THE NORMAL PALATE AND INDUCED CLEFT PALATE IN RAT EMBRYOS

AN IN VIVO, IN VITRO AND AUTORADIOGRAPHIC STUDY ON EMBRYOLOGICAL DEVELOPMENT

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IN RAT EMBRYOS

An in vivo, in vitro and autoradiographic study on embryological development
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INDUCED CLEFT PALATE
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STUDY ON EMBRYOLOGICAL DEVELOPMENT

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The etiology of congenital malformations in humans has interested embryo-logists, orthodontists, geneticists, pediatricians and workers in other fields of medicine or related sciences for the last century. Cleft palate is one of the most common congenital malformations, and its pathogenesis is not fully understood. Recently, several teratogenic agents have been used successfully in the production of cleft palate in laboratory animals. However, the conclusions drawn from these studies are, most of the time, diverging.

It was felt that an essential contribution to the knowledge on the embryological development of the normal secondary palate and experimental cleft palate could be presented by a combination of in vivo and in vitro experiments employing standardized material and techniques. Several teratogenic agents have been used in an aim to improve our understanding of the pattern of cleft palate formation. In addition, autoradiographic techniques have been employed to evaluate the changes at the cellular and intercellular level during normal or abnormal development of the secondary palate.
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CHAPTER I

GENERAL INTRODUCTION

The development of the secondary palate has interested many workers in several fields of medicine. The secondary palate constitutes the roof of the oral cavity and separates the oral from the nasal cavity.

When the separation between the oral and the nasal cavities formed by the secondary palate is incomplete at birth, this condition is referred to as cleft palate.

Cleft palate in human beings is found in a variety of types and is one of the common congenital malformations. It is a severe and handicapping anomaly. It mars the facial esthetics and disturbs the mastication, deglutition, respiration, and speech. It frequently leads to psychological problems too.

The statistics concerning the incidence of congenital clefts of the lip and palate in humans vary from 1: 770 (Cannon et al., 1951) and 1: 900 (Mac Mohan and McKeown, 1953) to 1: 1270 (Fogh-Andersen, 1942) live births. Its pathogenesis has been studied intensively on human abortus material and on laboratory animals with artificially produced cleft palate. However, still no conformity of opinion exists regarding the mechanism of cleft palate production.

In the present chapter an attempt will be made to put forward the most important theories and controversies regarding the normal embryological development of the secondary palate and the pathogenesis of cleft palate.

A. NORMAL PALATAL CLOSURE

The closure of the secondary palate as described in text-books can be summarized as follows:

The palatal processes in early embryonic stages appear as downward directed vertical projections. At this phase the tongue occupies the space between the two processes and fills also the oro-nasal cavity. At a certain embryonic stage the processes assume a horizontal position superior to the lowered tongue. Then the processes grow towards each other and fuse in the midline.

Uniformity of opinion exists regarding the above mentioned vital aspects of the palatal closure and is considered to be the same for humans and animals. On the other hand, most of the investigations employing human abortus em-
bryos and various laboratory animals as study material, are not in accord with each other regarding: how the palatal processes change their position from vertical to horizontal and from which region this change in position first commences.

Regarding the mode of the change in position of the processes, Peter (1924) presented the more or less generally accepted concept that the processes change their position by rotation. He proposed that the tongue plays an active part in the elevation of the processes and in the change from a vertical to a horizontal position. Schaeffer (1920) stated that differential growth of the processes leads to the change in position. Similarly, Lazzaro (1940) concluded that an increase in the intercellular substance of the processes prior to the change in position plays an important role.

An entirely new view in this respect has been proposed by Pons-Tortella (1937) who explained that the change in position of the palatal processes occurs by a regression at the inferior border of the processes and a concomitant outgrowth from the medial border. The authors mentioned above as well as Pons-Tortella (1937) arrived at their conclusions after studying human abortus embryos. The view of Pons-Tortella (1937) has been supported by Walker and Fraser (1956) who studied mouse embryos, and only regarding the inferior border of the posterior region by Coleman (1965) who studied rat embryos.

Recently several investigations have been conducted in a search for a physico-chemical basis for the movement of the palatal processes prior to the fusion (Larsson, 1960, '62a; Walker, 1961; Jacobs, 1964b, '64d). These studies concluded that mucopolysaccharides play a decisive role in the change in position of the palatal processes.

Regarding the region from which the change in position first commences, very few authors have specified the exact site. Walker and Fraser (1956) reported that the change in position of the processes occurs in a wave-like motion which commences in the posterior region and progresses anteriorly. Recently, a new view has been expressed by Andersen and Matthiessen (1967) that in the human embryos they studied the palatal processes were horizontal in the anterior region from the outset and the elevation movement mostly occurred in the posterior region.

Most of the relevant studies come to the conclusion that the area of initiation of the palatal fusion is located in the anterior region. However, disagreements do exist regarding the precise site of initiation (Stark and Ehrmann, 1958; Coleman, 1965).

It must be pointed out here that extreme caution should be taken into account regarding the comparison of studies conducted on human material and
animal embryos, in which in every species different strains of animals have to be distinguished. On the other hand, it was noted during the review of the present subject that the findings of most of the studies conducted on rats or mice agree with the findings obtained from human abortus embryos. However, contrary to this, the findings of several investigations differ from each other, even when similar material was employed.

Regarding the deviation in opinions created by the findings of the many investigations on the embryological development of the secondary palate, a number of factors are of interest. Most of these have also been recognized by other workers.

a. The differences in interpretation of the findings.
b. Deficient three dimensional survey of the regions (Patten, 1961).
c. Non-standardized histological techniques (Streeter, 1951).
d. Deficient or inaccurate terminology (Andersen and Matthiessen, 1967).

The first part of the present investigation was undertaken to study the events taking place before and during the fusion of the palatal processes in relation to the development of the surrounding areas, namely the nasal septum, the tongue, and the maxillary and mandibular regions. The normal palatal closure was studied in Wistar albino rat embryos both macroscopically and microscopically. Experiments were also conducted to study the palatal closure in vitro for a better understanding of some factors related with fusion which normally are unapproachable in the in vivo investigations. Radioactive isotopes were employed in the in vivo experiments to study the cellular and intercellular activity prior to and during the palatal closure. By combining the in vivo, in vitro and autoradiographic findings, an attempt was made to clarify some of the controversies and to throw an additional light on the subject of the closure of the secondary palate.

B. THE PATHOGENESIS OF CLEFT PALATE

Cleft palate has been studied in detail in human abortus embryos. But the findings of these studies are usually limited due to the small size of the sample. Further, they do not reveal information regarding the cause of the malformity. This holds also true for studies on living children with cleft palate. In humans it is difficult to determine whether the malformation is produced primarily by genetic or by environmental factors.

Much recent information on the pathogenesis of cleft palate has been obtained by animal experiments. It is possible to produce cleft palate in laboratory animals in several ways i.e. by administration of various chemicals and drugs, and by employing certain metabolic and mechanical agents. Further, the inci-
dence of cleft palate can be decreased or increased by inbreeding certain strains of mice. The investigations carried out on experimental animals have presented several conflicting theories regarding the pathogenesis of the induced cleft palate.

An attempt will be made here to describe the most relevant theories, attributed to the production of cleft palate. For a systematic appraisal the theories will be divided into three broad categories, based on the mode of the production of cleft palate.

a. Genetic factors

Warkany and Kalter (1961), reviewing the causes of congenital malformations, stated that the first order of causes is genetic. Fogh-Andersen (1942, '46) studying cleft palate in humans showed that in many cases of isolated cleft palate and cleft lip with or without cleft palate, a genetic predisposition was probably interacting with unidentified environmental circumstances.

Several studies on experimental animals have demonstrated close relationship between the production of cleft palate and the genotype of the mother or that of the foetus (Kalter, 1954, '57; Fraser et al., 1954, '57; Fraser, 1960; Walker and Crain, 1960).

Recently it has been reported that a single recessive gene, the short head, produces cleft palate in mice (Fitch, 1961a, '61b). Several other genes which produce cleft palate have been ascribed to the house mouse (Gluecksohn-Schoenheimer and Dunn, 1948; Bennet et al., 1959; Reed and Snell, 1931).

b. Mechanical factors

Several mechanical factors have been shown to lead to the production of cleft palate. Some of these are anoxia (Ingalls et al., 1950), uterine circulatory arrest (Field et al., 1960), hypothermia (Smith, 1957) and amniotic puncture (Trasler et al., 1956; Walker, 1959; Trasler and Fraser, 1963).

The possible causes of the production of cleft palate after the application of mechanical agents have been summarized by Walker (1959). The basic aspects of these studies will be described somewhere else.

[Maternal radiation has also been reported as one of the causative factors of cleft palate (Russell and Russell, 1952)].

c. Chemical factors

Since the present investigation deals with this aspect to a large extent, a more detailed review of the studies conducted in the past will be given. This will help in sorting out some of the existing controversies.
It has been reported that the majority of the chemical agents given to certain species during the first half of the pregnancy, result in the production of cleft palate (Fraser, 1964).

The most commonly employed teratogenic agents under this category are:


b. General maternal nutritional deficiency (Warkany and Nelson, 1940; Warkany, 1944).

c. Excessive doses of vitamin A (Cohlan, 1953) and also in combination with hormones (Millen and Woollam, 1957; Yamaguchi, 1967; Lotosh, 1968).

d. Cellular toxins such as azaserine (Murphy and Karnofsky, 1956), nitrogen mustard (Haskin, 1948), ethylurethane (Nishimura and Kuginuki, 1958) and 5-fluorouracil (Dagg, 1960).

e. Hormones such as cortisone (Fraser and Fainstat, 1951; Schwartz and Chaudhry, 1968), hydrocortisone (Ingalls and Curley, 1957), ACTH (Heiberg et al., 1959), equine serum gonadotrophin (Nishimura and Shikata, 1958), estrogens (Nishihara, 1958), partial thyroidectomy (Langman and van Faassen, 1955) and Dexamethasone sodium phosphate (Pinsky and DiGeorge, 1965).

f. Other agents such as caffeine (Nishimura and Nakai, 1960), 6-aminonicotinamide (Pinsky and Fraser, 1959), triamcinolone (Walker, 1965) and methylsalicylate (Warkany and Takacs, 1959).

All these agents, given in an effective dose and at a critical stage of the development of the embryo, lead to cleft palate and other associated anomalies. However, controversies do exist regarding how these teratogenic agents act and how they produce cleft palate.

Even in some instances where the same strain of animal and the same drug was used, the obtained results do not conform with each other. Cortisone acetate and hypervitaminosis A have been successfully employed as teratogenic agents to produce cleft palate just as well in the past by others as in the present investigation by the author. Therefore, a brief review of the existing relevant theories will be presented here.

Walker and Fraser (1956, 1957) stated that cortisone acetate, when given to the pregnant mice, interferes with the internal force within the palatal processes, which, in their view, forces them to change their position from a vertical to a horizontal plane. They localized this force in elastic fibers, present in the palatal processes. Later other workers could not support the findings of Walker and Fraser (1956) regarding the presence of elastic fibers (Stark and Ehrmann, 1958; Loevy, 1962; Frommer, 1968).
Larsson (1962b) found that compared to the normal situation the mucopolysaccharide synthesis was considerably less in the palatal processes from cortisone acetate treated mouse embryos.

Several divergent opinions exist regarding the mode of cleft palate production after the administration of high doses of vitamin A.

Giroud et al. (1957) reported that vitamin A has a direct effect on the foetus, as they found an increase in vitamin A content of the liver of foetuses from the treated pregnant rats.

Walker and Crain (1960) suggested that hypervitaminosis A causes delay in the initiation of the movement of the palatal processes in mice. This observation was not supported by Kochhar and Johnson (1965) in their study on rats. However, they confirmed the finding of Deuschle et al. (1959), regarding the presence of maxillo-mandibular ankylosis in mouse embryos with cleft palate. Kochhar and Johnson (1965) did not support the findings of Larsson (1962b), indicated above. Contrary to him, they found an increase in the content of sulphated mucopolysaccharides of the ground substance of the mesenchyme of the palatal processes of rat embryos with cleft palate after the administration of vitamin A.

Takekoshi (1964) concluded that it is impossible to draw any definite conclusion as to the mechanism of the teratogenic action of hypervitaminosis A. But he believed that there was a close relation existing between the teratogenesis and the fall in the blood concentration of the thyroid hormone in vitamin A treated rats.

Woollam and Millen (1960) conducted series of experiments in which they administered vitamin A to rats in combination with different hormones. They found that the incidence of embryos having cleft palate increased when vitamin A was given with cortisone or methylthiouracil, and was decreased when given with insulin and thyroxine. They concluded that their findings lend support to the view that hypervitaminosis A produces its effect by interfering with the carbohydrate metabolism of the developing embryo. Cohlan and Stone (1961), conducting similar experiments, could not support the findings of Woollam and Millen (1960).

This short review shows that the administration of various drugs in abnormally high doses, or the creating of severe dietary deficiencies in laboratory animals, have been studied basically for two main reasons. One is to find out the cause of the toxicity and the second is to study their effect on the various systems of the body including the secondary palate. The natural variation in the maternal protection of the embryo influences the reliability of the results of the in vivo studies. This aspect has been partly overcome by the use of in vitro techniques in the field of teratogenesis.
Only recently organ culture methods have been employed to study the palatal closure (Moriarty et al., 1963) and the effect of some teratogenic agents on the normal palatal closure in vitro (Myers, 1967; Pourtois, 1968). In vitro methods have many limitations, but, on the other hand, they make it possible to study the dynamic processes, associated with the palatal closure, under normal and abnormal conditions in a longitudinal way.

In the present investigation three drugs namely, vitamin A, cortisone acetate, and Dexamethasone, and the combination of the first two drugs were administered to pregnant Wistar albino rats to study their effect on the development of the secondary palate, and when, how and why, if at all, it deviates from the normal development. Histological techniques were used in the study of the in vivo and in vitro material. They were further applied in combination with autoradiographic methods.
CHAPTER II

MATERIAL AND METHODS

This chapter will deal with the general information regarding the material, methods, and techniques used in the present investigation. The details of the individual experiments will be given in the pertinent chapters.

GENERAL REMARKS

In the present investigation, Wistar albino rats were used. The rats were obtained from T.N.O. Delft, The Netherlands. The animals were kept under normal laboratory conditions in the Central Animal Laboratory (Head: Dr. vet. M. J. Dobbelaar) of the Faculty of Medicine, University of Nymegen, The Netherlands.

The Wistar strain was chosen because information was found to be lacking on this particular strain regarding the prenatal development of the normal secondary palate and the development and pathogenesis of the experimental cleft palate. Furthermore, additional information regarding behaviour, reproduction pattern, and growth and development of the craniofacial complex of this strain was available from previous investigations conducted in the Department of Orthodontics of the University of Nymegen (Duterloo, 1967; Prahl, 1968; Vilmann, 1968; Jeffers, 1969).

a. Embryo age determination

The female Wistar rats were placed together with the male rats of the same strain from 4.00 p.m. to the following 8.00 a.m. Upon this the vaginal plug was looked for and vaginal smears were made as an indication for copulation. It was assumed that in cases of positive recording, copulation had taken place at midnight. The day following was indicated as day 0 and subsequent days as 1, 2, 3, and so on.

The pregnant rats were put in separate cages, in groups of 2 or 3 and fed with Hope Farms rat biscuit. Water was available ad libitum.

1 De Centrale Organisatie voor Toegepast-Natuurwetenschappelijk Onderzoek.
1 The Central Organization for Applied Scientific Research in The Netherlands.
In all the experiments about half of the pregnant rats were sacrificed at a particular day by decapitation at 9 a.m. and the remaining ones at 4 p.m. This resulted in two groups of embryos with about 7 hours of difference in age, and, by this, the obtainable information was increased.

b. Selection of teratogenic agents

Vitamin A palmitate\(^1\) and two steroid preparations, namely cortisone acetate and Dexamethasone phosphate\(^2\), were employed in the present investigation to study experimental cleft palate. Vitamin A was preferred as one of the teratogenic agents for the production of cleft palate on the basis of the information presented by Cohlan (1953), Giroud and Martinet (1955, '56), Millen and Woollam (1957) and Kochhar and Johnson (1965). One of the other two drugs, cortisone acetate has also been used successfully by several workers for the same purpose as the one of this study (Fraser and Fainstat, 1951; Walker and Fraser, 1957; Chaudhry and Siar, 1967).

Vitamin A palmitate and both steroid preparations were administered to pregnant rats in different concentrations after dilution in normal saline.

c. Dosage of drugs

The proper dose to obtain cleft palate in experimental animals is rather critical. A too high dosage of a teratogen given to a pregnant mother can kill her or may lead either to resorption of the embryos or to excessive anomalies. Preliminary experiments showed that the production of congenital anomalies, including cleft palate, was usually effected by the dosage between 20,000 to 60,000 I.U. of vitamin A given once a day, and by 0.25 mg to 1.0 mg of Dexamethasone twice a day. Cortisone acetate did not appear to lead to the effects aimed for. The dosage investigated for that purpose ranged from 2.5 mg to 10 mg twice a day. Cortisone acetate has been used in this study mainly in combination with vitamin A.

d. Mode of drug administration

Excessive amounts of vitamin A produce many kinds of malformations when given to pregnant rats by gastric tube (Cohlan, 1954), but not intraperitoneally (Gebauer, 1954) or subcutaneously (Millen and Woollam, 1957).

After studying the consequences and effects of different ways of administra-

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1 Commercial name: 'Arovit', Roche, Basle, Switzerland.
tion of vitamin A, the gastric tube approach was preferred for the present study. Both steroid preparations were administered subcutaneously to obtain an effective level of circulating steroid and to avoid serious alterations in the maternal metabolism (Greenspan et al., 1953, Gunberg, 1957).

**e Period of drug administration**

Various studies in the past have shown that the age of the embryos at the first day of administration of the teratogenic agent and the length of the period of drug supply are of essential importance in controlling the type, the degree, and percentage of the anomalies produced (Giroud and Martinet, 1955, ’56, Baxter and Fraser, 1950, Fraser et al., 1954, Walker and Fraser, 1957).

On the basis of the findings reported in the literature and the results of our preliminary studies, the drugs were administered on either day 8, 9, or 10 of gestation and were given until day 11, 12 or 13.

**EXPERIMENTS IN VIVO**

The in vivo experiments were conducted to study the development of the normal secondary palate and the cleft palate macroscopically, microscopically and by employing autoradiographic techniques.

**a Administration of drugs**

Vitamin A was injected directly into the stomach by a rubber tube attached to an injection syringe. Both steroid preparations were administered subcutaneously. For the three drugs and in all concentrations, 0.5 ml of normal saline was used as dilutant. No anesthesia was employed.

**b Recovery of the embryos**

The pregnant rats were sacrificed by decapitation. The abdomen was opened and the embryos were recovered after taking the uterus out. They were freed of their membranes and placed in 90 x 15 mm Petri dish containing normal saline. The gross examination of the embryos was made first by naked eye and subsequently under the dissection microscope.

With the help of a sharp blade the tongue and the mandible of a few embryos of each rat were removed. This provided special access in the examination of the morphology of the palatal area.

**c Histologic techniques**

The embryo heads and, in some instances, the whole embryos were fixed in Bouin’s solution. After embedding in paraffin, sections of 7μ thickness were
cut in frontal and sagittal planes. The paraffin sections were stained with haematoxylin-eosin (Delafield), toluidine blue (0.05% in aqua dest), tri-chrome (Goldner), and Van Gieson's staining methods. The haematoxylin-eosin was used for the overall picture, Toluidine blue for the detection of mucopolysaccharides, and the last two mentioned as connective tissue stains.

d. Autoradiographic techniques

Radioactive sulphate\(^1\) \((^{35}\text{SO}_4)\): Radioactive sulphate was employed in the present investigation to study the metabolism of sulphated-mucopolysaccharides in the normal and abnormal development of the secondary palate. A single injection was given intraperitoneally to rats on the 13th, 14th and 15th day of pregnancy. The dosage used was 20 μC/gm body weight of the pregnant rat. The rats were sacrificed 48 hours after the administration and the embryos were fixed in Bouin's solution for one day.

