (1963), cultivated embryonic rat palatal shelves. Two major modifications were instituted, however, for improvement in the overall technique. They are as follows:

1. The sections used were without the tongue present, thus the palatal shelves had already assumed a horizontal position. This change insured a somewhat closer approximation of the shelves at the onset of the experimental procedure.
2. The use of a homologous type medium, namely the embryonic extract and the plasma were obtained from Sprague-Dawley rats. The tissue was cultivated for forty-eight to seventy-three hours and removal was based on the following:

(1) appearance of tissue; (2) tissue and clot deterioration; (3) apparent fusion. Histological appraisal showed that the tissue morphology and distortion were within normal limits.

Reeves et al (1966) cultivated embryonic rat palatal shelves using Eagle's Minimum Essential Medium (MEM) with 10% calf serum. He found that the palatal closure closely resembled Coleman's (1965) findings in vivo.

Pourtois (1966) cultivated embryonic rat palatal shelves in several semidefined media. They were essentially NCTC 109 and Medium 199. To avoid problems with horizontalization the shelves were dissected from the maxillary arch and approximated 1-2 mm apart. He noticed fusion occurred in the anterior and posterior areas first which was different from the normally reported fact that fusion occurred in the middle of the anterior third.

Chaudry et al (1967) cultivated thirteen and one-half day old embryonic A/Jax mouse palatal shelves in Eagle's Basal culture medium and obtained 75% growth and fusion after forty-eight hours incubation.

Myers et al (1967, 1968) cultivated embryonic rat palatal shelves, with the tongue removed, in semidefined and defined (synthetic) media. In the semidefined medium cultivation, 75-90% demonstrated fusion, while with the defined medium 75% showed closure and 50% showed fusion. It was also demonstrated that fusion occurred between the shelves if the

medial margins were manually approximated, regardless whether the shelves were from the same embryo or from littermates or non-littermates.

Pourtois (1967) utilizing a semidefined medium cultivated embryonic A/Jax mouse palatal shelves that were approximated and attained nearly 100% fusion. Even in one group that displayed the presence of a cleft lip fusion was demonstrated.

Vargas (1967) manually approximated and cultivated embryonic A/Jax mouse palatal shelves in a semidefined medium. The embryos were twelve and one-half, thirteen and one-half, and fourteen and one-half days old. The results showed all thirteen and one-half and fourteen and one-half day old embryos fused while the twelve and one-half day old explants did not fuse. He concluded that the potentiality for fusion occurred in vivo about forty hours before actual fusion.

Pourtois (1968) experimented with amniotic fluid and embryonic rat palatal shelves. The palatal shelves were approximated and placed in a purely micromolecular solution. Pourtois concluded that a pure micromolecular solution was not favorable for the achievement of palatal fusion in vitro. However, when amniotic fluid was added to the medium approximately 100% fusion did occur. Pourtois assumed that the proteins contributed by the amniotic fluid played a vital role in the fusion of the cultivated shelves.

Vargas (1968) manually approximated and cultivated fourteen and one-half day old embryonic mouse palatal shelves to complete fusion with

100% success, in a semidefined medium. He also fused embryonic rat palatal shelves with eyelids. Epithelial fusion occurred with great success while mesenchymal fusion was less frequent.

Pourtois (1966, 1968a) determined that fusion occurred in rats at sixteen and one-half days with the potential for fusion occurring in vivo at the fifteenth day.

Angelici et al (1968) by in vitro experimentation found no consistent difference in the manner of fusion of palatal shelves in rat or mouse embryos. The fusion process was discussed in four stages:

1. Differentiation of the cell layers at the edge of the shelves resulted in the formation of a zone of adhesiveness.
2. Fusion of these differentiated epithelial cells lead to the formation of a laminated wall between the shelves.
3. There was a rupture of the partition permitting contact between the elements of the mesenchyme from either side.
4. Finally, there was a degeneration of the epithelial remains of the seam marking completion of the fusion process. This is in agreement with the work of Hughes et al (1967), Pourtois (1966-1968) and Vargas (1967-1968).

Nanda (1969) dissected embryonic Wistar albino rat palatal shelves according to Moriarty et al (1963) and cultivated them to fusion in a



semidefined medium. Contrary to his finding in vivo, he found the palatal shelves approximated one another first in the posterior region and later, or at the same time, in the anterior region. Contrary to the initial site of contact of the palatal processes, fusion at the cellular level commenced in the anterior region as observed in vivo.