The embryo heads were embedded in paraffin and sectioned serially. To obtain easily comparable autoradiographic results, the paraffin sections of the corresponding areas of the normal embryos were placed with those of the experimental ones next to each other on the same slide. The autoradiographs were prepared using the stripping film, Kodak AR 10 (Wijffels, 1968). The exposure time was 3 weeks. Later the sections were stained with haematoxylin-eosin (Mayer) and toluidine blue (Bergeron, 1958). The findings were evaluated microscopically and by studying the photographs made of the areas of interest.

\textit{Tritiated thymidine}\(^2\) \((^{3}\text{H-Tdr})\): Tritiated thymidine was injected directly into the amniotic sac of the living embryos of normal and drug treated rats on days 16 and 17 of gestation to study the cell proliferation of the palatal processes before and after their fusion with each other.

For that purpose the rats were anaesthetized by ether and the abdomen was opened by making an about 3 cm long incision. The first three embryos from the right cornua of the uterus of each rat were gently taken out of the abdominal cavity and 15 μC of tritiated thymidine was injected into their amniotic sac. After this the embryos were placed back in the abdominal cavity and the incised area was sutured.

The rats were anaesthetized again after 4 hours and the sutures were opened. The embryos injected with tritiated thymidine were recovered and fixed in Bouin's solution. The method used prior to the autoradiographic treatment was the same as described for radioactive sulphate. The autoradiographs were

\(^1\) Radiochemical Center, Amersham, England. Sodium Sulphate-\(^{35}\text{S}, S.J. 162, 100 \text{mCi/mM.}\)

\(^2\) Radiochemical Center, Amersham, England. Thymidine (methyl-T) 5 Ci/mM, TRA. 120.
prepared using the dipping method with Ilford K-5 in gel form (Wijffels, 1968). The sections were stained with haematoxylin-eosin (Mayer).

EXPERIMENTS IN VITRO

The in vitro experiments were conducted to study the development and fusion of the palatal processes of the normal and drug treated rat embryos. The embryos were recovered at varying ages of gestation, which will be described in detail later in the study.

a. Operating procedures (Figs II-1, 2)

The pregnant rats were killed after ether anaesthesia. The abdomen was opened systematically, first by cutting the skin, and then the muscles. Under sterile conditions the uterus was gently taken out and was placed in a Petri dish containing Hank's balanced salt solution (BSS). The embryos were then removed from the uterus and after being freed of their membranes, were placed in another Petri dish containing Hank's BSS.

The technique of Moriarty et al. (1963) was employed to prepare expiants for organ culturing. One embryo at a time was taken out from the Petri dish and was placed in another one on top of a 4 × 4 cm gauze pad saturated with Hank's BSS. The mouth of the embryo was opened by pressing at the neck region with the help of a pair of tweezers. Through the oral opening, an incision was made with a thin blade, thus separating the head portion of the embryo from the mandibular region and rest of the body. The maxillary portion of the head was resected by making an incision parallel to the previous one at 1 to 1.5 mm more cranially. The tissue lying posterior to the palatal processes was cut and discarded. Finally, the tongue was removed if still found to be lying between the palatal processes.

The tissue obtained in the way just described for culturing included the primary palate, the maxillary ridges, the palatal processes and part of the nasal septum. It was about 3 to 4 mm in length, 3 to 4 mm in breadth and 1 to 1.5 mm in depth.

b. Organ culture method (Figs II-3, 4)

The basic medium used in the present study contained NCTC 109 mixed with bovine serum, to which Penicillin was added as suggested by Pourtois (1968). NCTC 109 is a sterile chemically defined medium, prepared according to the

1 Baltimore Biological Laboratory, BioQuest Division, Cockeysville, Maryland, U.S.A.
formula of Evans et al. (1964). The amounts of the three ingredients used in
the final culture medium were as follows:

a. NCTC 109 45.00 ml
b. Bovine serum 5.00 ml
c. Penicillin 5000 IU

The explants were cultured in Falcon\(^1\) plastic disposable organ culture dishes.
The dish has a trough on its outer periphery and a well in its center which
is 19 mm in diameter and 4 mm deep. One ml of culture medium was placed
in the center well of the dish and 5 ml of distilled water in the outer trough
containing an absorbant ring for humidity control. The explant was placed in
the center well on top of an Organ Culture Grid\(^1\), made of nontoxic stainless
steel. The shape of this grid is triangular and its legs extend approximately
13 mm from the center. The tips of the grid are stepped down to 1.5 mm to
fit over the center well. The complete organ culture procedure was carried out
under sterile conditions.

The dishes were closed and incubated at 37\(^\circ\)C. The period of incubation
varied from 24 hours to 8 days. The same culture medium was used throughout
the period of incubation, except in some exceptional instances.

c. Addition of teratogenic agents to the basic medium

Vitamin A and Dexamethasone were added to the basic medium to study the
direct action of both drugs on the development and fusion of the normal palatal
processes.

The drugs were added in the varying concentrations, ranging for vitamin A
from 10 to 50 I.U. and for Dexamethasone from 0.01 to 7.5 \(\mu\)gm, in 1 ml of
basic medium. Ethanol was used as dilutant for the drugs. In some cases
distilled water was also used as a vehicle. The final volume of the vehicle in
1 ml of basic medium was 0.0015 ml.

After the termination of the incubation period the cultured explants were
fixed in Bouin's solution, embedded in paraffin, sectioned serially, and were
stained with haematoxylin-eosin (Delafield) and toluidine blue (0.05\% in aqua
dest). The thickness of the sections was 7 \(\mu\).

\(^{1}\) Baltimore Biological Laboratory, BioQuest Division, Cockeysville, Maryland, U.S.A.
II-1. Diagrammatic representation of the cutting procedure employed for preparing explants. (A) denotes the first cut separating the head from the mandibular region and the rest of the body. (B) is the second cut, cranial to the previous one, separating the maxillary portion from the remaining part. (C) denotes the part used for culturing.

II-2. Oral view of the cut part (C) obtained by the dissection procedure indicated in the previous diagram. The tissue posterior to the palate was cut and discarded. The part used for culturing includes palatal processes (P), primary palate (PP), maxillary ridges (MR), and labial area (L).
II-3 Diagram showing organ culture dish. The dish contains a center well (A), absorbent ring (B) for humidity control, and stainless steel grid (C). For culturing, one ml of culture medium was placed in the center well (A). The explant was placed on top of the stainless steel grid (C) and 5 ml of water was poured on the absorbent ring (B).

II-4 Organ culture dish is closed, explant is in its place, and whole set-up is ready for incubation.
CHAPTER III

THE DEVELOPMENT OF THE NORMAL SECONDARY PALATE IN VIVO

INTRODUCTION

As described in Chapter I, several divergent theories have been presented on the prenatal development of the secondary palate. The differences of opinion are mainly concentrated on:

a. how the palatal processes assume a horizontal position prior to fusing with each other;
b. where the change from the vertical to the horizontal position of the palatal processes commences, and,
c. where the initiation of the fusion of the horizontally oriented palatal processes begins and how it proceeds.

The experiment was undertaken, firstly, to study these three aspects, and secondly, to obtain information on the normal palatal development in Wistar albino rat embryos. This will serve in the evaluation of the experimentally induced palatal abnormalities to be described later on.

MATERIAL AND METHODS

A total of 20 pregnant Wistar albino rats was used in the present experiment. Four rats were sacrificed each day, from day 14 to 18 of gestation. From each mother 4 embryos were obtained for histologic study and the remaining ones were analysed macroscopically. In total 16 embryos of each day were studied histologically and the number evaluated macroscopically ranged from 15 to 22.

As described in the previous chapter, the embryo heads used for histological study were fixed, embedded, serially sectioned, and stained with haematoxylin-eosin (Delafield) and toluidine blue.

FINDINGS

14 day old embryo (figs III-1 and 5)

The palatal processes were found to be positioned in a downward vertical direction, nearly parallel to each other. The space between them was occupied
by the main bulk of the tongue, which showed slight concavities on its lateral margins corresponding to the palatal processes. This phenomenon was more marked in the anterior than in the posterior region.

The mass of the palatal processes was composed of loose and undifferentiated mesenchymal cells. It was lined by 1 or 2 layers of cuboidal epithelial cells on its projected ends, increasing to 3 or 4 layers on the medial and lateral surfaces.

No essential systematic differences were observed between the embryos whose age differed by 7 hours. But, in a few of the older specimens, the processes in the anterior region were slightly inclined to the medial.

15 day old embryo (figs III-2, 6 and 7)

The palatal processes were found to be positioned inferio-medially in the anterior region (fig. III-6) and vertically in the posterior region (fig. III-7). The cellular picture of the processes was quite similar to the one observed at day 14 of gestation.

No systematic differences were observed between the embryos separated by a 7 hour difference in age.

Compared to the situation observed in the 14 days old embryos, the processes were bulbous, particularly in the anterior region. Furthermore, the concavities on the lateral borders of the tongue were more pronounced, particularly in the anterior region. Overall, the contours of the tongue, here also, corresponded to those of the oro-nasal cavity.

The size of the embryo heads was considerably larger in the older than in the one day younger animals. In the sagittal sections, this was clearly seen for the mandibular and nasal areas (fig. III-2). In these same sections, the tongue was found to be less curved than in the younger specimens.

16 day old embryo (figs III-3, 8, 9, 10a, b, c, d, e, and f)

Anteriorly, the palatal processes were found in a horizontal position and now superior to the tongue (fig. III-8). Posteriorly, the processes were also horizontally oriented but were small in size. There, the superior surface of the tongue was more curved and corresponded also to the contour of the processes (fig. III-9). The space present between the two palatal processes was the smallest in the middle third of the anterior half.

The mesenchymal tissue of the palatal processes was found to be slightly organized and the cell density was more intense near the future fusion area. The area of the processes facing each other was lined by 2 or 3 layers of columnar epithelial cells, while the nasal and oral surfaces were lined by 4 or 5 layers.
Mesenchymal condensation was clearly visible at the future maxillary area (fig. III-8).

Noticeable differences were seen in some of the specimens of 16.9 days and 16.16 days of age respectively. In 2 of the 8 embryos of 16.9 day group, the palatal processes were oriented horizontally in the anterior region but vertically in the posterior. On the other hand, one of the 8 embryos of 16.16 days showed fusion of the palatal processes in the middle third of the anterior half (figs III-10a, b, c, d, e, f). These variations, observed in 16 day old embryos, can be possible because of the lack of knowledge of the exact time of conception in the allotted mating period.

Compared to the picture observed in the 15 day old embryos, the palatal processes here were narrow medially and flaring out in the lateral region, instead of being bulbous. Furthermore, the size of the mandibular and nasal areas was considerably larger. In the sagittal sections, the tongue was found to be flat in the anterior half, while lying still curved in the posterior region (fig. III-3), as compared to the previous age.

17 day old embryo (figs III-4, 11 and 12)
The palatal processes were found to be directed horizontally over their entire length, and were fused. The fused palate was more arch-shaped in the posterior than in the anterior region. At the anterior end (future incisive foramina area) the union between the palatal processes and primary palate was incomplete. The tongue was observed to be well contained in the oral cavity.

The mesenchyme appeared to be organized and was composed of loosely arranged stellate cells. The fusion at the cellular level ranged from epithelial contact of the processes to complete disintegration of the epithelium. The epithelium prior to, and during contact was composed of oval or columnar epithelial cells. During the epithelial fusion of the processes, the cell layers numbered from 1 to 3. Epithelial remnants were observed in the areas where the complete mesenchymal fusion of the palatal processes had taken place. As compared to the previous day, an extensive osteogenic activity was observed at the future maxillary area, with bony projections into the lateral parts of the palate and into the future alveolar area (figs III-11 and 12).

In the sagittal sections, the superior surface of the tongue appeared to be flat overall (fig. III-4), and in a few embryos its tip was found to be projecting out of the oral opening. Furthermore, the nasal and mandibular areas were considerably larger, as compared to the previous day.

18 day old embryo (figs III-13 and 14)
The palatal fusion was found to be complete in all embryos. The palate appeared
to be more arch-shaped as compared to the previous day. A marked mesenchymal condensation was observed at the fusion area. The future maxillary and alveolar areas showed extensive osteogenic activity.

INTERPRETATION OF THE FINDINGS

The change of the palatal processes from the vertical to the horizontal position was found to take place in a relatively short period of time. Prior to this change, the processes, particularly in the anterior region, were more bulbous and the tongue showed concavities on its lateral surfaces, that corresponded with the contours of the processes. After the change in the position of the processes, their bulbous-like appearance was lost, and the superior surface of the tongue was more flat. Further, the nasal and mandibular areas were considerably larger than before, and initiation of osteogenic activity was now found in the future maxillary areas.

The change from the vertical to the horizontal position of the palatal processes appeared to have commenced at the anterior end and progressed posteriorly. This is based on the observation that in some embryos the processes were found to be directed medially in the anterior region while still vertical in the posterior one.

The fusion of the processes started at the middle third of the anterior half and proceeded anteriorly and posteriorly. This phenomenon was clearly seen in 2 embryos, one belonging to the group of 16.16 days and the other to 17.9 days. It was also noted that the complete fusion was first achieved in the anterior region.

At the cellular level, the fusion of the processes showed up in three distinct phases. Firstly, the epithelium of the opposing palatal processes fused in the midline. Shortly before this fusion, 2 or 3 layers of epithelial cells lined the approaching areas of both processes. After the fusion had taken place, only 1 or 2 cell layers of epithelium were present in the midline. The second phase was the breakdown of the epithelium at some places with subsequent penetration by the mesenchymal cells. In the third phase, a complete mesenchymal union was seen. However, epithelial remnants at some places in the fusion area were observed. No organized distribution of the epithelial remnants in the fusion area was noticed.

The morphology of the processes in the anterior and the posterior regions was different, both prior to and after the fusion. Before the fusion, anteriorly, the processes were narrow medially and flaring out laterally. Posteriorly, they appeared short and slightly medially rotated, where in the overall picture, the
processes followed the convex contour of the superior surface of the tongue. After the fusion, the palate was horizontal anteriorly and arch-shaped posteriorly. It seems that the arch-shaped appearance of the palate was associated with the increase in size of the future alveolar area.

DISCUSSION AND CONCLUSIONS

The normal prenatal development of the secondary palate has been investigated by many authors. Human abortus embryos have been studied, and various laboratory animals have been used in experimental work in palatal closure. Several theories have been presented regarding the way, in which the change in position of the palatal processes, leading to their ultimate fusion, takes place.

An attempt has been made in the present chapter to study the sequence of events resulting in the palatal fusion in Wistar albino rats. The discussion deals, first, with the change in position of the palatal processes, second, with the fusion and lastly with the role of the tongue.

Lazzaro (1940) studied the palatal fusion in human embryos and reported that the change in direction of the palatal processes from vertical to horizontal was very rapid. He even cited an embryo having one process vertical and the other horizontal. The same phenomenon was also observed by Walker and Fraser (1956) in their study on mice.

The present investigation also shows that the change in direction of the palatal processes takes place in a relatively short period of time. However, in our material no marked asymmetries were observed.

No uniformity of opinion exists in the literature regarding the mechanism that leads to the change in position of the processes. Most authors are of the opinion, that a number of factors are working together. However, varying significances have been attributed to each. Thus one theory proposes that a considerable increase in the intercellular substance of the connective tissue is the main factor (Lazzaro, 1940). Another theory assumes that the change is caused by an inward bulging of the medial side of the processes concurrent with a regression of their inferior border (Pons-Tortella, 1937; Walker and Fraser, 1956). The oldest theory is the one that considers a rotation of the processes as the essential factor in the change in position of the processes (Schorr, 1908; Peter, 1924; Asling et al., 1960). Furthermore, the opinion has been expressed that the transition of the processes occurs by a combination of the two last mentioned factors so that rotation of the processes takes place in the anterior region, with a concurrent medial outgrowth and regression of the inferior border in the posterior region (Coleman, 1965). Walker and Fraser (1956) were of the opinion that the change in position of the processes, that they described
as taking place in a wave-like motion, was due to an internal factor which they named as the elastic fibers. A search for the presence of the elastic fibers in the palatal processes by other authors did not reveal positive results (Loevy, 1962; Stark and Ehrmann, 1958; Andersen and Matthiessen, 1967). Later, the theory regarding the role of elastic fibers was withdrawn (Walker, 1961) and support was given to Larsson's (1960) point of view that the internal factor was due to mucopolysaccharide activity. A more recent theory states that the pronounced mitotic activity, the hydration of mucopolysaccharides and the ingrowth of blood vessels into the laterally oriented mesenchymal areas, together play an important role in the elevation of the processes (Andersen and Matthiessen, 1967).

The present investigation revealed that the processes undergo slight bulging at their inferior half prior to the change in the direction. This observation was noticed in nearly all of the 15 day old embryos, particularly in the anterior region. In the posterior region, this bulging in the medial direction took place relatively late, that is at day 16 of gestation. This conforms to Lazzaro’s observation on human abortus embryos. On the other hand, the present findings did not reveal either an increase in the intercellular substance (Lazzaro, 1940), or medial outgrowth and concurrent regression of the inferior border of the processes (Pons-Tortella, 1937; Walker and Fraser, 1956; Coleman, 1965) or an increase in the blood vessels (Andersen and Matthiessen, 1967). It may be mentioned here that the serial frontal sections of the 15 and 16 day old embryos showed that possibly the change in position of the processes was affected by rotation of the vertically directed processes (Peter, 1924).

Controversy also exists in the literature regarding the region from where the change in the position of the processes starts. In this respect there are two main opinions, first, that the transformation starts anteriorly and proceeds posteriorly (Pons-Tortella, 1937; Burdi and Faist, 1967; Coleman, 1965), and second, that it begins posteriorly and proceeds anteriorly in a wave-like manner (Walker and Fraser, 1956). Contrary to these theories, Andersen and Matthiessen (1967) reported that in human abortus embryos, the processes are horizontal in the anterior region from the outset, and later, the rest of the palate is formed by the elevation of the vertical processes.

The findings of the present study show that the elevation of the processes started in the anterior region and proceeded posteriorly. This observation is based on the fact that in some embryos on day 15 and 16 of gestation the processes were horizontally or medially oriented in the anterior region, while posteriorly they were still vertical. This finding is in accordance with other authors (Pons-Tortella, 1937; Coleman, 1965 and Burdi and Faist, 1967).
Regarding the initiation of fusion, the opinions are less deviating and agree on the point that fusion starts in the anterior region and proceeds posteriorly. On the other hand, different views have been expressed concerning the precise area of initiation in the anterior region. One is that the fusion starts in the incisive foramina area and proceeds posteriorly (Stark and Ehrmann, 1958) and another is that it commences in the middle third of the anterior region and then proceeds anteriorly and posteriorly (Coleman, 1965).

The results of the present investigation are in agreement with Coleman (1965), as two embryos were seen exhibiting clearly a fusion of the processes in the middle third of the anterior region and still unfused in areas anterior and posterior to this. In the same way, most of the specimens on day 17 of gestation showed that the posterior end of the palate was the last to fuse.

The morphology and the relationship of the tongue to the palatal processes has also been studied with great interest in the past. Lazzaro (1940) pointed out that in the human embryos he studied, the lowering of the tongue had taken place before the horizontal transformation of the vertical processes. He suggested that several mechanisms are involved in the lowering of the tongue which he found to be wedged between the processes prior to the events leading to the fusion. He assumed, that the lowering of the tongue was due to the forward displacement of the mandible, the lifting of the roof of the oral cavity, the changes in the form of the tongue caused by the muscular development, and, finally, to the muscular movements of the tongue. Walker and Fraser (1956), on the other hand, could not find any sign of the lowering of the tongue allowing the processes to become horizontal in mouse embryos. They were of the opinion that the general position of the tongue remains relatively constant, while its shape changes in response to the change in position of the processes. In their view the tongue played only a passive role.

It is considered meaningful to mention here the recently presented theory (Humphrey, 1968) that the lowering of the mandible pulls the tongue away from its position between the processes, thereby causing a lesser pressure in the primitive nasal cavities. This in turn, then, should result in a rapid upward movement of the processes to the horizontal position.

The findings of the present chapter leave it open as to the role of the tongue in the elevation of the processes prior to the fusion. However, from the embryos we studied prior to the fusion, a simultaneous lowering of the tongue and change in the position of the palatal processes could be observed. From the sagittal sections it became clear that the tongue was considerably less curved after the processes had assumed the horizontal position—a change that was accompanied by an increase in size of the nasal and mandibular areas and a lowering and forward displacement of the mandible.
More experiments must be carried out in order to allow a more definite statement and to take position regarding the different theories. The experiments to be described later will present more evidence, thus the discussion on this matter will be postponed until then.

Summarizing, it may be said that:

In the embryos of Wistar albino rats the palatal processes undergo bulging before assuming a horizontal position.
The processes change very rapidly from a vertical to a horizontal position starting from the anterior region and proceeding posteriorly.
A simultaneous lowering of the tongue and mandible together with an increase in size of the nasal and mandibular areas and a forward displacement of the lower jaw was observed at the time of elevation of the processes.
The fusion of the palate occurs between the days 16.16 to 17.9 of gestation and starts in the middle third of the anterior half and proceeds anteriorly and posteriorly.
III-1. Normal rat embryo, 14 days, sagittal view. Note the high position of the tongue (T) and the retroposition of the mandible. Toluidine blue. X 24.

III-2. Normal rat embryo, 15 days, sagittal view. Note the larger size of the mandible anteroposteriorly. The tongue is also considerably larger in size but lying still high in the oro-nasal cavity. Toluidine blue. X 22.

III-3. Normal rat embryo, 16 days, sagittal view. Note the larger size of the mandible anteroposteriorly and considerable flattening of the tongue. Also note the curved anatomy of the tongue in the posterior region and flat one in the anterior region. Haematoxylin-eosin. X 20.

III-4. Normal rat embryo, 17 days, sagittal view. Note the separation of the oral and nasal cavities by the fusion of the palate (P). The tongue is well contained in the oral cavity. Haematoxylin-eosin. X 15.
III-5. Normal rat embryo, 14 days, frontal view, middle region. The palatal processes are vertically directed. Note the tongue is occupying the oro-nasal cavity. Toluidine blue. X 56.


III-7. Normal rat embryo, 15 days, frontal view, posterior region. The palatal processes are conical, slender and directed inferiorly. Van Gieson. X 34.
III-8. Normal rat embryo, 16 days, frontal view, anterior region. The palatal processes are lying horizontal but unfused. The tongue is lying well contained in the oral cavity and is following the contours of the oral surface of the palate. Mesenchymal condensation (MC) can be seen in the maxillary area. Note the considerably large mandible and nasal septum, compared to the previous ages. Haematoxylin-eosin. X 30.


III-10a. Normal rat embryo, 16.16 days, frontal view, anterior region. The palatal processes are horizontal but unfused. Haematoxylin-eosin. X 32.

III-10b. Anterior region (slightly posterior to Fig. III-10a). The palatal processes are epithelially fused. Note the ostegenic activity (OS) in the maxillary region. Haematoxylin-eosin. X 32.

III-10c. Middle region (posterior to Fig. III-10b). The palatal processes are epithelially fused. Haematoxylin-eosin. X 32.

III-10d. Posterior region. Note the palatal processes are unfused. Haematoxylin-eosin. X 32.

III-10e. Higher magnification of the fusion area of Fig. III-10b. The palatal processes of both sides are in the stage of fusion. X 300.

III-10f. Higher magnification of the fusion area of Fig. III-10c. The palatal processes are fused but separated by a single strand of epithelial cells. X 300.
III-11. Normal rat embryo, 17 days, frontal view, anterior region. The palatal processes are fused with each other and with the nasal septum. Note osteogenic activity (OS) in the maxillary region. Van Gieson. X 28.

III-12. Normal rat embryo, 17 days, frontal view, posterior region. The palatal processes are well fused. Note the osteogenic activity in the lateral regions of the palate. Haematoxylin-eosin. X 28.


III-14. Normal rat embryo, 18 days, frontal view, posterior region. Note the palate is of concave shape towards the oral side. Also note the osteogenic activity (OS) in the palate. Haematoxylin-eosin. X 15.
THE DEVELOPMENT OF CLEFT PALATE IN VIVO

INTRODUCTION

On basis of the studies presented in the past few decades, it is generally accepted that many congenital malformations can be produced in experimental animals by creating alterations in the environmental conditions during pregnancy.

The extensive review of Kalter and Warkany (1959) of metabolic alterations resulting in congenital malformations shows, that cleft palate is one of the most commonly observed anomalies. It can be caused by several chemical and mechanical factors acting during pregnancy. Cortisone acetate, hypervitaminosis A, folic acid deficiency, anoxia, maternal radiation and several other agents have been employed, to study the production of experimental cleft palate.

Though many studies have been carried out on the effect of hypervitaminosis A by Cohlan (1953, '54), Giroud and Martinet (1955, '56, '59, '60), Millen and Woollam (1957, '58), Kalter (1960), Hartel and Hartel (1960), Kalter and Warkany (1961), Kamei (1962), Takekoshi (1964), Kochhar and Johnson (1965), Kalter and Deuschle (1966), Lotosh (1968), Yamaguchi (1967), and Abramovich and Devoto (1967), the embryological development and the mechanism of cleft palate production is still rather obscure. Most of these studies dealt with the incidence of cleft palates recovered, the description of the anomaly produced, and effects of other agents employed in combination with vitamin A. In only a small number of studies has an attempt been made to determine the chemical and mechanical changes leading to the abnormal development of the secondary palate after the administration of high doses of vitamin A, cortisone acetate and other teratogens.

The details of the various studies on the production of cleft palate employing hypervitaminosis A, cortisone acetate, and a combination of both drugs, will be omitted here, as a comprehensive review has already been given in Chapter I.

The investigation reported in this chapter was undertaken to study the pathogenesis of cleft palate after the administration of higher doses of vitamin A, cortisone acetate, and a combination of the two drugs, as well as Dexamethasone sodium phosphate.
MATERIAL AND METHODS

A total of 142 pregnant Wistar rats was employed in the present investigation. The rats were divided into one control and four experimental groups.

Control group

Twelve pregnant rats were given 0.5 ml of normal saline by gastric tube from day 8, 9, or 10 to day 11, 12 or 13. Three other rats received the same amount of normal saline subcutaneously, twice a day. The rats were sacrificed on day 18 or 19. Embryos were examined both macroscopically and microscopically as indicated in Chapter II.

Experimental groups

Four experimental groups were formed according to the type of drug administered.

Group A 61 rats Vitamin A
Group B 14 rats Cortisone acetate
Group C 30 rats Combination of A and B
Group D 22 rats Dexamethasone

Group A: (Table IV-1)

Vitamin A palmitate was given to 61 pregnant rats via gastric tube after dilution in 0.5 ml of normal saline. To obtain an optimal dose for the production of cleft palate with minimal associated anomalies, the drug was administered first in small quantities and subsequently increased to higher ones.

Group B: (Table IV-2)

Cortisone acetate was administered in different doses, and for varying periods by subcutaneous injections. The drug was given twice a day after dilution in 0.5 ml of normal saline to obtain a good level of circulating steroid in the pregnant rats.

Group C: (Table IV-3)

In this experiment the pregnant rats were given vitamin A once a day and cortisone acetate twice a day, as described in Chapter II.

Group D: (Table IV-4)

Dexamethasone sodium phosphate, was administered subcutaneously after dilution in 0.5 ml of normal saline. In most instances the drug was given twice a day.
TABLE IV-I GROUP A. Teratogenic effect of hypervitaminosis A observed after different dosages and periods of administration

<table>
<thead>
<tr>
<th>Experiment (series)</th>
<th>Dosage</th>
<th>Period of administration</th>
<th>Mothers no.</th>
<th>Embryos no.</th>
<th>Living embryos no.</th>
<th>Resorbed embryos no.</th>
<th>Surviving embryos with cleft palate no.</th>
<th>%</th>
<th>Exencephaly and other anomalies no.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>20.000</td>
<td>9-12</td>
<td>4</td>
<td>33</td>
<td>31</td>
<td>2</td>
<td>9</td>
<td>29.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>20.000</td>
<td>10-13</td>
<td>5</td>
<td>39</td>
<td>38</td>
<td>1</td>
<td>10</td>
<td>26.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.</td>
<td>30.000</td>
<td>9-12</td>
<td>4</td>
<td>28</td>
<td>25</td>
<td>3</td>
<td>6</td>
<td>24.0</td>
<td>1</td>
<td>16.6</td>
</tr>
<tr>
<td>4.</td>
<td>30.000</td>
<td>10-13</td>
<td>7</td>
<td>57</td>
<td>48</td>
<td>9</td>
<td>28</td>
<td>58.3</td>
<td>1</td>
<td>3.5</td>
</tr>
<tr>
<td>5.</td>
<td>40.000</td>
<td>8-11</td>
<td>4</td>
<td>33</td>
<td>19</td>
<td>14</td>
<td>18</td>
<td>94.7</td>
<td>2</td>
<td>11.1</td>
</tr>
<tr>
<td>6.</td>
<td>40.000</td>
<td>9-12</td>
<td>8</td>
<td>69</td>
<td>58</td>
<td>11</td>
<td>46</td>
<td>79.3</td>
<td>4</td>
<td>8.6</td>
</tr>
<tr>
<td>7.</td>
<td>40.000</td>
<td>10-13</td>
<td>7</td>
<td>59</td>
<td>51</td>
<td>8</td>
<td>47</td>
<td>92.1</td>
<td>3</td>
<td>6.3</td>
</tr>
<tr>
<td>8.</td>
<td>50.000</td>
<td>10-13</td>
<td>3</td>
<td>28</td>
<td>19</td>
<td>9</td>
<td>18</td>
<td>94.7</td>
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<td>11.1</td>
</tr>
<tr>
<td>9.</td>
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<td>9-12</td>
<td>5</td>
<td>43</td>
<td>26</td>
<td>17</td>
<td>24</td>
<td>92.3</td>
<td>8</td>
<td>33.3</td>
</tr>
<tr>
<td>10.</td>
<td>60.000</td>
<td>9-12</td>
<td>6</td>
<td>51</td>
<td>36</td>
<td>15</td>
<td>32</td>
<td>88.8</td>
<td>6</td>
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</tr>
<tr>
<td>11.</td>
<td>60.000</td>
<td>10-13</td>
<td>8</td>
<td>64</td>
<td>35</td>
<td>29</td>
<td>30</td>
<td>85.7</td>
<td>9</td>
<td>30.0</td>
</tr>
</tbody>
</table>
The rats used in the four experimental groups were sacrificed on day 18, 19 or 20, with the exception that some animals were killed on days 14, 15, 16 and 17 of pregnancy. The embryos were examined as described in Chapter II.

The embryos obtained showed clearly whether fusion of the palatal processes had taken place or not. As outlined in Chapter III, this occurs in normal Wistar albino rats on day 17.

FINDINGS

a. Control group

All embryos were found to be normal and in accordance with the description given in Chapter III.

b. Experimental groups

In the description of the findings of these experiments, the general aspects will be dealt with first followed by the macroscopic and microscopic observations.

Group A: Vitamin A palmitate

General aspects: Table IV-1 shows that 20,000 to 30,000 I.U. administered between the days 9 to 13 resulted in cleft palate in 24 to 58.3% of the surviving embryos, and a small number of resorbed sites. An interesting observation was, that more than half of the living embryos showed cleft palate when 30,000 I.U. was given from day 10 to 13, in comparison to day 9 to 12 when only 24% of the embryos were found to be affected. A high ratio of affected embryos and a low percentage or resorbed sites occurred in series 7, where 40,000 I.U. was given from day 10 to 13. On the other hand, the same dose given from day 8 to 11 produced a comparable high percentage of embryos with cleft palate but a large number of resorbed sites. With a daily dosage of 50,000 or 60,000 I.U. of vitamin A, the percentage of embryos with cleft palate was high, but, at the same time, many resorbed sites and excessive anomalies were found.

Macroscopic findings (Figs IV-2, 3 and 5): No attempt was made in the present investigation to record systematically the number of anomalies other than the cleft palate and exencephaly. The most common abnormalities observed in embryos showing cleft palate were, steep frontal region of the face including the snout, micrognathia, retrognathia, open eyes, exophthalmus, malpositioned ears, short or absent fore-or hindlimbs, syndactyly, and a short or completely missing tail. No systematic occurrence of these anomalies was noticed.

The finding most pertinent to this study was the frequent observation of
microstomia in embryos with cleft palate. In some cases the oral opening was only the size of a pinpoint.

**Microscopic findings:** It was considered meaningful, to present a detailed description of the features associated with cleft palate on day 19 of gestation and to compare these to the normal situation of the same age. As an introduction to this, a short description of the differences observed on days 14, 15, 16 and 17 will be given.

**14 day old embryo:** No noticeable anatomical differences were observed compared with the corresponding control embryos.

**15 day old embryo** (Fig. IV-7): Slight differences could be distinguished on this day. The palatal processes were found to be comparatively small and less bulbous, both in the anterior and posterior regions. The mandibular and nasal areas were also relatively small. The cellular picture of the palatal processes did not deviate from the normal embryos. A slight mesenchymal condensation was also noticed at the lateral extremities of the palatal processes.

**16 day old embryo** (Fig. IV-8): Contrary to the normal controls, the palatal processes were orientated inferio-medially in the anterior region and vertical in the posterior one. The processes were of abnormal shape; in some embryos they were conical and in others bulbous or thick with ruffled borders.

The nasal area was small. The nasal septum was bulbous and short in vertical direction.

In the maxillary region mesenchymal condensation was seen with heterotopic chondrogenesis. This was also observed at the corners of the oral cavity.

The mandibular development appeared to be retarded, and an unorganized bone formation was observed around the Meckel’s cartilage.

The tongue was in a high position and was lying between the palatal processes.

**17 day old embryo** (Figs IV-9a and b): On this day distinct signs were seen indicating cleft palate.

In the majority of the embryos of this age the palatal processes were found in a horizontal position superior to the tongue (Fig. IV-9a). In some cases they were lateral to the tongue in the anterior region. Posteriorly, the processes were short and showed no sign of a change from a vertical to a horizontal position (Fig. IV-9b). In a few embryos the processes were completely missing in the extreme posterior region. The tongue occupied the oro-nasal cavity in most of the embryos.
In vitamin A treated 19 day old embryos, the cleft palate was clearly visible. The palatal processes were unfused. In some instances, the processes were found to be in contact with each other only in the extreme anterior region. The gap between the unfused processes was greater in the posterior region as compared with the anterior. In a few embryos the processes appeared as rudiments in frontal sections at the level of the eye (Fig. IV-13). Overall, the processes were big, thick, and wide in the middle and towards the maxillary region, but medially they were pointed or conical in shape (Fig. IV-11b). Posteriorly they were small and presented no consistent picture.

The nasal septum was found to be thick, vertically short and bulbous (Fig. IV-11b). The bone formation around the nasal area was abnormal and appeared to be continuous with the bony areas of the maxillary and zygomatic regions.

The mandibular area was found to be deformed. In the anterior region the body of the lower jaw was small. The ramal area of the mandible was more severely affected. Meckel's cartilage appeared to be deformed in several instances and ectopic cartilages were seen in the bony areas of the mandible. In the majority of the embryos the condylar cartilage could not be located.

A most interesting observation seen at this age was the presence of heterotopic cartilage (Figs IV-11a, b; and 13), extending from the maxillary area to the condylar region of the mandible. In some embryos the heterotopic cartilage from the maxillary region, appeared to be in contact with another similar cartilage arising from the zygomatic and condylar regions. This heterotopic cartilage was larger in the sections posterior to the middle third of the anterior half of the palate (Fig. IV-13).

In the embryos with cleft palate, the tongue was of a completely abnormal form. It was found to be lying between the palatal processes and projecting into the nasal cavity (Fig. IV-11b). The tongue appeared to be small particularly in the embryos with microstomia.

An overall examination of the head revealed small vertical and wide lateral dimensions. In most of the embryos showing cleft palate, the upper molar buds were missing (Fig. IV-13), rudimentary or malformed. In a few instances, several molar and incisor buds were seen in the same frontal section indicating a malposition of the teeth. The development of the lower molar buds was normal, however, they were missing in some cases.

The cellular picture of the embryo head was far from normal. Anteriorly the epithelium of the palatal processes was of 2 to 3 cell layers thick, and in some instances, it was in contact with the epithelium of the opposing process or the primary palate. Posterior to this the thickness of the epithelium varied
from 3 to 4 layers. The mesenchyme presented no uniform picture. In some specimens, it was slightly organized and in others it was not. The lateral parts and superior halves of the palatal processes showed slight osteogenic activity which was in contact with the bony area around the nasal periphery.

Some additional information was gained by the examination of the sagittal sections (Figs IV-14 and 15). The tongue was found to be bulging into the opening between the processes in the mid-sagittal sections (Fig. IV-15). In other sections, lateral to the middle ones, the superior surface of the tongue was lying against the nasal and pharyngeal regions. The mandibular area showed small antero-posterior dimensions.

**Group B: Cortisone acetate**

*General aspects* (Table IV-2): A total of 14 pregnant rats were given cortisone acetate subcutaneously, in different concentrations starting on either day 9 or 10. No cleft palates or other associated anomalies were seen in the recovered embryos.

*Macroscopic findings*: The embryos appeared to be normal and no anomalies were found.

*Microscopic findings*: The development of the palatal processes followed the normal events as described in the previous chapter. The cellular picture also appeared to be normal.

**Group C: Vitamin A and cortisone acetate**

*General aspects* (Table IV-3): When 30,000 I.U. of vitamin A, once a day, was given with 5 mg of cortisone acetate, twice a day, from day 8 to 12, only 20.6% of the embryos showed cleft palate. When only the dosage of vitamin A was increased, the percentage of living embryos with cleft palate and also that of the resorbed sites increased considerably. When the doses of both drugs were increased as in series 4 and 5, the percentage of the embryos showing cleft palate was 76.4 and 78.9, and of the resorbed sites 50.0 and 38.7 respectively. On the other hand, when only the dose of the cortisone acetate was lowered, the percentage of the cleft palates and resorbed sites was low. The incidence of exencephaly was higher when both drugs were given in large doses.

*Macroscopic findings* (Fig. IV-6): The macroscopic examination revealed the same picture as observed in the embryos obtained from rats treated with vitamin A only. However, more embryos with severe excessive anomalies were found.
### TABLE IV-2 GROUP B. Teratogenic effect of cortisone acetate observed after different dosages and periods of administration

<table>
<thead>
<tr>
<th>Experiment (series)</th>
<th>Dosage (mg.)</th>
<th>Period of administration</th>
<th>Mothers no.</th>
<th>Embryos no.</th>
<th>Living embryos no.</th>
<th>Resorbed embryos no.</th>
<th>%</th>
<th>Surviving embryos with cleft palate no.</th>
<th>%</th>
<th>Exencephaly and other anomalies no.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2.5</td>
<td>9-11</td>
<td>4</td>
<td>31</td>
<td>30</td>
<td>1</td>
<td>3.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>5.0</td>
<td>9-12</td>
<td>5</td>
<td>39</td>
<td>32</td>
<td>7</td>
<td>17.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>3.</td>
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<td>10-13</td>
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<td>12</td>
<td>11</td>
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</tr>
<tr>
<td>4.</td>
<td>10.0</td>
<td>9-11</td>
<td>3</td>
<td>27</td>
<td>21</td>
<td>6</td>
<td>22.2</td>
<td>0</td>
<td>0</td>
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<td></td>
</tr>
</tbody>
</table>

### TABLE IV-3 GROUP C. Teratogenic effect of combination of vitamin A and cortisone acetate observed after different dosages and periods of administration

<table>
<thead>
<tr>
<th>Experiment (series)</th>
<th>Dosage</th>
<th>Period of administration</th>
<th>Mothers no.</th>
<th>Embryos no.</th>
<th>Living embryos no.</th>
<th>Resorbed embryos no.</th>
<th>%</th>
<th>Surviving embryos with cleft palate no.</th>
<th>%</th>
<th>Exencephaly and other anomalies no.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5.0</td>
<td>30.000</td>
<td>8-12</td>
<td>4</td>
<td>31</td>
<td>2</td>
<td>6.4</td>
<td>6</td>
<td>20.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>5.0</td>
<td>40.000</td>
<td>9-12</td>
<td>5</td>
<td>36</td>
<td>24</td>
<td>12</td>
<td>33.3</td>
<td>75.0</td>
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<td>22.2</td>
</tr>
<tr>
<td>3.</td>
<td>5.0</td>
<td>60.000</td>
<td>9-12</td>
<td>4</td>
<td>33</td>
<td>27</td>
<td>6</td>
<td>18.1</td>
<td>100.0</td>
<td>11</td>
<td>40.7</td>
</tr>
<tr>
<td>4.</td>
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<td>40.000</td>
<td>9-11</td>
<td>4</td>
<td>34</td>
<td>17</td>
<td>17</td>
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<td>76.4</td>
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<td>60.000</td>
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<td>4</td>
<td>31</td>
<td>19</td>
<td>12</td>
<td>38.7</td>
<td>78.9</td>
<td>6</td>
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<tr>
<td>6.</td>
<td>2.5</td>
<td>40.000</td>
<td>9-12</td>
<td>4</td>
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<td>14.7</td>
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<tr>
<td>7.</td>
<td>2.5</td>
<td>60.000</td>
<td>9-12</td>
<td>5</td>
<td>41</td>
<td>35</td>
<td>6</td>
<td>14.6</td>
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<td>11.1</td>
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</tbody>
</table>
Microscopic findings (Figs IV-16a, b, c, and d): Basically, the histological picture of the embryos showing cleft palate was similar to the one described before, when vitamin A was given alone. Only in some embryos the mandible was more severely deformed. In all specimens, the tooth buds – both upper and lower – were either missing or malformed. The heterotopic cartilage was observed in almost all the embryos with cleft palate.