### C. The Direct Effect of Various Agents on Palatal Growth In Vitro

Recently, investigations have been conducted in regards to the direct action of certain agents on palatal fusion in vitro. Many of these investigators grew embryonic rat and mouse palatal shelves in a semidefined medium and showed that an inhibition occurs in growth as a result of the actions of teratogenic agents.

In 1967 Lahti and Saxen, using embryonic mouse palatal shelves, added hydrocortisone in concentrations varying from .001 to 20.0 mg/ml to essentially a chemically defined medium fashioned after Biggers et al (1961). The only change was the addition of ascorbic acid in a concentration of 5 mg/ml. It was observed that fusion was retarded, but complete fusion did occur one to three days later.

Myers et al (1967) investigated the effects of methotrexate, a folic acid antagonist, on embryonic rat palatal shelves in a semidefined medium. The majority of the shelves showed closure and fusion, thus no apparent evidence for a teratogenic action of methotrexate is assumed to exist.

Myers et al (1967a) studied the effect of 6-aminonicotinamide and Vitamin A on embryonic rat palatal shelves in a semidefined medium. They noticed a failure of the palatal shelves to fuse upon the addition of 6-aminonicotinamide, even if the shelves were approximated, fusion would not take place. When Vitamin A was added, the failure to fuse at the normal time was a result of retarded development. The tissue competency was not effected as in the 6-aminonicotinamide investigation.

Myers et al (1968) again studied embryonic rat palatal shelves in a semidefined medium. Galactoflavin, a riboflavin antagonist, was investigated and added to the medium. It was noted that a decrease in complete fusion accompanied by an increase in partial fusion occurred in the majority of the samples.

Pourtois (1968a) did an extensive study on the effect of various teratogenic agents on embryonic rat palatal shelves in a semidefined medium. Vitamin A, folic acid and the hydrocortisone, hemisuccinate were studied. The introduction of Vitamin A in excess produced defects in fusion associated with histopathologic changes. This is contrary to the study by Myers et al (1967a). When folic acid was added, the palatal shelves fused normally. The hydrocortisone, hemisuccinate prevented mesenchymal fusion but epithelial fusion was present in isolated areas. This is contrary to the finding of Lahti et al (1967). When hydrocortisone, hemisuccinate and Vitamin A were added together to the medium in concentrations not strong enough alone to produce a defect, a narrowing in the areas of fusion was observed.

In 1969 Nanda noted the effects of Vitamin A and dexamethasone on embryonic rat palatal processes in a semidefined medium. He demonstrated that when Vitamin A is added directly to the culture medium the processes were stunted, retarded in growth and unfused. He suggested that the failure of the processes to fuse was primarily due to the direct action of Vitamin A on the cellular components of the palatal processes. These findings are in agreement with those of Pourtois (1968a). The introduction of dexamethasone caused the shelves to appear small and stunted. The majority of the shelves did not fuse.

Thompson et al (1969), using embryonic rat palatal shelves, concentrated their efforts on introducing Vitamin A and Vitamin A with cortisone into the culture medium. Those cultures treated with an excess of Vitamin A showed a very high percentage of non-fusion. It suggested an alteration in the basement membrane or the intercellular cementing substance. The incidence of histological change relates to the findings of Pourtois (1968a), Nanda (1969). Vitamin A and cortisone when added to the medium caused non-fusion of the palatal shelves corresponding to the results reported by Woollam and Millen (1957). When cortisone was added without Vitamin A, no clefts occurred. This substantiated the majority of the findings in vivo, that maternal injections of cortisone do not produce clefts in rats. In contrast, Nanda (1969) utilizing the steroid Dexamethasone displayed a high incidence of clefts in rats in vivo.

CHAPTER III  
MATERIALS AND METHODS

A. Animals

Mice

Forty-five A/Jax mice (30 female, 15 males) were obtained from the Jackson Laboratory in Bar Harbor, Maine, when they were ten weeks old. These mice were selected because of their previous use in similar studies and their susceptibility to cortisone-induced cleft palate (Fraser and Fainstat, 1951).

Mating

At first during the period when the mice were being mated, males and females were grouped in cages in various ratios. They were grouped together at 4:00 p.m. to the next 8:00 a.m. in a totally darkened environment. At this time, the females were examined for vaginal copulation plugs. The females that demonstrated the vaginal copulation plugs were isolated. The date was recorded on the isolation cage as was the animal's weight. This date was designated as day zero of the pregnancy and subsequent days as one, two, three, etc. When calculating the exact age of the embryos, fertilization was assumed to occur at 2:00 a.m. (Snell et al, 1940).

A second method of mating was employed in order to make our procedure more efficient. Ten to twelve cages were set up and one male

mouse placed in each. They remained in their respective cages isolated until the third day, at that time one female was introduced into each cage. They remained together for five days. The females were examined for vaginal copulation plugs at 8:00 a.m. each morning. If the female demonstrated a plug, the isolation procedure as described above was followed.

## B. Dissecting Procedure

### Recovery of the Embryos

The pregnant A/Jax mice were sacrificed at thirteen and one-half days and at fifteen and one-half days by decapitation. The abdomen was incised and the uterus placed in a standard size sterile Petri dish containing Earle's balanced salt solution (BSS). The embryos were then removed from the uterus and all excess material was discarded. The embryos were grossly observed first by the naked eye and then under a dissection microscope.

### Dissection of the Palatal Shelves

One embryo at a time was placed in a sterile piece of lens paper which was placed in a standard Petri dish of Earle's solution (BSS). The Petri dish was situated under the dissection microscope. The procedure of Moriarty et al (1963) was followed closely for the first group that contained the thirteen and one-half day old embryos. The mouth of the embryo was opened with the help of a pair of tweezers by a light but sufficient force in area of the neck. An incision was made through the

oral area using a fine dissecting scissors separating the maxillary area and cranium from the rest of the body. About 2 mm cranially from the last incision a parallel incision was made with a thin sharp blade, leaving the primary palate, maxillary ridges, palatal processes and part of the nasal septum. In instances where the tongue was still intact, it was removed to allow the palatal shelves to assume a horizontal relationship. Finally, all excess tissue posterior to the palate was incised and removed.

The second group contained nine, fifteen and one-half day embryos. Seven of the embryos were dissected using Moriarty's method (1963). The other two embryos were dissected using Pourtois' (1966) and Vargas' method (1967-1968); i.e., the shelves were dissected out and approximated manually on the grid.

### C. Organ Culture Procedure

#### Organ Culture Medium

The medium utilized for this experiment was Minimum Essential Medium (see Appendix) with a 10% fetal calf serum (Eagle, 1959).

#### Organ Culture Technique

The palatal shelves were cultivated using a sterile Petri dish 8 x 5 mm on to which a nontoxic stainless steel grid cut in a triangular shape was placed. On the stainless steel grid a piece of lens paper 3 mm in diameter was situated which served to prevent the palatal

shelves from attaching to the grid. The lens paper was used in the study involving the fifteen and one-half day old embryos after problems occurred in removing the palatal shelves of the thirteen and one-half day old explants from the grid upon completion of cultivation. The explants were placed on the lens paper in most cases oral side up. One to two ml of medium was placed in the 8 x 5 mm Petri dishes containing the palatal shelves. A falcon plastic disposable organ culture dish 100 x 15 mm was used as a container for the smaller sterile Petri dishes. The dishes were placed in a bell jar and then sealed. An environment of 95% oxygen and 5% carbon dioxide was employed. Incubation was at 37<sup>o</sup> C. and varied from 72 to 120 hours. The culture medium was changed daily in the first study involving the thirteen and one-half day old embryos while the same culture medium was used throughout the period of incubation for the fifteen and one-half day old embryos.

#### D. Histological Procedure

The specimens were fixed in 10% normal formalin upon completion of the culture procedure. After embedding in paraffin, sections were cut in the frontal plane at 6 microns. The sections were stained utilizing haematoxylin and eosin.

## CHAPTER IV

### FINDINGS

A gross evaluation of the explants was made by the naked eye and the dissection microscope, prior to, during and after incubation. Before incubation the thirteen and one-half day old explants displayed very fine and delicate palatal shelves with a considerable separation between them. The fifteen and one-half day old explants demonstrated firmer and thicker palatal shelves with less separation between them as compared to the thirteen and one-half day old explants. In some instances the palatal shelves displayed approximation in the anterior region.

Macroscopically, it was also observed that the embryos from the same mother were not all at the same stage of palatal development. In one such instance two embryos demonstrated undeveloped palatal shelves with a considerable amount of separation between them; while another was partially approximated in the anterior region, and a fourth embryo displayed an anterior bilateral cleft palate. Situations such as those just described were seen quite frequently throughout this study.

The explants were also examined microscopically for: (1) fusion and non-fusion of the palatal shelves; (2) cell appearance and continuity of the mesenchyme, nasal cartilage, nasal epithelium and oral epithelium; (3) shelf position; (4) presence and appearance of Jacobson's organ; and (5) presence and appearance of the eye.