Several individual variations were also noticed in the embryos showing cleft palate. In a few, the malformed palatal processes appeared to have fused in the anterior region. These fused processes showed a central groove on their oral side (Fig. IV-16b). Moving posteriorly, the groove became deeper and deeper and ended in a separation of the processes. An interesting observation here was the absence of the tongue in the anterior region where the processes were found to be fused or cleaved (Fig. IV-16b). The tongue was present where the processes were found to be unfused.

Group D: Dexamethasone sodium phosphate

General aspects (Table IV-4): Dexamethasone produced a high incidence of cleft palate, ranging from 57.1% to 77.7%. The percentage of the resorbed sites was relatively large when 0.5 or 1.0 mg was given twice a day. In series 4 where only a single dose of 0.5 mg was administered, none of the embryos showed cleft palate. Very few excessive anomalies were noticed, compared with the use of other drugs described earlier.

Macroscopic findings: Only a limited number of other anomalies were observed, which included open eyes, syndactyly, microstomia, wrinkled skin and short tail.

Microscopic findings (Figs IV-17a and b): A follow up of the embryos from day 14 to 17 showed no differences from the findings when vitamin A was given alone to the pregnant rats. The embryos observed on day 19 showed minor differences.

The palatal processes were generally slender and less deformed. In the posterior region the processes were either missing or were rudimentary.

The nasal area was small and more bulbous. The mandibular bone formation was disorganized with the presence of ectopic cartilages.

Interpretation of the Findings

Of the three different drugs and the combination of vitamin A and cortisone acetate employed in the present investigation, only cortisone acetate did not
TABLE IV-4 GROUP D. Teratogenic effect of Dexamethasone phosphate observed after different dosages and periods of administration

<table>
<thead>
<tr>
<th>Experiment (series)</th>
<th>Dosage (mg.)</th>
<th>Time/day</th>
<th>Period of administration</th>
<th>Mothers no.</th>
<th>Embryos no.</th>
<th>Living embryos no.</th>
<th>Resorbed embryos no.</th>
<th>Surviving embryos with cleft palate no.</th>
<th>Surviving embryos with cleft palate %</th>
<th>Exencephaly and other anomalies no.</th>
<th>Exencephaly and other anomalies %</th>
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<td>1.</td>
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<td>8–13</td>
<td>4</td>
<td>33</td>
<td>29</td>
<td>4</td>
<td>18</td>
<td>62.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>2×</td>
<td>9–12</td>
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<tr>
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<td>8–13</td>
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<td>26</td>
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<td>2</td>
<td>7.6</td>
<td>0</td>
<td>0</td>
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<tr>
<td>5.</td>
<td>1.0</td>
<td>2×</td>
<td>8–13</td>
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<td>39</td>
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<td>25.6</td>
<td>21</td>
<td>72.4</td>
<td>3</td>
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<tr>
<td>6.</td>
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<td>2×</td>
<td>9–12</td>
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<td>5</td>
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</tbody>
</table>
produce cleft palate or any other observable anomaly. Administration of vitamin A to the pregnant rats revealed that the period from day 10 to 13 of gestation was most critical for the development of the palate. When the administration of vitamin A was started on day 8 or 9 of gestation, more resorbed sites and fewer embryos with cleft palate were obtained.

The combination of vitamin A and cortisone acetate failed to increase the incidence of cleft palate. Comparatively fewer embryos with cleft palate and a large number of resorbed sites, as well as excessive anomalies, were seen. Only in one series the combination of drugs did produce 100% of the embryos with cleft palate, but 40.7% of the embryos had exencephaly and other excessive anomalies.

Dexamethasone was found to be a successful teratogen. A small dose of 0.25 mg twice a day given from day 8 to 13 produced cleft palate in 62% of the living embryos with only 12.1% resorbed sites.

The macroscopic observations revealed various malformations, of which the most frequent were micrognathia, retrognathia, open eyes, syndactyly and microstomia. Vitamin A alone and in combination with cortisone acetate produced several other severe anomalies, which were rarely noticed in embryos obtained from Dexamethasone treated rats.

Microscopically, the differences between the development of the palatal processes of the drug treated groups and that of normal rats were visible after day 15. On day 16, the differences were well established. The palatal processes were still found to be positioned in an inferio-medial or vertical direction, and the tongue was interposed between them as compared to the normal picture observed in 16 day old embryos. On day 19, when the processes in the majority of the embryos were found to be horizontal in the anterior region, the tongue was contained in the oral cavity as seen in the normal embryos. But posteriorly, where the processes were small, in nearly all the embryos with cleft palate the tongue was positioned high in the oro-nasal cavity with its superior lateral corners extending into both nasal cavities. The nasal septum was found to be swollen, and was short in the vertical direction. The mandibular development was also abnormal. Condylar cartilage was not found in most of the embryos. A heterotopic cartilage was observed in the maxillary region extending into the mandibular area, or in some instances, fusing with a similar cartilage arising from the temporo-mandibular and zygomatic regions.

The cellular picture of the palatal processes did not present systematic differences from the normal situation. The rest of the cellular picture was different, showing abnormal development of bone and cartilage formation. A conceivable explanation which can be given regarding the presence of cleft palate in our drug treated sample is that the heterotopic cartilage leads to ankylosis of
maxillo-mandibular regions, thus preventing the mandible to develop in a
downward and forward direction, and the nasal septum to grow downward.
This lack of developmental mobility may have lead to microstomia which in
turn with other features prevented the tongue from lowering down and sliding
forward to allow the palatal processes to assume a horizontal position. All
these changes which started from day 15 showed a deviating-picture on day 16
when the processes were still found to be lying vertical and the tongue inter­
posed between them. On day 17, movement of the abnormal processes was
observed in the horizontal direction, especially in the anterior region.

DISCUSSION AND CONCLUSIONS

The present findings agree with the observations of Giroud and Martinet
(1955, '56) that there is a critical period in the development of every organ
and that a teratogenic agent given in the first half of the pregnancy can produce
light to severe malformations, depending on the stage of development of the
particular organ. The incidence of the cleft palate observed after the admin­
istration of vitamin A was found to be higher in the present investigation than
the one reported by Cohlan and Stone (1961), under similar conditions in CF
Wistar rats. The difference could be attributed to the different strains employed
in the two investigations.

Various explanations have been offered as to the cause of the production of
cleft palate after the administration of several teratogenic agents, including vi­
tamin A and cortisone acetate. The present findings will be discussed in relation
to the ones offered by others.

One of the frequently reported observations is the failure of the processes to
attain a horizontal position on day 16 of gestation in rats (Asling et al., 1960)
and on day 15 in mice. Walker and Fraser (1957) attributed this failure to a
loss of 'internal force' of the palatal processes, when they administered cortisone
acetate to mice to produce cleft palate. Later this internal force was identified
by Larsson et al. (1959) and Walker (1961) as the mucopolysaccharides of the
mesenchymatous ground substance of the palatal processes. Walker and Crain
(1960), studying the effect of hypervitaminosis A on mice, also found a delay
in the movement of the processes.

Contrary to these findings, Kochhar and Johnson (1965) could not observe
a delay in the movement of the palatal processes from a vertical to the hori­
zontal position, when vitamin A was given to pregnant black-hooded rats.
However, the majority of the obtained embryos showed cleft palate.

In the present investigation a delay was observed in the transposition of the
processes from vertical to horizontal in 16 day old embryos. Only at a later
date elevation of the processes was seen partially in the anterior region, while the palatal processes in the posterior region were found to be too short and rudimentary. These findings agree with those of Walker and Fraser (1957), Walker and Crain (1960), and Kamei (1962), even though they used mice, and Asling et al. (1960) who used folic acid deficiency to produce cleft palate in Long-Evans rats. The disagreement with Kochhar and Johnson (1965) might be attributed to differences between the strains used in the present and their study.

A number of theories have been presented explaining the role of the tongue in combination with one or more than one of the other factors. These are foreshortening of the maxilla and mandible (Cohlan, 1954), microstomia and micro- or retrognathia (Giroud and Martinet, 1959; Deuschle et al., 1959; Kalter, 1960) and the presence of heterotopic cartilage within the preosteoblastic tissues of the maxilla leading to maxillo-mandibular ankylosis (Deuschle et al., 1959; Kochhar and Johnson, 1965; Kalter and Deuschle, 1966; Abramovich and Devoto, 1967). This latter finding has only been reported after the administration of vitamin A to both rats and mice. It has been suggested that all these abnormalities lead to an inhibition of the downward and forward displacement of the tongue, thereby not allowing the processes to assume a horizontal position and thus making a fusion impossible. The role of the tongue and mandible has been further emphasized by the experiments of Trasler et al. (1956), Walker (1959) and Trasler and Fraser (1963). They studied the effects of amniotic puncture in mice and reported that it caused the uterus to compress against the foetus. They hypothesized, that the compression of the foetal head against the thorax resulted in the lower jaw pressing the tongue against the nasal septum and subsequently not allowing the processes to assume a horizontal position.

Besides these theories, several others have been presented which are supposed to occur in conjunction with the ones already mentioned above. One is the direct increase of vitamin A in the foetal content after its administration to the pregnant rats (Giroud et al., 1957). This was later disagreed with by Cohlan and Stone (1961), on the basis that the recorded increase was so low that it could have been caused by the error in counting.

Another theory is that the teratogenic action of the drugs can be due to the interference at some level in the carbohydrate metabolism of the mother, placenta or foetus (Woollam and Millen, 1960).

The present investigation showed almost no differences in the way in which cleft palate was produced when vitamin A or a combination of vitamin A and cortisone acetate or Dexamethasone was administered to the pregnant Wistar albino rats. The other theories, mentioned in relation to the mechanical or
cellular changes, were out of scope of the methods and materials used in the present experiment. Later in the study, radioactive isotopes will be employed to study the role played by mucopolysaccharides in the production of cleft palate.

Summarizing, it can be stated that Dexamethasone, hypervitaminosis A, and hypervitaminosis A in combination with cortisone acetate produced cleft palate and other associated anomalies, when given to pregnant Wistar albino rats in different doses and during the critical period of embryonic development. Cortisone acetate did not produce cleft palate. Only minor differences in morphology, both at macroscopic and microscopic level, were found in the embryos with cleft palate obtained after the administration of the different drugs.

The present study shows that due to the teratogenic agents, a delay occurs in the elevation of the processes from vertical to horizontal on day 16 of gestation. This movement occurred 24 hours later, and then only partially, while in the meantime the tongue stayed between the palatal processes.

The findings also revealed the presence of heterotopic cartilage arising from the maxillary region and leading to maxillo-mandibular ankylosis. This was mostly associated with the presence of microstomia.

The findings of this chapter show that the following factors, working separately but more probably in combination, play a role in the production of cleft palate.

Microstomia; not allowing the tongue to lower down and to slip outside the oral opening, as observed normally on day 16.

Maxillo-mandibular ankylosis.

Retarded downward growth of the nasal septum.

Retarded downward and forward movement of the mandible associated with maxillo-mandibular ankylosis and non-lowering of the tongue, on day 16 of gestation.

Disturbance in the palatal processes at the cellular or intercellular level.

The last point will be investigated later in the study with experiments employing radioactive isotopes.
IV-1. Normal rat embryo, 18 days. Note the inclination of the forehead, nose, oral opening, and the size of the mandible. X 2.5.

IV-2. Rat embryo, 18 days. Vitamin A, 40,000 I.U./day from 9th to 12th day of gestation. Several abnormalities can be seen such as, open eyes, displaced ears, microstomia, retrognathia, foreshortening of the fore- and hindlimbs, syndactyly and short tail. Note especially the inclination of the forehead and the nasal area. X 2.5.

IV-3. Rat embryo, 18 days. Vitamin A, 60,000 I.U./day from 9th to 12th day of gestation. Note exencephaly, open eyes and abnormal position of the ears. X 5.


IV-5. Rat embryo, 19 days. Vitamin A, 40,000 I.U./day from 9th to 12th day of gestation. Note unfused palatal processes. In the anterior region the palatal processes are near to each other compared to the posterior area. The nasal opening can be seen. X 5.

IV-6. Rat embryo, 19 days. Vitamin A, 40,000 I.U./day and cortisone acetate 5 mg 2X/day from 9th to 12th day of gestation. A severe cleft palate, the palatal processes are too short and nasal openings are large. X 5.

IV-7. Rat embryo, 15 days, frontal view, middle region. Vitamin A 40,000 I.U./day from 9th to 12th day of gestation. Note the palatal processes are lying vertical and slightly mesially inclined. Mesenchymal condensation (MC) can be seen in the maxillary area. Haematoxylin-eosin. X 26.

IV-8. Rat embryo, 16 days, frontal view, posterior region. Vitamin A 40,000 I.U./day from 9th to 12th day of gestation. The palatal processes are still lying vertical. Haematoxylin-eosin. X 22.
IV-9a. Rat embryo (S-5), 17 days, frontal view, anterior region. Vitamin A 40,000 I.U./day 8th to 11th day of gestation. The left palatal process is completely horizontal and the right one partly horizontal due to the abnormal position of the tongue (arrows). Note among other malformations, the presence of heterotopic cartilage (HC) on both sides and the abnormal osteogenic activity (OS) in the maxillary region. Haematoxylin-eosin. X 25.

IV-9b. The same embryo (S-5), posterior region. The palatal processes are of abnormal shape and are lying still vertical. Note the heterotopic cartilage (HC), the absence of the upper molar buds, and the disturbed osteogenic activity in the maxillary and mandibular regions. Haematoxylin-eosin. X 20.

IV-10a. Normal rat embryo (N-9), 19 days, frontal view, anterior region (Section through the lower incisor area). The palatal processes are in contact with the primary palate. Haematoxylin-eosin. X 21.

IV-10b. The same embryo (N-9), middle region (Section through the level of anterior orbit). Note the palate is fused with the nasal septum and extensive osteogenic activity (OS) in the maxillary region. Haematoxylin-eosin. X 17.

IV-11a. Rat embryo (S-23), 19 days, frontal view, anterior region. Vitamin A 40,000 I.U./day from 9th to 12th day of gestation. The palatal processes are horizontal and in contact with the primary palate. Note the bulbous and abnormal shape of the palatal processes, the presence of heterotopic cartilage (HC) on both sides, malpositioned tongue (T) and the vertically short and bulbous nasal septum (NS). Haematoxylin-eosin. X 22.

IV-11b. The same embryo (S-23), middle region. Note that the left palatal process is horizontal, compared to the right one. Both palatal processes (P) are malformed and have unsMOOTH margins. The tongue (T) is protruding into the nasal cavity. Note the malformed mandible (M), the heterotopic cartilage (HC) on both sides, the abnormal osteogenic activity (OS) in the maxillary region and the vertically short and bulbous nasal septum. Haematoxylin-eosin. X 22.

IV-13. Rat embryo (S-23), 19 days, frontal view, posterior region. Vitamin A 40,000 I.U./day from 9th to 12th day of gestation. The palatal processes are rudimentary with abnormal osteogenic activity (OS). Large heterotopic cartilage (HC) can be seen on both sides extending from the maxillary and the mandibular region and also in contact with the cartilage around the nasal area. The upper molar buds are missing. Note the malformed shape of the mandible. Toluidine blue. X 20.

IV-14. Normal rat embryo, 19 days, sagittal view. Note the almost horizontal palate (P) and the flat tongue. Haematoxylin-eosin. X 13.

IV-15. Rat embryo, 19 days, sagittal view. Vitamin A 40,000 I.U./day from 9th to 12th day of gestation. Note the cleft palate and the middle region of the tongue projecting into the nasal cavity. Haematoxylin-eosin. X 13.

IV-16a. Rat embryo (D-7), 19 days, frontal view, extreme anterior region (Section at the level of upper incisors UI). Vitamin A 40,000 I.U./day and cortisone acetate 5 mg 2×/day from 9th to 12th day of gestation. Note microstomia (MI) and the thick oral epithelium. Haematoxylin-eosin. X 24.

IV-16b. The same embryo (D-7), anterior region. The malformed and malshaped palatal processes fused with the thick and bulbous nasal septum. A groove can be seen dividing the palate into two halves. Note the invaginated oral epithelium (OE), the heterotopic cartilage (HC), and the small oral cavity with the absence of the tongue in the section. Haematoxylin-eosin. X 22.
IV-16c. The same embryo (D-7), anterior region. The palatal processes are missing and on one side the tongue is protruding into the nasal cavity. Note: several tooth buds in one plane, the heterotopic cartilage (HC), the bulbous and vertically small nasal septum, the small mandible and other abnormalities. Haematoxylin-eosin. X 21.

IV-16d. The same embryo (D-7), posterior region. Note complete absence of the palatal processes, the heterotopic cartilage (HC), the malformed Meckel's cartilage (Mc) and mandible (M). Haematoxylin-eosin. X 19.

IV-17a. Rat embryo (P-55), 19 days, frontal view anterior region. Dexamethasone 0.25 mg 2×/day from 9th to 12th day of gestation. Note the unfused, thin and the slender palatal processes, the heterotopic cartilage (HC), the missing upper molar buds, the stunted nasal septum and other abnormalities. Haematoxylin-eosin. X 21.

IV-17b. The same embryo (P-55), middle region, Note the osteogenic activity in the palatal processes, the heterotopic cartilage (HC) and other malformations. Haematoxylin-eosin. X 17.
CHAPTER V

PALATAL CLOSURE IN VITRO

INTRODUCTION

In vitro experiments are of great value for studying certain aspects, that cannot be approached by other methods. In vitro experiments have been widely used to study cells, tissues, and organs in artificial environments under normal and abnormal conditions. Especially, the organ culture method is indispensable for two groups of investigations:

a. those dealing with the interaction of cells and tissues and
b. studies of the effect of biologically active compounds such as hormones, vitamins, carcinogens, and antibodies on different organs (Fell, 1965).

Only recently the organ culture method has been introduced in the study of the pattern of normal palatal closure and the associated cellular changes (Moriarty et al., 1963). By this method it is possible to study the active fusion process on a longitudinal basis and follow it over its whole course. At the same time, it must be taken into account that the cutting operation to prepare palatal explants disrupts the normal relationship between the palatal processes and the overall growth of the head and eliminates influences of tongue and jaw movements. On the other hand, the in vitro method opens a way to study the palatal closure without the presence of the tongue, head, and other surrounding structures which may have certain effects, advantageous or disadvantageous, on it. With the in vitro method the developmental changes, associated with or caused by the effects of isolation, operations, and varying physiological conditions, can be studied by direct observation.