The results regarding fusion were calculated and classified into three groups: (1) no fusion; (2) epithelial fusion; and (3) mesenchymal fusion. Nine of the thirteen and one-half day old explants were incubated for seventy-two hours and one for one hundred and twenty hours. While being removed from the grid after incubation, two of the thirteen and one-half day old explants were badly torn and not included in this study. Of the eight remaining thirteen and one-half day old explants, three demonstrated fusion involving only the epithelium, while the remaining five specimens displayed no fusion.

Of the eight, fifteen and one-half day old explants, one demonstrated an anterior bilateral cleft and was not included in the final analysis. Of the remaining seven explants, three demonstrated complete fusion involving the epithelium and mesenchyme, while the remaining four explants demonstrated no fusion.

In the thirteen and one-half day old and fifteen and one-half day old explants that demonstrated no fusion, it should be mentioned that growth did occur accompanied with an apparent decrease in the original distance between the palatal shelves.

Cell appearance and continuity were also examined. It was demonstrated that the mesenchyme, nasal cartilage and nasal epithelium displayed little or no necrosis suggesting that these tissues responded favorably to the culturing procedure. In contrast, the oral epithelium and its basement membrane were demonstrated in only two specimens.

Microscopically, the palatal shelves of the thirteen and one-half day old explants demonstrated distorted tissues with only those that eventually fused displaying any conformity. Contrastingly, the palatal shelves of the fifteen and one-half day old explants demonstrated much less deviation.

The presence and appearance of the eye and Jacobson's organ which exists as paired tubes, one on the median wall of each nasal cavity (Arey, 1966), were also evaluated microscopically. The eye was quite apparent and occurred with 90% frequency. When the eye was demonstrated its cytologically appearance was unaltered. Jacobson's organ was displayed in approximately 50% of the explants, and when demonstrated it was also cytologically unchanged.

#### Summary of Findings

<u>Age at Onset of Incubation (days)</u>	<u>Explant Number</u>	<u>Incubation Period (hours)</u>	<u>No Fusion</u>	<u>Epithelial Fusion</u>	<u>Mesenchymal Fusion</u>	<u>Percentage of Fusion</u>
13½	8	72	5	3	0	37.5
15½	7	72	4	3	3	42.8

## CHAPTER V

### DISCUSSION

It is possible to grow embryonic A/Jax mouse palatal shelves to fusion in vitro. Fusion occurred in vitro after approximately seventy-two hours of incubation. It was also demonstrated that the cell continuity was maintained in an artificial environment.

The age of the explants before incubation was quite important in attempting to grow the shelves to successful fusion. The thirteen and one-half day old explants displayed palatal shelves that were more delicate and immature than the fifteen and one-half day old specimens. When fusion was observed in the thirteen and one-half day shelves, it was only epithelial in nature. Cell continuity was easily maintained in these specimens but because of the undeveloped palatal shelves and separation between them, fusion was difficult to obtain. Vargas (1967) achieved almost 100% fusion in his study with thirteen and one-half day old explants when he dissected the right and left palatal shelves and approximated them. In the present study involving thirteen and one-half day old explants, the technique of Moriarty et al (1963) was used, which more closely simulates in vivo conditions.

In the study involving the fifteen and one-half day old explants, the palatal shelves demonstrated further development and less separation between the shelves. When fusion was demonstrated, it was more complete than the thirteen and one-half day old explants and involved not only

the epithelium but also the mesenchyme. Cell continuity of the other areas was maintained with little or no necrosis except for the oral epithelium. In contrast, Moriarty et al (1963) and Konegni (1965) reported degeneration of the epithelium of the enclosed nasal chamber after culturing.

Even in cases of no fusion, the thirteen and one-half day old and fifteen and one-half day old explants demonstrated growth of the palatal shelves and a decrease in the separation between the shelves. Nanda (1969) reported a similar finding, and he concluded that the shelves may have not fused because of the large distance between the two processes at the moment of incubation. According to Nanda (1969), another explanation for their non-fusion is the relatively fast downward growth of the nasal septum which could have prevented the palatal shelves from approximating. However, we do not believe this is a factor because the shelves could then fuse to the nasal septum.

In this present study it was observed that embryos from the same mother were at different stages of palatal development. This complicates a meaningful study of fusion, and also makes it difficult to predict with any accuracy the potential for fusion in any given litter. This is contrary to what Vargas stated in 1967. Contrastingly, Toto (personal communication) suggested that the variability in palatal development may be protective in that not all the developing palatal shelves are simultaneously vulnerable to agents or conditions temporarily leading to growth disturbances.