The purpose of the investigation presented in this chapter was to study

a. the time of onset of acquired potentiality for the fusion of the palatal processes of Wistar albino rat embryos;

b. the pattern of fusion of the palatal processes in comparison to the in vivo findings presented before;

c. the histological aspects of the cellular fusion in vitro and to compare these with the observations of the previous in vivo experiments.
MATERIAL AND METHODS

In total 92 embryos were obtained at different periods of gestation from 39 pregnant Wistar albino rats. Two or three embryos from one pregnant mother were used. The remaining embryos were studied macroscopically and microscopically, some of them were incorporated in the experiments described in Chapter III. The explants containing the palatal processes were prepared according to the technique of Moriarty et al. (1963), which has been described in Chapter II. The explants were incubated for varying periods of time. Details concerning the age of embryos, the number of explants, and the duration of the incubation periods are presented in Table V-1. To evaluate macroscopically the process of fusion, photographs of two explants from each series were taken prior to the incubation and, thereafter, at 12 to 24 hour intervals. For this procedure the explants were taken out of the incubators for only a few minutes. Explants were used of embryos of 14.9 to 16.9 days of age. Day 16.16 was not included because several of these specimens showed approximation of their palatal processes or a start of the fusion process.

At the end of the incubation period the cultured tissues were fixed in Bouin's solution, embedded in paraffin, stained, and were then examined microscopically.

FINDINGS

a. Macroscopic findings (Figs V-1a, b, c; 3a, b, c):

The macroscopic evaluation of the explants prior to, during and after the incubation was made by naked eye, with the help of a dissection microscope and by means of photographs.

On the moment of incubation the explants of 14 and 15 days of age showed a different picture than the explants of 16 days of age, as in the latter, the tongue was not found to be lying interposed between the palatal processes prior to the dissection. The differences between the ones of 14 and 15 days of age were also evident, as in the older specimens the processes were mesially inclined and thick, while in the younger ones the processes were small, vertically directed and slender.

During the dissection procedure on day 14 and 15, when the tongue was removed from its position between the palatal processes, the two processes moved subsequently to an almost horizontal position. This movement was more evident in the older embryos than in the younger ones and resulted in a considerable decrease of the distance between the two palatal processes. However, a contact between the two processes was not observed in any specimen.
After an incubation period of 24 hours, the explants were more difficult to examine due to an increasing opaque appearance.

Contact between the two processes was first noticed in the posterior region, followed by a separate approximation in the anterior region. In some explants, contact in the anterior and posterior regions was found on the same moment of observation.

b. Microscopic findings (Table V-1, figs V-2a, b, c; 4 and 5):

The findings regarding the fusion were classified in three groups:

a. no fusion: the palatal processes did not exhibit any form of fusion;

b. epithelial fusion: the processes were found to be in contact with each other epithelially and no break in the continuity of the epithelium was noticed. No differentiation was made here between one or more layers of epithelium at the area of fusion;

c. mesenchymal fusion: the mesenchyme of both sides was united. This involved complete mesenchymal fusion, but there were cases in which some broken epithelial strands were present at the fusion site.

The palatal processes dissected on day 14.9 and incubated for 24 hours showed no epithelial or mesenchymal fusion. Of the ones studied after 48 and 72 hours

<table>
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<th>Incubation time (hrs.)</th>
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of incubation, 6 out of 16 showed epithelial fusion and only one mesenchymal fusion. From the 20 explants dissected on day 14.16, five showed epithelial and 3 exhibited mesenchymal fusion.

From the material dissected on days 15.9, 15.16, and 16.9, relatively more explants showed mesenchymal fusion. Of a total of 92 explants incubated, 29.3% showed no fusion, 23.9% showed epithelial fusion, and 46.8% showed mesenchymal fusion. The majority of the no fusion's were obtained from the explants dissected on day 14 of gestation.

It must be pointed out here that explants classified as 'no fusion' showed, as a rule, considerable growth of the palatal processes and a decrease in the gap between them.

The epithelium of the unfused palatal processes was either one or two cell layers thick on the medial surfaces. The lateral surfaces of the processes towards the oral side were 2 or 3 cell layers thick. The cells were oval or columnar in shape. The mesenchyme was composed of stellate shaped cells. After an incubation period of 48 hours the cells were more dense in the medial third of the processes. Eleven explants out of 40 dissected on days 14.9 and 14.16 showed epithelial fusion involving a union by one or two strands of cells, or an epithelial bridge running from one process to the other. The mesenchymal fusion observed in 4 explants was limited to a small area, mainly in the anterior region.

An overall examination of the frontal sections showed that the fused palatal processes had a flat oral surface and a curved nasal surface. The size of the fused palate appeared relatively smaller than the one observed in vivo. Several explants presented an epithelial fusion posteriorly, a mesenchymal fusion anteriorly, and an unfused area in the middle region of the palate. The processes were lined by 1 or 2 epithelial cell layers on the nasal and oral borders. The cells were oval or columnar in shape. A migration of the epithelial cells towards each other was noticed, after which the processes fused epithelially. Subsequently the mesenchyme of both the processes was found to be separated by one or two epithelial cell layers. The epithelial cells were later found to be in different stages of lysis. At places where complete mesenchymal fusion was observed, few epithelial remnants remained in the fusion area. Prior to this, the epithelial fusion was preceded by a condensation of the mesenchymal cells in the medial region of the processes. The mesenchymal penetration started near the oral surface and progressed towards the nasal one. Several mitotic figures were seen in the fusion area prior to and during the fusion of the processes.

The cartilaginous parts of the explants appeared cytologically unaltered. In several unfused 14 day old explants, a comparatively large distance was found between the nasal septum and the palatal processes. The nasal septum showed...
considerable growth in lateral and vertical directions during the incubation period from 24 to 72 hours.

**INTERPRETATION OF THE FINDINGS**

Fusion of the palatal processes could be achieved in an artificial environment. Relatively greater numbers of fusions were obtained with explants of older age. The palatal processes generally fused after 24 to 72 hours of incubation when explanted on or later than day 15 of gestation. The fusions obtained with younger explants were in general only of epithelial nature.

The gap between the processes was greater in the explants dissected on day 14, as on that age the processes were found to be still vertical and the tongue was interposed between them. At the moment the tongue was removed the processes became horizontal. The palatal processes showed histo-differentiation as noticed prior to fusion *in vivo*, and also, in the older explants cultured *in vitro*. The reason that no fusion took place in the 14 day old explants after an incubation period of 72 hours, may primarily be due to the large distance between the two processes at the moment of incubation. Another explanation for their non-fusion is the relatively fast growth of the nasal septum which appeared between the two processes and prevented them to grow towards each other. In such instances the processes fused with the nasal septum and demonstrated their potentiality to fuse.

The histological picture of the fusion *in vitro* was, in general, similar to the one observed *in vivo*. The only difference noted was regarding the area of approximation of the processes. The cultured processes came into contact first posteriorly, but in some cases posteriorly and anteriorly at the same time. This is in contrast to the observations on the *in vivo* material where contact was first established and fusion was found to have commenced at the middle third of the anterior region, and from there progressed anteriorly and posteriorly.

**DISCUSSION AND CONCLUSIONS**

The onset of the potentiality of the processes to fuse *in vitro* has also been studied by Pourtois (1966) and Vargas (1967). Pourtois (1966) reported that in Sprague Dawley rat embryos, the potentiality was present in all the explants at the age of 15½ days, which is 24 hours prior to the actual time of fusion of the processes. He based this conclusion on the fact that he did not observe fusion of the processes explanted prior to day 15 of gestation, and incubated in close contact with each other. Vargas (1967) found that the potentiality
of the palatal processes, dissected from A/Jax mouse embryos, to fuse was present approximately 40 hours before the actual time of closure. He attributed the difference between his results and those from Pourtois (1966), to specific differences in the two types of rodents employed.

The results of the present investigation showed that in the explants dissected on, or after day 15 of gestation, the palatal processes in general achieved fusion. This result is in agreement with the one reported by Pourtois (1966). On the other hand the present findings differ from his, as some fusions were obtained in our study in 14 day old explants. This can be related with the incomplete knowledge of the time of conception of our rats. However, the histological findings lead to the assumption that at least at the cellular level the processes of 14 day old explants were capable of fusing with each other after 24 hours of incubation. Furthermore, in some of the longer incubation experiments the processes were found to be in contact with each other or epithelially fused in some areas, or were found to be in the stage of fusion with the nasal septum. Another fact that renders support for our assumption is that the 14 day old processes were found to have increased considerably in their horizontal length towards each other.

A possible reason why the process did not achieve fusion at this age in the majority of the cases might be the big gap between the two processes which was difficult to fill even after an incubation period of 72 hours. Also, in the meantime, the enclosed nasal area appeared to have grown considerably in lateral and oral directions, thereby not allowing the processes to reach each other.

The difference between our observations and those of Pourtois (1966) could be due to the difference in method of dissecting the explants. Pourtois (1966, '68) and Vargas (1967, '68) dissected the left and right palatal processes in two separate parts from the embryos and explanted them together on culture media facing each other in close proximity. This differs in many aspects from the technique of Moriarty et al. (1963), employed in the present study, where the processes stayed attached to the maxillary area. The effect of surface tension in approximating the processes is diminished in that way. The method used by us has the advantage of corresponding more to the situation in vivo. The development of the processes towards each other, and the subsequent fusion, is well comparable. In the experiments of Pourtois (1966, '68) and Vargas (1967, '68) the first factor was missing and regarding the second one, surface tension played a helping role, as reported by Pourtois (1966).

It must be remarked here that the comparison of our findings with those
obtained by others, is subjected to certain limitations due to differences in the methods of dissection and the strains of rats used.

Moriarty et al. (1963) found different patterns for the approximation of the palatal processes in vitro and in vivo. In their in vitro experiments, they observed that the palatal processes approximated first in the posterior region and then in the anterior and middle regions. But the fusion itself first started in the anterior region when examined microscopically.

Also Reeve et al. (1966) found, by employing the time-lapse photographic technique, that the palatal processes approximated first posteriorly in the explants dissected from 14 day old rat embryos. Since they did not specify the strain of rat or method of noting the day of conception of the female rats, their findings could not be carefully compared with ours.

Our findings are well comparable and in agreement with those of Moriarty et al. (1963). The explants, examined macroscopically after 24 to 72 hours of incubation, showed the processes to be in contact with each other in the posterior region or in some specimens more or less at the same time in the anterior region. This finding is contrary to our observations of 15 and 16 day old embryos in vivo. There, the processes achieved approximation and fusion first in the middle third of the anterior region and from there it progressed anteriorly and posteriorly.

The different initiation pattern of the approximation in the in vitro and in vivo experiments may be explained as due to the absence of the tongue in the cultured material. In vivo the lowering of the tongue started in the anterior region with a simultaneous change in the position of the palatal processes from vertical to horizontal. This pattern allowed the processes to assume contact first in the anterior region. In the in vitro experiments the tongue was removed in the posterior region at an earlier developmental stage and a premature horizontal position of the palatal processes was established.

It is remarkable that the cellular fusion started not in the area where the first contact was made, but in the anterior region, as was the case in the in vivo situation. This leads to the assumption that the first site of fusion is dependent on inherent information available to the palatal processes.

It was also noted in the in vitro experiments that the fused palatal processes had a flat oral surface in the posterior region. This is different from the observations in the in vivo experiments where the processes showed a concave oral surface in that area. This difference is probably also due to the absence of the tongue in the in vitro experiments. It is not unlikely that the tongue plays an active part in the moulding of the shape of the palatal processes. This remark can be substantiated by the results of in vivo experiments where the tongue was found to have followed the contours of the palate and other oral surfaces, both before and after the fusion.
An interesting finding found both in the *in vitro* and *in vivo* experiments is the presence of a mesenchymal condensation in the medial third of the palatal processes prior to and during the mesenchymal fusion.

Several mitotic figures were observed in the midline epithelium of the palatal processes and the nasal septum. Konegni *et al.* (1965) also reported the presence of mitotic figures in the midline epithelium. Barry (1961) stated that an actively, high rate of proliferation of the epithelial cells in the midline is also a possible etiologic factor in the production of the cleft palate. The findings of Chapter IV do not support this observation, as the mode of the produced cleft palate was found to be different in our experiments. The observations of Barry (1961) may well be true for the abortus human embryos he studied.

Moriarty *et al.* (1963) and Konegni *et al.* (1965) reported degeneration of the epithelium of the enclosed nasal chamber after culturing. Contrary to this, the specimens of the present investigation showed several mitotic figures in the nasal epithelium.

Summarizing, it may be said that the potentiality of the palatal processes to fuse *in vitro* depends on two main factors: a. the age of the embryo at the time of dissection of the explants, and b. the time of incubation provided for the explants.

The main conclusions drawn from the experiment discussed in this chapter are:

The palatal processes are able to fuse *in vitro.*

The palatal processes prior to the fusion with each other approximate first in the posterior region and later, or at the same time, in the anterior region. This finding is contrary to the one observed in the *in vivo* experiments. It is suggested that the tongue plays an important role in this phenomenon. Contrary to the initial site of contact of the palatal processes, the fusion at the cellular level commenced in the anterior region as observed *in vivo.* The pattern of cellular fusion was found to be similar to the one observed *in vivo.*
V-1. Photographs of the same explant, showing the fusion of the palatal processes on a longitudinal basis.

a. Explant of a 15.16 day old normal rat embryo (M-5). Incubation period 0 hours. Note the unfused palatal processes. X 22.

b. The same explant (M-5) after an incubation period of 48 hours. The palatal processes are in contact with each other. Note the opaque appearance of the explant. X 22.

c. The same explant (M-5) after an incubation period of 72 hours. The palatal processes are in contact with each other over a larger area of the palate than in the previous age. X 22.
V-2. A typical example of an explant showing all the stages of palatal fusion in vitro.

a. Explant of a 15.9 day old normal rat embryo (M-ç) after an incubation period of 72 hours, frontal section. The palatal processes are in epithelial contact with each other. Haematoxylin-eosin. X 150.

b. The same explant (M-ç). Frontal section slightly posterior to the previous one. The palatal processes are in contact with each other. The union is of one epithelial strand. Haematoxylin-eosin. X 150.

c. The same explant (M-ç). Frontal section cut through the posterior region of the palate. Note mesenchymal fusion of the palatal processes. Haematoxylin-eosin. X 150.
V-3. Photographs of the same explant, showing fusion of the palatal processes on a longitudinal basis.

a. Explant of a 15.9 day old rat embryo (M-24). Incubation period 0 hours. Note the unfused palatal processes; they are nearest to each other in the posterior region of the palate. X 22.

b. The same explant (M-24) after an incubation period of 24 hours. Note the gap between the palatal processes. X 22.

c. The same explant (M-24) after an incubation period of 48 hours. Note the contact of the palatal processes in the anterior region. X 22.
V-4. Explant of a 16.9 day old normal rat embryo after an incubation period of 72 hours, frontal section. The midline fusion of the palatal processes is indicated by arrows. Note that the shape of the palate is similar to the normal one in vivo. Haematoxylin-eosin. X 150.

V-5. Explant of a 14.16 day old normal rat embryo after an incubation period of 72 hours, frontal section. The midline fusion of the palatal processes is indicated by arrows. Toluidine blue. X 150.
CHAPTER VI

IN VITRO DEVELOPMENT OF THE PALATAL PROCESSES OF EMBRYOS FROM DRUG TREATED MOTHERS

INTRODUCTION

The results obtained in Chapter IV indicated that two main factors may play an important role in the artificial production of cleft palate in Wistar albino rats: a. the approximate 24 hour delay in the change from vertical to horizontal position of the palatal processes, and b. the continued presence of the tongue between the medially inclined palatal processes on day 16 of gestation. These factors were associated with retarded downward growth of the nasal septum, decreased downward and forward growth of the mandible, maxillo-mandibular ankylosis and microstomia. It was suggested that one, or more, of these phenomena acting together can prevent the lowering of the tongue, and thereby not allowing the palatal processes to assume a horizontal position.

The study of the cleft palate in vivo presented three important questions:

a. did the palatal processes loose their tendency to become horizontal on day 16 of gestation in embryos of drug treated animals?

b. were the palatal processes of normal composition, or was the persistent presence of the tongue between the processes on day 16 of main importance?

c. did the palatal processes loose their potency for fusion?

The in vitro findings of the previous chapter provided another method to investigate whether or not the palatal processes of embryos of drug treated rats will fuse in vitro. The findings also led to new information regarding the mode of normal palatal closure. It was felt that among other things the above mentioned questions could be studied meaningfully by in vitro experiments.

The main purpose of the investigation described in this chapter was to study in vitro:

a. the effect of the treatment with vitamin A and Dexamethasone on the palatal processes, and

b. whether the occurrence of cleft palate is primarily due to the action of the drugs on the palatal processes and/or due to other factors.

MATERIAL AND METHODS

A total of 51 explants were dissected from the embryos of 17 pregnant Wistar
TABLE VI-1. *In vitro* fusion of palatal processes from embryos of vitamin A treated mothers

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<tr>
<th>Age at the time of operation</th>
<th>Series</th>
<th>Expiants no.</th>
<th>Incubation time (days)</th>
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<th>Epithelial fusion</th>
<th>Mesenchymal fusion</th>
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TABLE VI-2. *In vitro* fusion of palatal processes from embryos of Dexamethasone treated mothers

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<td>6</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>1</td>
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<td>24</td>
<td>22</td>
<td>2</td>
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</table>

albino rats. The mothers had been treated with either vitamin A or Dexamethasone.

Out of these 17 pregnant rats 9 had received 40,000 I.U. of vitamin A once a day from day 10 to 13 of gestation. The remaining ones were given 0.5 mg of Dexamethasone twice a day from day 9 to 12. The rats were sacrificed on day 15.16 under sterile conditions as already mentioned in Chapter II. Twenty-seven embryos from the vitamin A group and 24 embryos from the Dexamethasone group were used. The normal explants of the experiment underlined in the previous chapter cultured on day 15.16 served as controls.

The duration of the culturing period varied as shown in Table VI-1 and 2. It was extended to 8 days, because no marked changes occurred in the first 72 hours.

Photographs of some explants were taken with an interval of 24 hours. The explants were studied both macroscopically and microscopically.
FINDINGS

a. Macroscopic findings

*Vitamin A group* (Figs VI-1a, b, c): On the fourth day of incubation some movement was noticed in the posterior region of the processes and in one explant contact was observed. After the 4th day more explants were found showing approximation of the processes. On days 7 and 8 contact was also present in the anterior region of a few explants.

After 48 hours of incubation the explants were cloudy in appearance, the other features were similar to the ones already reported in Chapter V.

*Dexamethasone group* (Figs VI-5a, b, c): The cultured processes of the Dexamethasone group did not show appreciable growth of the palatal processes in the 8 day incubation period. In the beginning the palatal processes were small, as was the case in the vitamin A group, but here the gap remained in general the same. Only 2 explants out of 24 showed a slight movement at the posterior end.

b. Microscopic findings

*Vitamin A group* (Figs VI-2; 3; 4a and b): No fusions were observed till the third day of incubation (Table VI-1). An extended incubation period resulted in a number of fused palatal processes. On the 8th day of incubation 6 processes out of 8 showed either epithelial or mesenchymal fusion. Out of a total of 27 explants from 15.16 day old embryos, incubated for varying periods of time, 7 showed mesenchymal and 7 epithelial fusions.

The overall histological examination showed relatively more degeneration of the mesenchyme in comparison to the control series, and the palatal processes appeared to be smaller after 3 days of incubation. On the other hand, the nasal septum appeared to be relatively bigger in all the fused or unfused incubated explants.

Before the 4th day of incubation all explants showed a gap between the processes. The epithelial lining of the palatal processes was 2 to 3 cell layers thick, and in some older explants at the medial border it was 3 to 4 cell layers thick. The underlying mesenchyme in the medial third of the palatal processes had fewer cells than the normal. The cell density was almost normal in the middle and lateral regions of the palate. The epithelium and the underlying mesenchyme of the enclosed nasal area showed the same characteristics as seen for the palatal processes.

From the 4th day of incubation several explants showed varying degrees of
fusion, increasing with the time of incubation. Several 7 or 8 day old explants exhibited both epithelial and mesenchymal fusions and areas of non fusion, as was observed in the control series at the third day. The fusions were mostly seen in the posterior and anterior areas.

Prior to fusing with each other the epithelium of the processes was 3 to 4 layers thick. This was different from the control series where the epithelium in the fusion area was only 1 to 2 cell layers thick. The underlying mesenchyme in the medial third of the palatal processes showed less cells than the control series but more than the unfused processes.

Several mitotic figures were seen in the epithelium of the palatal processes and nasal septum prior to their fusion with each other (Figs VI-4a and b).

*Dexamethasone group* (Fig. VI-5d): Only 2 palatal processes from 24 cultured explants obtained epithelial fusion (Table VI-2).

The overall picture was entirely different from the control series and the vitamin A group. No mesenchymal fusions were seen. Only in 2 explants the processes had fused with each other epithelially in a small area. Excessive degeneration of both epithelial and mesenchymal cells was noticed, particularly in the explants incubated for longer periods.

The processes had a stunted appearance. The epithelium was at some places 4 to 5 layers thick. The underlying mesenchyme was scarce and undifferentiated.

The epithelium of the nasal septum appeared less affected than the palatal processes. In places it was also of 4 to 5 layers thick where only 1 or 2 layers were seen in the control series.

**INTERPRETATION OF THE FINDINGS**

In the present experiment the palatal processes in explants from embryos of vitamin A treated mothers did not fuse on the 3rd day of incubation. However, when incubated for longer periods, they showed the ability to fuse.

From this finding it may be concluded that the palatal processes were affected prior to the explanting procedure due to the teratogenic action of the drug. The fact that fusion *in vitro* took place in some specimens may have to do with the absence of the tongue and the elimination of the lateral growth effect of the head.

The palatal processes from the Dexamethasone treated group – as a rule – did not show any growth or tendency to fuse *in vitro*. The tissues were affected directly by the administration of the drug to the pregnant rats.
DISCUSSION AND CONCLUSIONS

Since, to our knowledge, no comparable study has been carried out, the discussion will be limited to the findings of the present chapter and those of the previously reported chapters.

An interesting finding was that the palatal processes from the vitamin A group did not show growth during the first three days of incubation. After that, changes took place, and in many cases fusion occurred in vitro. These observations can with some reservation be transmitted to the findings of the in vivo vitamin A experiments reported in Chapter IV, where an approximately 24 hour delay in change from a vertical to a horizontal position of the palatal processes was found. When these processes did become horizontal, they were found to be malformed and the gap between them increased with age. The present findings show that in vitro the palatal processes in the vitamin A group still maintained or could develop their potency for fusion. It is most likely that the continued increased growth of the head in the lateral direction in the in vivo experiments prevented the establishment of contact of the two processes, so that fusion could not occur.

The histological picture showed some changes in the processes in the vitamin A group during earlier periods of incubation, in comparison to the control series. But as the time of incubation increased a 'catch up' phenomenon was noticed and the cellular picture became more similar to the control group.

Another interesting fact, which was noticed in normal palatal processes both in vivo and in vitro, was also seen in the processes of the vitamin A group in vitro. This was the presence of mesenchymal condensation in the medial third of the palatal processes prior to the fusion. This was found to be missing in all the unfused palatal processes, thus signifying its role in achievement of the fusion.

Contrary to the findings of the vitamin A group, the palatal processes obtained from embryos of Dexamethasone treated rats did not show appreciable growth or fusion in vitro. This finding suggests that the palatal processes of this group were so severely damaged that they could not grow in vitro. This is substantiated by the histological findings where the explants were found in various stages of degeneration, and on the basis of this it may be assumed that they lose their potency for fusion.

The results of the experiments described in this chapter show clearly that although hypervitaminosis A and Dexamethasone both produce cleft palate in vivo and almost with similar morphological features, the anomaly is reached by two different mechanisms.

To elaborate these observations further, experiments with cultivating palatal
processes in vitamin A and Dexamethasone added culture medium will be described in the next chapter. Such experiments should be able to show whether both drugs inhibit or retard the development of the processes by a direct or intermediary action.

Summarizing, it can be stated that:

- The palatal processes from embryos of vitamin A treated rats, showed delayed fusion in vitro.
- The palatal processes of vitamin A treated groups are capable of fusing with each other in vivo, even after a 24 hour delay in the horizontalization of the processes but, due to the retarded development of the processes and the continued lateral growth of the head, this can not be achieved.
- The palatal processes from embryos of Dexamethasone treated rats, in general did not show any appreciable growth and also no fusion.
- The histological picture of the Dexamethasone group material suggests that the drug acts directly on the palatal processes leading to cellular degeneration.
VI-1. Photographs of the same explant, showing the fusion of the palatal processes on a longitudinal basis.

a. Explant of a 15.16 day old rat embryo (T-16), mother treated with vitamin A 40,000 I.U./day from day 10 to 13 of gestation. Incubation time 0 hours. Note that the palatal processes are nearest to each other in the posterior region. X 22.

b. The same explant (T-16) after an incubation period of 4 days. The palatal processes are in contact with each other. X 22.

c. The same explant (T-16) after an incubation period of 7 days. Note the area of contact of the palatal processes is larger than in the previous photograph. X 22.
VI-2. Explant of a 15.16 day old rat embryo, frontal section, mother treated with 40,000 I.U./day from 10th to 13th day of gestation. Incubation period 6 days. Note the persistence of the epithelial cell layers in the fusion line (arrow). Haematoxylin-eosin. X 150.

VI-3. Explant of a 15.16 day old rat embryo, frontal section, mother treated with 40,000 I.U./day from 10th to 13th day of gestation. Incubation period 8 days. Note the mesenchymal fusion of the palatal processes. Haematoxylin-eosin. X 150.

VI-4a. Explant of a 15.16 day old rat embryo, frontal section, mother treated with 40,000 I.U./day from 10th to 13th day of gestation. Incubation period 6 days. The palatal process of the right side is not fused with the nasal septum. Note the cell degeneration in the palatal processes. The mesenchymal cells from the nasal area can be seen lying between the palatal processes and nasal septum. Haematoxylin-eosin. X 150.

VI-4b. Higher magnification of the outlined area of the nasal septum, showing cells in different stages of mitosis. Haematoxylin-eosin. X 490.
VI-5. Photographs of the same explant, showing the changes of the palatal processes on a longitudinal basis.

a. Explant of a 15.16 day old rat embryo (D-13), mother treated with Dexamethasone 0.5 mg 2×/day from 9th to 12th day of gestation. Incubation period 0 hours. Note the large size of the gap between the two palatal processes. X 22.

b. The same explant (D-13) after an incubation period of 4 days. No appreciable decrease in the gap between the two palatal processes can be seen. X 22.

c. The same explant (D-13) after an incubation period of 8 days. Note that the palatal processes are still wide apart. X 22.

d. The same explant (D-13) after an incubation period of 8 days. Frontal section through the middle region of the palate. Note the palatal processes are wide apart. Haematoxylin-eosin. X 50.
CHAPTER VII

IN VITRO DEVELOPMENT OF THE PALATAL PROCESSES IN A DRUG ADDED MEDIUM

INTRODUCTION

Since Gaillard (1932) made the first attempt to study the action of a vitamin on the skeletal tissues in vitro, various studies have been conducted to analyse the direct action of vitamins and hormones on differentiating tissues in culture. Organ culture has been widely used for investigations in the fields of nutrition, endocrinology and teratology.

Fell (1956) reviewed several studies on the effect of vitamin A on various skeletal tissues in vitro. She concluded that vitamin A has a direct action on a wide variety of tissues grown under conditions which preclude indirect effects mediated through other systems or organs.

Other investigations came to the conclusion that excess vitamin A in vitro causes rarefaction of bone, accelerates resorption of cartilage (Fell and Mellanby, 1952), stimulates mitosis of fibroblastic cultures (Lasnitzki, 1955a, '55b), and leads to hypoplasia and some cell hyperthrophy with lobulation of the skin (Hardy, 1967).

Similarly, cortisone, hydrocortisone and related compounds have been studied. It has been reported that they inhibit the growth of cartilagenous femurs (Buno and Goyena, 1955; Whitehouse and Lash, 1961), retard the growth of the adult human epithelium and embryonic chick epithelium (Geiger et al., 1956), and inhibit DNA and polysaccharide synthesis (Reynolds, 1966).

Recently a few investigations have been presented regarding the direct action of drugs on the palatal closure in vitro. Several drugs were found to have an inhibitory effect on the palatal closure in rat embryos, e.g. galactoflavin (a riboflavin antagonist), 6-aminonicotinamide, excess vitamin A (Myers, 1967; Myers and Lee, 1967; Myers et al., 1967a; Myers et al., 1967b), hydrocortisone (Lahti and Saxén, 1967) and hypo- and hypervitaminosis A, and hydrocortisone (Pourtois, 1968).

The findings of the previous chapters call for an experiment to find out whether vitamin A and Dexamethasone act directly on the palatal processes leading to retardation or inhibition of closure. This has been studied in the experiment presented in this chapter by adding these substances to the culture medium.
MATERIAL AND METHODS

A total of 86 explants containing the palatal processes were dissected from the 15.16 day old embryos of 28 normal pregnant Wistar rats. The explants were divided over 3 separate groups of experiments.

1. **Control group**: (Table VII-1). 19 explants were cultured in a medium containing sterile water or ethanol in a concentration of 0.0015 ml/ml of medium. Both water and ethanol were added prior to explanting the tissue.

2. **Vitamin A group**: (Table VII-2). 35 Explants were cultured in a medium to which vitamin A was added in the concentrations of 10, 25 or 50 I.U./ml.

3. **Dexamethasone group**: (Table VII-3). 32 Explants were cultured in a medium containing Dexamethasone in the concentrations of 0.01, 0.1 or 7.5 μgm/ml.

Both vitamin A and Dexamethasone were added in their proper concentrations after diluting them with water or ethanol.

As indicated in Tables VII-1, 2, and 3, the explants were incubated for different periods. The same culturing medium was employed in the explants incubated for more than 3 days. The histological techniques employed for the present experiment have already been described in Chapter II.

FINDINGS

The findings of the three groups will be described separately.

a. **Macroscopic findings**

1. **Control group** (Table VII-1): The explants showed movement and approximation of the processes after an incubation period of 24 hours. The pattern of approximation and other factors were similar to the ones observed in Chapter V when explants were cultured in a normal medium.

<table>
<thead>
<tr>
<th>Added agents</th>
<th>Series</th>
<th>Explants no.</th>
<th>Incubation time (days)</th>
<th>No fusion</th>
<th>Epithelial fusion</th>
<th>Mesenchymal fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water concent. 0.0015 ml/ml of final medium</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ethanol concent. 0.0015 ml/ml of final medium</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
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<td></td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>19</td>
<td>4</td>
<td>5</td>
<td>10</td>
<td></td>
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</tr>
</tbody>
</table>
2. *Vitamin A group* (Table VII-2; Figs VII-1a and b): Only a slight approximation of the palatal processes was observed when 10 I.U. of vitamin A was added in the culture medium. The processes came in contact in the posterior region only in a limited area. In the explants cultured in media where 25 or 50 I.U. was added, no appreciable growth of the palatal processes was noticed. They appeared after 3 days small and stunted.

3. *Dexamethasone group* (Table VII-3): Only 5 explants out of 32 showed a slight approximation of the processes in the posterior region. The majority of

<table>
<thead>
<tr>
<th>TABLE VII-2. Palatal processes dissected on day 15.16 of gestation and cultured in a vitamin A supplemented medium</th>
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<tbody>
<tr>
<td>Concentration of vitamin A (I.U./ml of medium)</td>
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<td>------------------------------------------------</td>
</tr>
<tr>
<td>10 I.U.</td>
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<tr>
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<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>25 I.U.</td>
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<tr>
<td></td>
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<tr>
<td>50 I.U.</td>
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<table>
<thead>
<tr>
<th>TABLE VII-3. Palatal processes dissected on day 15.16 of gestation and cultured in a Dexamethasone supplemented medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Dexamethasone (μgm/ml of final medium)</td>
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<tr>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>0.01 μgm</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>0.1 μgm</td>
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<td></td>
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<tr>
<td>7.5 μgm</td>
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the explants, cultured in the media containing 0.1 or 7.5 μgm did not show a noticeable growth of the palatal processes. No appreciable decrease in the gap between the palatal processes was observed. Even after an incubation period of 6 to 8 days, the processes appeared small and stunted.

b. Microscopic findings

1. Control group: Table VII-1 shows that 8 explants out of 10 achieved some form of fusion either epithelial or mesenchymal when 0.0015 ml of water was added in 1 ml of final medium. Only two explants did not fuse.

Similarly, out of a total of 9 explants, only 2 did not achieve fusion when ethanol was added in the strength of 0.0015 ml/ml of culture medium.

In more than half of the cultured explants mesenchymal fusions were obtained. Most of the explants fused after 24 hours of incubation.

The histological appearance of the both fused and unfused processes was similar to the one observed in the normal group of Chapter V.

2. Vitamin A group (Figs VII-1c and 2): Table VII-2 presents the number of fusions or no fusions according to the different amounts of vitamin A added to the culture medium. When 10 I.U. was added, only 3 epithelial and 2 mesenchymal fusions were obtained out of 12 explants. These fusions were obtained only when the incubation period was prolonged to 6 or 8 days.

Contrary to these findings is the fact that when 25 or 50 I.U. of vitamin A was added, no mesenchymal fusions were obtained and only one explant out of a total of 23 showed epithelial fusion. In both cases the explants were incubated for varying periods of time to a maximum of 8 days.

Slight differences were observed in the histological findings of the explants cultured in three different media containing vitamin A in the concentrations of 10, 25, and 50 I.U.

The explants cultured in a medium having 10 I.U. of vitamin A, showed palatal processes with intact epithelium of cuboidal or columnar cells arranged in one or two layers. The mesenchyme was found to be slightly dense at the medial third of the processes (Fig. VII-1c). Overall, a considerable number of degenerating cells were noticed, both in the epithelium and mesenchyme. The area in the posterior region, where 3 epithelial fusions were observed, was small.

The cellular picture of the palatal processes cultured in the presence of 25 or 50 I.U. of vitamin A was quite different from that of the one mentioned above. The epithelial covering of the palatal processes was found to be broken at several
This degeneration or peeling off of the epithelium was only observed at the oral and medial surfaces. The epithelium facing the nasal chamber appeared intact but was oedematous. In some instances, the outer layers of the epithelium were pyknotic and the inner layers were oedematous.

The mesenchymal cells were densely populated in the medial third of the palatal processes but most of these cells appeared pyknotic or in a degeneration stage. The mesenchymal cells in the middle and lateral regions were comparatively more healthy. It was also noted that the degeneration of the mesenchyme started from the medial area of the palatal processes and progressed laterally.

Compared to the palatal processes, the epithelium of the nasal septum was intact in the majority of the explants. However it did not fuse with the palatal processes. The mesenchymal cells of the nasal septum towards the oral side appeared oedematous and were, in some cases, in stages of degeneration. Here too, degeneration seemed to have started from the area facing the palatal processes. The cartilage of the nasal septum appeared comparatively healthy.

3. Dexamethasone group (Figs VII-3, 4 and 5): Table VII-3 indicates that when 0.01 μgm was added in 1 ml of final medium only 4 explants out of 13 showed epithelial fusion. The fusion was limited to a very small area in the posterior region. With 0.1 and 7.5 μgm/ml of final medium, only one epithelial fusion was observed in 19 explants. In this group no explant showing mesenchymal fusion was observed.

The epithelium of the processes cultured in different concentrations of Dexamethasone appeared intact and slightly oedematous. At some places it was of 3 to 4 layers, especially in the medial part facing the nasal septum. The mesenchymal cells in the medial part of the palatal processes were considerably less in number than in the middle and lateral parts of the processes. With increase in the concentration of Dexamethasone, more cellular degeneration was noticed in the explants.

The nasal septum also appeared affected like the palatal processes. The cartilage looked comparatively healthy as observed in the controls.

**INTERPRETATION OF THE FINDINGS**

The palatal processes from 15.16 day old embryos showed in general no fusion when they were cultured in media in which different concentrations of vitamin A and Dexamethasone were added. The processes cultured in the higher doses of both drugs appeared to be severely damaged. The gap between the palatal processes remained widely open. The vitamin A added medium led to oedema and peeling off of the epithelium of the palatal processes. The mesenchymal cells
appeared pyknotic and degenerative, which appeared to have started from the areas of the processes facing each other.

In the media to which Dexamethasone was added, the mesenchyme was mostly affected. The medial third of the processes was scarce in cells in comparison to the middle and lateral thirds. The cells appeared to be pyknotic. The epithelium on the whole was intact, but appeared oedematous when compared with the control material.

**DISCUSSION AND CONCLUSIONS**

The closure of the palatal processes appeared to be normal in the water and ethanol added culture medium. This finding is in agreement with Hardy (1967) who cultivated embryonic mouse skin in an ethanol added medium of the same concentration as ours. She found that it improved the health and growth of the explants.

Myers *et al.* (1967b) reported that when vitamin A was added to the culture medium, this resulted in a high incidence of failures of processes to fuse. On the other hand, they found no difference between the cellular picture of the processes of vitamin A group *in vitro* and their control material. They concluded that the failure of the processes to fuse in a vitamin A supplemented medium was due to the retarded development of the palatal processes.

Pourtois (1968) found histopathic effects on the palatal development when vitamin A was added to the culture medium. He observed signs of turgor in the intercellular spaces of the marginal mesenchyme, detachment of the epithelium from the mesenchyme, or an oedema in the marginal mesenchyme of the palatal processes. Still, he reported noticing epithelial fusions to some extent.

The findings of the present investigation are in agreement with those of Pourtois (1968), as several histopathologic changes were recorded when processes were cultured in a vitamin A added medium. The present findings only agree with those of Myers *et al.* (1967b) to the extent that a high incidence of unfused processes was obtained and palatal growth was found to be inhibited or retarded. However, our findings do not agree with theirs in the respect that there was no difference between the cellular picture of the normal and vitamin A groups. We can suggest from our material that the failure of the processes to fuse was primarily due to a direct action of vitamin A on the cellular components of the palatal processes.

Lahti and Saxén (1967) studied the effect of hydrocortisone on the closure of the palatal processes. They added hydrocortisone to the culture medium in concentrations ranging from 0.001 to 20.0 g/ml of culture medium. After in-
cubating palatal explants from 3 to 12 days, they reported the occurrence of complete fusion in all instances, irrespective of the concentration of hydrocortisone in the culture medium. Contrary to this, Pourtois (1968) found that 0.1 mg/ml of hydrocortisone led to necrosis of the mesenchyme. However, he was able to find epithelial fusions in small areas. Smaller concentrations of added hydrocortisone led to an oedema in the marginal epithelium. Pourtois stated that several palatal processes fused epithelially, but mesenchymal fusion was seldom observed.

The findings of the present study suggest that the Dexamethasone is a very toxic substance. This is in agreement with Pinsky and DiGeorge (1965) as they stated that Dexamethasone was several times more teratogenic than hydrocortisone and cortisone acetate when given to pregnant mice. The histological findings partly agree with those of Pourtois (1968) but the majority of the processes in our experiment did not show epithelial or mesenchymal fusions.

The in vivo findings of Chapter IV and, in vitro findings of Chapter VI and those of the present chapter suggest that vitamin A acts directly on the palatal processes.

In Chapter VI it was noted that when palatal processes from vitamin A treated rat embryos were incubated for longer periods, that is, up to 8 days, the palatal processes showed a tendency to fuse. This phenomenon was not observed in the present experiment.

The experiments employing Dexamethasone, both in vivo and in vitro, show that this steroid acts directly on the palatal processes, by retarding their development and leading to cleft palate. The in vitro findings of Chapter VI suggest that the action of Dexamethasone is irreversible. This fact is further substantiated by the present findings which showed that when added directly to the media it causes necrosis and inhibits the growth of the mesenchyme.

Summarizing, it can be stated that:

Vitamin A, when added directly to the culture medium, causes oedema and peeling off of the epithelium and necrosis of the mesenchyme and epithelium both. The majority of the processes appeared stunted or retarded in growth and were unfused.