Two of the eight, fifteen and one-half day old explants were manually approximated according to Pourtois (1966, 1967, 1968) and Vargas (1967, 1968). The right and left palatal shelves were dissected from the embryo and placed in the culture medium 1-2 mm apart. Growth was demonstrated in both explants after seventy-two hours of incubation. One explant demonstrated complete fusion of the palatal shelves, while the second specimen was not completely evaluated due to poor orientation when embedded in the paraffin.

The medium utilized was MEM (Eagle, 1959) with 10% fetal calf serum. It was illustrated that this medium was unable to provide sufficient physical support for the palatal shelves of the thirteen and one-half day and fifteen and one-half day old explants when they were placed nasal surface down on the stainless steel grid. Perhaps the use of a semisolid agar medium might prevent the collapse of the palatal shelves. In utilizing a semisolid agar medium, the stainless steel grid with its disadvantageous side effects can be eliminated.

An attempt was made to evaluate the medium as being able to sustain growth for seventy-two hours. In the study involving the thirteen and one-half day old explants the medium was changed every twenty-four hours. In the experiment involving the fifteen and one-half day old explants the medium was inspected daily and left undisturbed for the entire period of incubation. There was a favorable but not appreciable difference in the growth of the fifteen and one-half day old explants

using this technique. It is my opinion that the medium should not be disturbed during the incubation period to allow for the establishment of a proper milieu. Also, a suitable ratio of medium volume to size of the explant is an important concept to consider if growth and fusion are to be achieved.

When difficulty was encountered in removing the thirteen and one-half day old explants from the grid upon completion of incubation, lens paper 3 mm in diameter was placed on the grid before the cultivation of the fifteen and one-half day old explants. The lens paper absorbed the medium and acted as a base for the palatal shelves to grow. The placement of the lens paper between the grid and explant allowed easy removal of the specimen and thus eliminated much distortion of the tissues.

## CHAPTER VI

### SUMMARY AND CONCLUSIONS

Pregnant female A/Jax mice were sacrificed at thirteen and one-half and fifteen and one-half days of pregnancy. The palatal shelves obtained from these mice were cultivated in MEM (Eagle, 1959), with 10% fetal calf serum. The incubation period was seventy-two hours with one explant being incubated for one hundred and twenty hours.

Fusion was demonstrated in vitro in 37.5% of the thirteen and one-half day old explants and 42.8% of the fifteen and one-half day old explants. The thirteen and one-half day old specimens were smaller and much more delicate and promotion of fusion was quite difficult. It is my opinion that in order to enhance fusion the fifteen and one-half day old explants should be utilized with an incubation period of at least seventy-two hours. The explant should be left undisturbed with the initial medium maintained throughout the study.

As a result of observing many embryos from the same mother at different stages of palatal development, a greater percentage of fusion can be obtained by dissecting the right and left palatal shelves and approximating them together.

In general, growth still occurred in explants that did not fuse. Cell continuity was maintained with the exception of the oral epithelium which was generally difficult to identify.

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APPENDIX

COMPONENTS OF MEM, MINIMUM ESSENTIAL MEDIUM (EAGLE)

<u>Component</u>	<u>mg/L</u>
NaCl.....	6800.0
KCl.....	400.0
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O.....	140.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	200.0
CaCl <sub>2</sub> (anhyd.).....	200.0
Glucose.....	1000.0
L-Arginine.....	105.0
L-Cystine.....	24.0
L-Histidine.....	31.0
L-Valine.....	46.0
Choline Cl.....	1.0
Folic Acid.....	1.0
i-Inositol.....	2.0
Nicotinamide.....	1.0
D-Ca pantothenate.....	1.0
L-Glutamine.....	292.0
L-Isoleucine.....	52.50
L-Leucine.....	52.40
L-Lysine.....	58.0
L-Methionine.....	15.0
L-Phenylalanine.....	32.0
L-Threonine.....	48.0
L-Tryptophan.....	10.0
L-Tyrosine.....	36.0
Pyridoxal HCl.....	1.0
Riboflavin.....	0.10
Thiamine HCl.....	1.0
Phenol Red.....	10.0
NaHCO <sub>3</sub> .....	2200.00

## APPROVAL SHEET

The thesis submitted by Gerald A. Lamberti 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.

April 24, 1970

Date

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Advisor