Dexamethasone when added directly to the culture medium leads to necrosis of the mesenchyme and the processes appeared scarce of mesenchymal cells. Nearly all the cultured processes were small and stunted. The majority of the processes did not fuse.

Vitamin A and Dexamethasone, when added to the culture medium, act directly on the palatal processes leading to their necrosis and retardation in the development.
VII-1. Photographs of the same explant showing the effect of vitamin A, supplemented to the medium, on the palatal closure on a longitudinal basis.

a. Explant of a 15.16 day old normal rat embryo (X-9). 10 I.U. of vitamin A was added to 1 ml of culture medium. Incubation time 0 hours. Note that the palatal processes are in contact in the posterior region. X 22.
b. The same explant (X-9) after an incubation period of 6 days. Note increase in size of the contact area. X 22.
c. The same explant (X-9), frontal section. Note the epithelial contact of the palatal processes. The epithelial lining of both palatal processes is intact. Haematoxylin-eosin. X 150.

VII-2. Explant of a 15.16 day old normal rat embryo, frontal section. 25 I.U. of vitamin A was added to 1 ml of culture medium. Incubation time 6 days. Note the unfused palatal processes and the degeneration of the cells. Haematoxylin-eosin. X 150.
VII-3. Explant of a 15.16 day old normal rat embryo, frontal section. 0.01 μgm of Dexamethasone was added to the culture medium. Incubation period 6 days. Note that the palatal processes are unfused; the epithelial lining is not intact. Haematoxylin-eosin. X 150.

VII-4. Explant of a 15.16 day old normal rat embryo, frontal section. 0.1 μgm of Dexamethasone was added to the culture medium. Incubation period 6 days. Note the degeneration of the epithelial and mesenchymal cells of the palatal process; the epithelial lining of the nasal septum facing the palatal process is intact. Haematoxylin-eosin. X 150.

VII-5. Explant of a 15.16 day old normal embryo, frontal section. 7.5 μgm of Dexamethasone was added to the culture medium. Incubation period 3 days. Note the degeneration of the epithelial and mesenchymal cells of the palatal process, and the nasal septum. Haematoxylin-eosin. X 150.
CHAPTER VIII

AUTORADIOGRAPHIC STUDY OF NORMAL AND CLEFT PALATE EMBRYOS

INTRODUCTION

The findings, mentioned in the previous chapters, suggest that vitamin A and Dexamethasone produce cleft palate by acting directly on the palatal processes. This means that both drugs, when given to pregnant rats, disturb the palatal processes of the embryos either at the intra-or/and the intercellular level. Both factors in turn may be dependent upon biochemical, genetic, or other inborn factors. Others have examined the palatal processes at the intercellular level prior to and during the fusion. As mentioned earlier, Walker and Fraser (1956) have suggested that an internal force should be present between the palatal processes that produces an active elevation of the processes from a vertical to a horizontal position, and also should overcome the resistance provided by the relatively stationary tongue. Later Larsson et al. (1959), Larsson (1960) and Walker (1961) employing $^{35}$S-labelled sulphate suggested that elastic mucopolysaccharides containing gels of the mesenchymatous ground substance may be the source of the 'internal force'. These authors also reported that a disturbed metabolism of sulphated mucopolysaccharides may lead to insufficiency of the 'internal force', thereby resulting in a failure of the processes to change their position and to subsequently fuse with each other.

The studies employing cortisone acetate as a teratogenic agent for the production of cleft palate, suggested that it inhibits the utilization of inorganic sulphate in the synthesis of mucopolysaccharides (Larsson, 1962b; Jacobs, 1964a, '64c, '64d). Kochhar and Johnson (1965) employed vitamin A as a teratogenic agent and concluded that it disturbs the synthesis of mucopolysaccharides of the mesenchymatous ground substance of the palatal processes. But contrary to the findings of the studies employing cortisone acetate, they found the synthesis of sulphated mucopolysaccharides to have increased considerably in the palatal processes of embryos with cleft palate.

The autoradiographic experiments using $^{35}$S-labelled sodium sulphate, were undertaken to throw more light on our own findings presented in the previous chapters and also on the contradictory findings reported by others regarding the role of mucopolysaccharides in the palatal closure and in the production
of cleft palate. In these experiments, only vitamin A was employed as a teratogenic agent.

It was also considered meaningful to study the effect of vitamin A on the cell proliferation and DNA synthesis. To the best of our knowledge no experiments of this type have been conducted before in the study of the normal secondary palate development and in the evaluation of the cause of cleft palate production.

MATERIAL AND METHODS

According to the material and methods employed, the present experiment can be divided into two main groups.

**Group I:** $^{35}$S-labelled sodium sulphate.

**Group II:** $^{3}$H-thymidine.

**Group I**

This group consisted of both normal and vitamin A treated animals. The pregnant rats were injected with 20 µCi/gm body weight of $^{35}$S-sulphate on days 13, 14, and 15 of gestation. In total 9 vitamin A treated rats and 9 normal animals were used. The details are shown in Table VIII-1. The rats were sacrificed systematically 48 hours after the administration of $^{35}$S-labelled sulphate. The autoradiographic evaluation was limited to 3 embryos from each rat. The details of the histologic and autoradiographic techniques are described in Chapter II.

**TABLE VIII-1. Distribution of material for autoradiographic studies employing $^{35}$SO$_4$**

<table>
<thead>
<tr>
<th>Series</th>
<th>Embryonic age (days)</th>
<th>Normal mothers</th>
<th>Embryos</th>
<th>Vitamin A* mothers</th>
<th>Embryos</th>
<th>Isotope dose µCi/gm body wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>3</td>
<td>23</td>
<td>3</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>3</td>
<td>26</td>
<td>3</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>3</td>
<td>22</td>
<td>3</td>
<td>18</td>
<td>20</td>
</tr>
</tbody>
</table>

* Mothers received 40,000 I.U. of vitamin A/day on days 9, 10 and 11 of gestation.

**Group II**

Tritiated thymidine was injected directly into the amniotic sac of the embryos of untreated and vitamin A treated 16 and 17 day old rats. Details are shown in Table VIII-2. Series 3 is formed by sham operations. Instead of the isotope,
TABLE VIII-2 Distribution of material for autoradiographic studies employing $^3$H-thymidine

<table>
<thead>
<tr>
<th>Series</th>
<th>Embryonic age (days)</th>
<th>Normal mothers</th>
<th>Embryos</th>
<th>Vitamin A* mothers</th>
<th>Embryos</th>
<th>Isotope dose μCi/embryo</th>
<th>Killed after (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>24</td>
</tr>
</tbody>
</table>

* Mothers received 40,000 I U of vitamin A/day on days 9, 10 and 11 of gestation
** 15 μl of saline

15 μl of saline was injected into the amniotic sac of 6 normal and 6 vitamin A treated embryos. The rats of this particular series were sacrificed after 24 hours.

**Findings**

**GROUP I** The incorporation of $^{35}$S-labelled sulphate was studied microscopically and by evaluating the photographs of the areas pertinent to the experiment. Care was taken to photograph the interesting areas of the normal embryos and the ones of the treated animals at the same level in the frontal plane. By the combination of the microscopic findings and those obtained from the photographs, an estimate was made regarding the density of the incorporated $^{35}$S-labelled sulphate in the palatal processes and other tissues.

No cleft palates were observed in the normal embryos while all the embryos of vitamin A treated animals recovered on day 17 of gestation had their processes unfused, stunted, and deformed.

**Normal embryos (Figs VIII-1, 3, 5, 7):**

The autoradiograms of the sections of the embryos obtained on days 15, 16, and 17 of gestation showed uptake of $^{35}$S-labelled sulphate primarily in the tissues of the mesenchymal origin. The mesenchymatous ground substance of the palatal processes showed a marked incorporation of the labelled sulphate in the 16 and 17 day old embryos. Compared to the older embryos the labelling was slightly less in the 15 day old embryos.

For the sake of a more detailed description of the findings of the palatal processes in frontal sections, they were arbitrarily divided into three parts medial, the part facing the process of opposite side, lateral, the part of the process towards the maxillary area, middle, the part of the process between the medial and lateral parts.
In the 15 and 16 day old embryos, more labelling was observed in the transverse sections in the medial third of the palatal processes than in the middle and lateral parts. Such a localization effect was not noticed in the palatal processes of the 17 day old embryos. No difference was observed in the uptake of $^{35}$S-labelled sulphate between the anterior and posterior regions of the palate in any of the days studied.

Compared to the palatal processes, the nasal cartilage showed a considerable higher uptake of $^{35}$S-sulphate (Figs VIII-3 and 7). This effect increased with the age of the embryos.

The areas where labelling was noticed corresponded with the regions where toluidine blue metachromasia was observed.

*Embryos of vitamin A treated animals* (Figs VIII-2, 4, 6, 8):

Noticeable differences were observed in the uptake of $^{35}$S-labelled sulphate in the palatal processes of vitamin A treated rat embryos as compared with the controls. The palatal processes of the 15, 16, and 17 day old treated embryos showed a manyfold increased labelling.

In the 16 and 17 day old embryos the labelling was more intense in the medial part of the palatal processes than in the middle and lateral parts. This localization effect was not observed in the 15 day old embryos. The intensity of labelling of the nasal cartilage was considerably more as compared to the palatal processes (Figs VIII-4 and 8).

*GROUP II: No cleft palate was observed in the normal embryos injected with $^3$H-thymidine or saline water. All the embryos of the vitamin A treated rats showed cleft palate.*

To evaluate the cell proliferation and DNA synthesis of the epithelial and mesenchymal cells of the palatal processes, the labelled cells and mitoses were counted. The labelling index of the epithelium and the mesenchyme of both the 16 and 17 day old palatal processes was determined separately. This was also done for certain areas of the tongue.

*Normal embryos* (Figs VIII 9; 11a, b; 13a, b; 15a, b):

Considerable labelling was observed both in the epithelium and mesenchymal cells of the 16 and 17 day old embryos. In the younger ones, more labelled epithelial and mesenchymal cells were noted in the anterior half of the palate, than in the posterior one. On day 16 more epithelial cells than mesenchymal cells were undergoing DNA synthesis. As compared to the lateral parts of the
palatal processes, more labelling was noted in the medial and middle regions. Several labelled and unlabelled mitotic figures were found in the epithelial and in the mesenchymal cells of the palatal processes. The number of grains per nucleus ranged from 30 to 40.

The 17 day old embryos showed fused palatal processes. The labelled cells were counted in the anterior and posterior regions as well as in the center part of the palate. The mesenchymal cells of the palatal processes were sparse compared to the younger animals. The number of grains per nucleus was 20 to 30. No localized increase in the density of labelled cells was noticed. Approximately three times more labelled mitotic figures were seen in the tongue epithelium as compared to the palatal epithelium. The tongue mesenchyme showed comparatively few labelled mitoses.

*Embryos of vitamin A treated animals (Figs VIII-10, 12a, b, 14a, b, 16a, b):*

The results of vitamin A treated embryos will be described in relation to those of the normal embryos.

Both in the 16 and 17 day old embryos the labelling per nucleus was more intense. This was true for the epithelium as well as for the mesenchyme. Compared to the normal embryos, the treated embryos of both age groups presented less labelled epithelial and mesenchymal cells. The differences tested with the Chi-square test were found to be significant in most of the cases (Table VIII-3).

**Table VIII-3 Labelling indices of palatal processes of normal and treated embryos injected with tritiated thymidine**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Area</th>
<th>Age (days)</th>
<th>Labelling index</th>
<th>X²</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
<td>Vit A</td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>Anterior</td>
<td>16</td>
<td>41 3</td>
<td>21 7</td>
<td>609</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>16</td>
<td>34 2</td>
<td>18 7</td>
<td>455</td>
</tr>
<tr>
<td>Mesenchyme</td>
<td>Anterior</td>
<td>16</td>
<td>32 1</td>
<td>22 7</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>16</td>
<td>23 6</td>
<td>12 4</td>
<td>348</td>
</tr>
<tr>
<td>Epithelium</td>
<td>Anterior</td>
<td>17</td>
<td>31 9</td>
<td>15 3</td>
<td>583</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>17</td>
<td>28 8</td>
<td>17 9</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>17</td>
<td>38 0</td>
<td>16 9</td>
<td>812</td>
</tr>
<tr>
<td>Mesenchyme</td>
<td>Anterior</td>
<td>17</td>
<td>40 4</td>
<td>12 8</td>
<td>14 31</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>17</td>
<td>32 5</td>
<td>9 0</td>
<td>13 33</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>17</td>
<td>28 1</td>
<td>12 4</td>
<td>6 00</td>
</tr>
</tbody>
</table>
In 16 day old embryos the epithelium appeared more disturbed than the mesenchyme. Contrary to this, in 17 day old embryos the mesenchyme was more severely affected.

In both younger and older embryos relatively little labelling of the epithelium and considerable labelling of the mesenchyme was noticed in the medial part of the palatal processes, as compared to the more lateral positioned areas.

No labelled or unlabelled mitotic figures were seen either in the epithelium or mesenchyme of the palatal processes. The mesenchymal cells of the palatal processes of both younger and older animals were big in size, lying apart from each other and relatively small in number. The grains per nucleus were found to be 50 to 60.

In the epithelium and mesenchyme of the tongue, no labelled mitotic figures were found. However unlabelled mitotic figures were seen at several places.

INTERPRETATION OF THE FINDINGS

The experiments employing $^{35}$S-labelled sulphate revealed that its incorporation was slightly less in the palatal processes of the 15 day old normal embryos, in comparison to the 16 and 17 day old embryos. In the younger embryos the incorporation was more in the medial part of the palatal processes, as compared to the middle and lateral parts. In the 17 day old embryos more even distribution of the label was observed.

The uptake of $^{35}$S-sulphate was considerably greater in the palatal processes of embryos from vitamin A treated rats, as compared to the normal embryos. The typical distribution of the labelling, as noted in the 15 and 16 day old normal specimens, was only observed here in the 16 and 17 day old embryos.

The significance of these findings will be described in the discussion of the present chapter to avoid repetition.

The findings of the experiments employing $^{3}$H-thymidine showed that labelling was more intense per cell in the palatal processes of treated embryos than in the controls. These processes showed a smaller number of labelled cells per area than the normal ones.

The findings of the embryos from vitamin A treated rats revealed remarkable differences in comparison with the normal ones. The labelling was significantly less in the vitamin A treated specimens than in the controls. This holds true for both ages studied and for all the compared regions of the palate. This finding suggests that the DNA synthesis and the cell proliferation of the palatal processes was disturbed in the vitamin A treated embryos. The absence of labelled and unlabelled mitotic figures in the palatal processes of treated embryos does support this.
Another interesting observation in the treated group was that on day 16 the epithelial cells were comparatively more affected than the mesenchymal cells, whereas in the 17 day old embryos the mesenchyme was more disturbed than the epithelium.

A comparison of the cells of the tongue with those of the palatal processes showed almost no differences in the normal embryos. On the other hand, in the treated embryos several unlabelled mitotic figures were seen in the epithelium of the tongue and none in the palatal processes. In the tongue epithelium of the normal embryos both unlabelled and labelled mitotic figures were found. So it may be concluded that the generation cycle of the epithelial cells of the tongue and palatal processes in the treated group was considerably prolonged. The total absence of mitotic figures in the epithelium and mesenchyme of the palatal processes leads us to believe that the generation cycle of the cells is either inhibited or temporarily retarded.

The findings also showed that vitamin A caused a decrease in the number of the mesenchymal cells per area. The individual cells were comparatively large in size and had more grains per nucleus.

**DISCUSSION AND CONCLUSIONS**

Dziewiatkowski (1949) demonstrated that radioactive sulphur in the form of sulphur is incorporated as ester sulphate into the sulpho-mucopolysaccharides of cartilage and other mesenchymal tissues. Since this pioneer work, several investigations have been conducted to determine the role played by sulpho-mucopolysaccharides in normal and abnormal tissues, both *in vivo* and *in vitro*.

A number of factors can influence the sulphate exchange of sulpho-mucopolysaccharides. Dziewiatkowski (1951) reported a sulphate fixation in the mesenchymal ground substance after treatment of rats with thyroxine, whereas cortisone was found to have decreased the rate of uptake (Layton, 1951). Boström (1957) reviewing the studies on the sulphur metabolism of sulpho-mucopolysaccharides in mesenchymal tissues stated that these compounds, like most of the other body constituents, are metabolically active. He reported that the metabolism of these compounds is influenced by C-avitaminosis, cortisone, salicylic acid, and hypophysectomy.

That mucopolysaccharides play a role in the development of the secondary palate was first suggested by Walker and Fraser (1956), on the basis that they found metachromasia after toluidine blue staining and network after staining with Gomori's aldehyde fuchsin. They concluded that the movement of the palatal processes from a vertical to a horizontal position was either due to swelling
of the processes by binding of water to hyaluronic acid, or due to the development of an elastic network producing a force that could carry the processes superior to the tongue.

Larsson et al. (1959) and Larsson (1962a) confirmed the above mentioned observations of Walker and Fraser (1956) but did not agree with their interpretations. Later on, it was suggested that the presence of sulpho-mucopolysaccharides before and during the active change in direction of the palatal processes gives the ground substance the ability to undergo changes in shape of the processes and also leads to the development of force which causes the processes to assume a position superior to the tongue (Walker, 1961; Larsson, 1962a).

Larsson (1962b) reported a decrease in the uptake of $^{35}$S-sulphate by the mesenchymal tissue of the palatal processes prior to their change in position in embryos obtained from cortisone treated mice. He concluded that cortisone decreases the synthesis of sulpho-mucopolysaccharides during the period of change in the direction of the palatal processes, and suggested it as one of the causes of cleft palate.

Gaudino (1954) stated that tissues containing predominantly sulphated mucopolysaccharides may have a low content of water, while those in which hyaluronic acid predominates are hydrophilic. From the observations of Gaudino (1954), Jacobs (1964b) concluded that an increase in the hyaluronic acid content of the palatal processes leads to edema in embryos obtained from cortisone treated mice. He concluded that this disturbance in the water metabolism of the processes leads to a decreased cohesion capacity of the palatal processes. Later, Jacobs (1967) reported that in mice an access of vitamin A produces cleft palate in a similar fashion as cortisone acetate.

Kochhar and Johnson (1965) reported a considerable increase in the $^{35}$S-sulphate prior to and during the change in the position of the palatal processes from vertical to horizontal in normal embryos. Although, contrary to the results of Larsson (1962a, '62b) and Jacobs (1964d, '67), they found a considerable increase in the $^{35}$S uptake in the mesenchymatous ground substance of the excess vitamin A induced cleft palate.

The findings on the $^{35}$S-sulphate uptake of the present experiment employing normal embryos are slightly different from those of other investigators. Our results did not reveal a remarkable increase in the uptake of $^{35}$S-sulphate in the mesenchymatous ground substance prior to and during the change in direction of the palatal processes. However, an increased uptake of $^{35}$S-label was noticed in the medial part of the palatal processes as compared to the middle and lateral parts of the vertically oriented palatal processes of day 15 and the horizontal positioned ones of day 16. This observation is in agreement
with the findings of our *in vivo* and *in vitro* experiments in which a condensation of the mesenchymal cells was noted prior to and shortly after the change in the position of the processes.

How far the increase in $^{35}$S-uptake plays a primary role in the change in position of the processes is difficult to determine with the means employed in the present study.

Our results relating normal embryos may be discussed in the light of the explanation given by Larsson (1962a). He stated that the larger uptake of $^{35}$S-sulphate corresponds to the increased synthesis of sulpho-mucopolysaccharides in the mesenchymatous ground substance of the palatal processes. This increased synthesis, in his view, causes a change in the plasticity of the ground substance and enlarges its tension, thereby building up a force which makes it possible for the palatal processes to change from a vertical to a horizontal position. He believed, as Walker and Fraser (1956), that during this period the tongue remains stationary and does not play any active role. Particularly on the basis of this last remark regarding the tongue, we are obliged to consider his findings with reservation as we have shown that the tongue descends down with an increase in size and a growth of the nasal septum and a growth of the mandible in a downward and forward direction (Chapter III).

In our opinion it is difficult to determine the factors playing a primary or secondary, direct or indirect role in the change in position of the palatal processes. In the later half of the pregnancy of the rat several dynamic processes are occurring fast and simultaneously. The closure of the palate is associated with a rapid development of the maxilla, mandible, tongue, nasal septum, and other surrounding tissues. The isolation of the cause or causes of the change in position of the palatal processes is therefore difficult to accomplish.

Our findings concerning the manyfold increased uptake of $^{35}$S-sulphate in the vitamin A induced cleft palates are in complete agreement with those of Kochhar and Johnson (1965) and contrary to those reported by Larsson (1962b) and Jacobs (1964b) who administered cortisone acetate to the mice. Jacobs (1967) also worked with excess vitamin A and showed a considerable decrease in the uptake of $^{35}$S in the non-fused palatal processes. In our material the change from a vertical to a horizontal position was observed in all the treated embryos. However, this occurred approximately 24 hours later than normal and was not followed by the fusion of the processes. The contradictory findings reported here lead us to believe that the increase in the synthesis of sulpho-mucopolysaccharides is not the only essential factor in the change in position of the palatal processes.

The effect of excess vitamin A on the sulpho-mucopolysaccharides in the other
body tissues has also been investigated. However, there too, no uniformity in findings and opinions is demonstrated.

Experiments conducted on skin, employing excess vitamin A revealed an increase in the $^{35}$S-uptake (Barker et al., 1964; Pelc and Fell, 1960). On the other hand, it has been shown that excess of vitamin A impairs the ability of chondrocytes in the articular and epiphyseal cartilage and dermal cells to synthesize sulpho-mucopolysaccharides (McElligot, 1962; Chung and Houck, 1964). Frape et al. (1959) had already reported that vitamin A deficiency caused an increased sulphate uptake into the connective tissue mucopolysaccharides, whereas a decrease occurred when excess vitamin A was given. Wolf and Varandani (1960) suggested that vitamin A plays a role in the biosynthesis of polysaccharides. Among other things, they showed that a vitamin A destroying enzyme lipoxidase can lower or abolish the incorporation of $^{35}$S into mucopolysaccharides.

All the above mentioned studies, frequently showing conflicting results, do not offer any explanation regarding the relation of the increase in uptake of $^{35}$S and vitamin A produced cleft palate. Kochhar et al. (1968) offered the explanation that the increased uptake of $^{35}$S may be related to the great demand for the sulphate created by the presence of heterotopic cartilage in vitamin A treated embryos. Their other suggestion was that it may be due to what they called 'a direct access' of vitamin A to the embryo as found by Giroud et al. (1957). The later explanation is unlikely as Cohlan and Stone (1961), in testing this, could not show an appreciable increase of vitamin A concentration in rat fetuses. The first explanation is also not substantiated, as the cause and effect relationship has not been established.

The present findings on embryos from normal and vitamin A treated animals show that the sulpho-mucopolysaccharides do undergo some changes prior to the change in direction of the palatal processes. However, on the basis of the present and other investigations no definite statement can be made on the role of sulpho-mucopolysaccharides in the change in position of the palatal processes. We will limit ourselves to the remark that it is not unlikely that the increase in $^{35}$S-sulphate prior to the fusion may be a part of an overall increase in the synthesis of sulpho-mucopolysaccharides of the mesenchymal tissues of the embryo at that particular time. This may not then be a specific characteristic for the proper timed change in direction of the palatal processes leading to their ultimate fusion.

To the best of our knowledge, the experiments employing $^3$H-thymidine are the first ones conducted on experimental cleft palate with reference to normal
palatal closure. The results clearly showed that vitamin A causes prolongation of the DNA synthesis phase or retardation of the cells to undergo proliferation.

Marin-Padilla (1966) reported early changes in the mesodermal tissues of hamster embryos following hypervitaminosis A which consisted of shrinkage of mesodermal cells and dilatation of the intercellular and vascular spaces. These changes, to some extent, were also found in our material where a less number of mesenchymal cells per area was observed in the palatal processes of the embryos of vitamin A treated rats.

Our results can be compared with those of Langman and Welch (1966, '67). They administered excess vitamin A to rats during different periods of pregnancy and reported that it caused abnormalities of the neuro-epithelial cells of the lateral ventricle of the cerebral cortex. Using tritiated thymidine to study the cell proliferation of these abnormal epithelial cells they concluded that excess vitamin A interferes with mitosis as well as DNA synthesis. They stated that the total generation time of these cells was prolonged by approximately 40%. They suggested that vitamin A interferes with the mitosis and the function of the cell membranes of the neuro-epithelial cells. In the present study, the material that received tritiated thymidine 6 hours prior to sacrifice, no labelled mitosis could be observed in the palatal processes and in the tongue epithelium of the vitamin A group, whereas several labelled mitotic figures were noted in the palatal and tongue epithelium of the normal embryos. Moreover, we also did not observe any unlabelled mitotic figures in the palatal processes of the vitamin A group, whereas in the tongue several such figures were seen. This suggests that the palatal processes were more severely affected than the tongue during the period investigated.

Our results in the experiments using tritiated thymidine are further supported by the in vitro findings reported in Chapter VII where it was noted that an excess of vitamin A in the medium caused a degeneration of the mesenchymal cells and also a peeling off of the epithelial cells of the palatal processes.

Langman and Welch (1967) further reported that the neuro-epithelial cells, despite the damage caused to them by excess vitamin A, survived and proceeded with their proliferation pattern, though at a decreased rate. The difference in period of availability of tritiated thymidine to embryos in our experiment and those of Langman and Welch (1967), eliminates a comparison in this respect. However, our in vitro findings presented in Chapter VI offer a basis for a comment. There, it was shown that the palatal processes of embryos of vitamin A treated mothers, when cultured for longer periods, do show an ability to fuse but one day later than the normal palatal processes. This leads us to the assumption that the detrimental effect of excess vitamin A is most marked during the critical period of closure. This effect may be partly recovered, but
due to the continued lateral growth of the head, palatal processes are unable to meet each other for fusion in the *in vivo* experiments.

Summarizing, it can be stated that the palatal processes do undergo intercellular changes prior to their change from a vertical to a horizontal position. However, the evidence from our findings and those reported in the literature does not allow the conclusion that sulpho-mucopolysaccharides play an essential role in the horizontalization of the palatal processes. On the same basis it could not be ascertained that a change in the metabolism of sulpho-mucopolysaccharides is essential in the production of cleft palate.

The findings of the experiments with tritiated thymidine, allowed more definite and remarkable conclusions:

Comparatively more cellular activity was observed in the medial part of the palatal processes prior to and during the fusion of the palatal processes of normal embryos.

The cells of the palatal processes from the treated group showed dense labelling and a less number of cells per given area.

No labelled mitotic figures were seen in the palatal processes of the vitamin A treated group, as was the case in the normal group.

In the vitamin A treated group the palatal processes appeared to be more affected than the tongue, regarding the cellular proliferation.

The teratogenic effect was most marked on the epithelial cells of the 16 day old and mesenchymal cells of the 17 day old palatal processes.

It has been suggested that vitamin A retards the generation cycle of the cells of the palatal processes. It is also suggested that this retarded cellular proliferation may lead to the aforementioned delay in change in the position of palatal processes from a vertical to a horizontal position.
VIII-1. Normal rat embryo, 15 days. Area from the palatal process. $^{35}\text{SO}_4$ was given to the mother on 13th day of gestation. Focus is on the particles above the section. Toluidine blue. $X$ 490.

VIII-2. Rat embryo from vitamin A treated mother, 15 days. Area from the palatal process. $^{35}\text{SO}_4$ was given to the mother on 13th day of gestation. Note considerably more labelling than in Fig. VIII-1. Toluidine blue. $X$ 490.

VIII-3. Normal rat embryo, 16 days. Area from the palatal process. $^{35}\text{SO}_4$ was given to the mother on 14th day of gestation. Note the more intense labelling than in Fig. VII-1. Toluidine blue. $X$ 490.

VIII-4. Rat embryo from vitamin A treated mother, 16 days. Area from the palatal process. $^{35}\text{SO}_4$ was given to the mother on 14th day of gestation. Note more intense labelling than in Figs VIII-2 and 3. Toluidine blue. $X$ 490.

VIII-5. Normal rat embryo, 17 days. Area from the palatal process. $^{35}\text{SO}_4$ was given to the mother on 15th day of gestation. Note no appreciable increase in labelling compared to Fig. VII-3. Toluidine blue. $X$ 490.

VIII-6. Rat embryo from vitamin A treated mother, 17 days. Area from the palatal process. $^{35}\text{SO}_4$ was given to the mother on 15th day of gestation. Note the considerable more intense labelling compared to Figs VIII-4 and 5. Toluidine blue. $X$ 490.

VIII-7. Normal rat embryo, 16 days. Area from the nasal cartilage. $^{35}\text{SO}_4$ was given to the mother on 14th day of gestation. Note the considerable more intense labelling than in the palatal process of this age shown in Fig. VII-3. Toluidine blue. $X$ 150.

VIII-8. Rat embryo from vitamin A treated mother, 16 days. Area from the nasal cartilage. $^{35}\text{SO}_4$ was given to the mother on 14th day of gestation. Note the more intense labelling compared to Fig. VIII-7. Also compare it with the labelling in the palatal process of the same age in Fig. VII-4. Toluidine blue. $X$ 150.
VIII-9. Normal rat embryo, 16 days. Area of the palatal process. $^3$H-Tdr injected 6 hours before the sacrifice. Note the labelling of the epithelial and mesenchymal cells and the general distribution of the labelling. Haematoxylin-eosin. X 490.

VIII-10. Rat embryo, vitamin A treated mother, 16 days. Area of the palatal process. $^3$H-Tdr injected 6 hours before the sacrifice. Note the smaller number of the labelled cells and the intensity of the labelling. Haematoxylin-eosin. X 490.

VIII-11a. Normal rat embryo, 17 days. Frontal section, anterior region. $^3$H-Tdr injected 6 hours before the sacrifice. The palatal process is in contact with the primary palate. Haematoxylin-eosin. X 49.

VIII-11b. Higher magnification of the outlined area in Fig. VIII-11a. Note the labelling of the cells. X 490.
VIII-12a. Rat embryo from vitamin A treated mother, 17 days. Frontal section, anterior region. $^3$H-Tdr injected 6 hours before the sacrifice. The palatal process is in contact with the primary palate. Haematoxylin-eosin. X 49.

VIII-12b. Higher magnification of the outlined area in Fig. VIII-12a. Note the intensity of the labelling and compare this with Fig. VIII-11b. X 490.

VIII-13a. Normal rat embryo, 17 days. Frontal section, anterior region. $^3$H-Tdr injected 6 hours before the sacrifice. Note the fused palate. Haematoxylin-eosin. X 49.

VIII-13b. Higher magnification of the outlined area of the palatal process in Fig. VIII-13a. Note the distribution of the labelling. X 490.

VIII-14a. Rat embryo from vitamin A treated mother, 17 days. Frontal section, anterior region. $^3$H-Tdr injected 6 hours before the sacrifice. Note unfused palatal processes. Haematoxylin-eosin. X 49.

VIII-14b. Higher magnification of the outlined area of the palatal process in Fig. VIII-14a. Note the intense labelling and compare this with Fig. VIII-13b. X 490.
VIII-15a. Normal rat embryo, 17 days. Frontal section, posterior region. $^3$H-Tdr injected 6 hours before the sacrifice. Note the fused palate. Haematoxylin-eosin. X 49.

VIII-15b. Higher magnification of the outlined area of the palatal process in Fig. VIII-15a. Note the distribution of the labelling. X 490.

VIII-16a. Rat embryo from vitamin A treated mother, 17 days. Frontal section, posterior region. $^3$H-Tdr injected 6 hours before the sacrifice. Note the unfused palatal processes. Haematoxylin-eosin. X 49.

VIII-16b. Higher magnification of the outlined area of the palatal process in Fig. VIII-16a. Note the intensity of the labelling and compare it with Fig. VIII-15b. X 490.
The significance of the findings of the various experiments conducted in the present study has already been discussed in detail in the pertinent chapters. Therefore, the concluding remarks can be limited to the integration of some of the results of the different experiments, and to general aspects related to the problems dealt with in the study.

Several views have been expressed in the past regarding the pathogenesis of experimental cleft palate. It has been suggested that disturbance of the 'internal force' of the palatal processes (Walker and Fraser, 1957) or in the metabolism of sulfo-mucopolysaccharides of the palatal processes (Walker, 1961; Larsson, 1962b; Jacobs, 1964) can lead to the production of cleft palate.

Besides this, much attention has been focused upon mechanical factors assumed to be responsible for the non-closure of the palatal processes. Trasler et al. (1956) stated that an increase in the resistance of the tongue against the palatal processes leads to cleft palate in mouse embryos.

Trasler and Fraser (1963) reported that too narrow processes are unable to meet each other in the midline, thereby causing a direct inhibition of the fusion resulting in cleft palate. Similarly, microstomia, micrognathia, retrognathia, and maxillo-mandibular ankylosis, the malformations mostly found to be associated with cleft palate, have been pointed out as the causative agents (Asling et al., 1960; Deuschle et al., 1959; Schwartz and Chaudhry, 1968). It has also been hypothesized that the head of the embryos may be too wide, thereby not allowing the processes to fuse with each other (Trasler and Fraser, 1963).

Many of the malformations mentioned above showed up in the embryos with cleft palate in the present investigation. However, their mere presence does not indicate that they are primarily responsible for the production of cleft palate. A coordination of the findings of various experiments employing different techniques revealed that the teratogenic agents used in the present investigation cause disturbance in the development of the palatal processes at the cellular level. This, in our view, is one aspect of an overall disturbance of the embryonic development which was due to the administration of the teratogenic drugs.

The morphogenesis of the craniofacial area is of great complexity. Many factors have to interact with each other in a proper balanced way (Van der Linden, 1966). It is possible that a disturbance in one of the constituting components of the embryo may lead to a malformation that shows up also in other
structures. On this basis, it is possible to hypothetize that microstomia, maxillo-mandibular ankylosis, or other associated malformations may interfere with the lowering of the tongue, and, by this, prevent an establishment of contact between the two palatal processes. Even, if in that situation the two palatal processes were cytologically normal, no fusion could be expected.

In our study, it became clear that the teratogenic agents affected the cellular component of the palatal processes directly, as shown by in vitro experiments where the environmental factors, present in vivo, were missing. This was of a temporary nature only in vitamin A treated animals. These processes were capable of fusing with each other after a 24 hour delay. It was explained that this did not occur due to the fact that the continued lateral growth of the head had increased the gap between the two processes.

Further, it may be remarked that the study of the normal secondary palate formation revealed that the elevation of the palatal processes occurs while their differential growth is taking place. A mesenchymal condensation and marked cellular activity was observed with slight bulging of the processes at the inferior half of the vertically directed palatal processes prior to the change in position. With the elevation of the palatal processes, a simultaneous lowering of the tongue, a downward and forward growth of the mandible, and downward growth of the nasal septum took place. It may be that at the same time the palatal processes are changing their direction, the mandible undergoes a growth spurt which displaces the tongue from its position between the palatal processes. This growth spurt has also been proposed by others (Asling et al., 1960; Zeiler et al., 1964).

The present study does by no means answer all the questions regarding the pathogenesis of cleft palate. Various aspects of the normal embryological development of the secondary palate and the pathogenesis of induced cleft palate have been studied by several techniques, and new findings can be presented in both fields. It is suggested here that further investigation may be directed towards the role played by the so called associated malformations in the production of experimental cleft palate.

The prenatal development is well balanced and delicate in nature. Many environmental disturbances can lead to congenital abnormalities. Studies in this field will increase our knowledge of the pathogenesis of congenital malformations and of the mode of action of teratogenic agents. It may be expected that these studies will contribute to the prevention of the occurrence of such malformations in mankind.
SUMMARY

The purpose of the present study was to investigate the normal embryological development of the secondary palate, and to study the pathogenesis of the experimentally induced cleft palate. The Wistar albino rat was selected as the experimental animal and a total of 1534 embryos were studied with different methods.

The first chapter mainly deals with the general aspects and theories regarding the normal palatal closure and cleft palate production. The material and methods employed in this work are presented in the second chapter.

The normal embryological development of the secondary palate has been described in Chapter III. A total of 185 embryos were studied for that purpose. The findings revealed that the palatal processes undergo slight bulging before their change from a vertical to a horizontal position. The change in position starts in the anterior region and progresses posteriorly. The fusion takes place between 16.16 and 17.9 days of gestation and commences in the middle third of the anterior half of the palate and progresses anteriorly and posteriorly. The palatal processes change their position from vertical to horizontal simultaneously with the lowering of the tongue, the downward and forward development of the mandible, and the growth of the nasal septum in downward direction.

The development of cleft palate was studied after administering Dexamethasone, vitamin A, and vitamin A in combination with cortisone acetate. (Chapter IV). Cortisone acetate was also used separately but it did not induce cleft palate. The drugs were administered in varying doses, on different days, and during different ages of gestation. The amounts used, and the obtained results are shown in Tables IV-1, 2, 3, 4. The cleft palates produced by different teratogenic agents were morphologically quite similar and were usually associated with microstomia, micrognathia, retrognathia, syndactyly, open eyes, and exencephaly. The combination of vitamin A and cortisone acetate produced the most severe anomalies and led to many resorbed sites.

An almost 24 hour delay in the change in position of the palatal processes from vertical to horizontal was noted in the drug treated embryos. The tongue
stayed between the processes during this delay. Nearly all the embryos with cleft palate exhibited a heterotopic cartilage extending from the maxillary region to the mandibular area. This cartilage was found to be instrumental in the production of maxillo-mandibular ankylosis. It was concluded that a disturbance in the palatal processes at the cellular level or intercellular level and several other factors namely: microstomia, maxillo-mandibular ankylosis, retarded downward growth of the nasal septum, and retarded downward and forward growth of the mandible may lead to cleft palate, either acting alone or more probably in combination with each other.

To gain more information on the normal development and the pathogenesis of cleft palate, 92 explants of the area of interest were cultured in an artificial environment for varying periods of time. Besides normal material, explants derived from embryos of vitamin A and Dexamethasone treated mothers were studied (Chapter V). The results were recorded in three categories: explants showing no palatal fusion, epithelial fusion, and mesenchymal fusion (Table V-1). The experiment demonstrated that the palatal processes have the ability to fuse in vitro. Relatively more fusions were obtained from explants cultured on or after day 15 of gestation. The palatal processes first approximated in the posterior region of the palate. This was explained by the absence of the tongue in the in vitro experiments. The cellular fusion, however, started in the anterior region, as was the case in the in vivo study.

The fused palatal processes were found to be slender and different in shape from their normal in vivo counterparts. It was suggested that the latter aspect may be due to the absence of the tongue. No essential differences were recorded between the pattern of cellular fusion of the palatal processes in the in vitro and in vivo material.

In a comparable way as indicated above, 51 explants from embryos of vitamin A or Dexamethasone treated mothers were cultured (Chapter VI, Table VI-1 and 2).

The palatal processes from embryos of vitamin A treated rats showed a delayed fusion in vitro. The explants from the Dexamethasone group did not exhibit fusion. It was concluded that the palatal processes of embryos of vitamin A treated mothers are capable of fusing with each other in vivo, even after a 24 hour delay in the horizontalization of the processes, but due to the continued lateral growth of the head and the retarded development of the processes, this could not be accomplished. Furthermore, it was noted that although vitamin A and Dexamethasone produce cleft palate with quite similar morphological characteristics, the mode of cleft palate production of the two agents was
Dexamethasone affected the palatal processes permanently, while vitamin A did so only temporarily.

To examine the local action of vitamin A and Dexamethasone on the palatal closure in vitro, 86 explants of normal embryos were cultured in media to which one of these drugs, or a control substance, was added in different concentrations (Chapter VII, Tables VIII-1, 2 and 3). The results showed that vitamin A when added directly to the culture medium causes peeling off and oedema of the epithelium and necrosis of both the mesenchyme and epithelium. Similarly, Dexamethasone also led to the necrosis of the mesenchymal and epithelial cells. Nearly all the cultured processes in the drug added media were small and stunted. The majority of the processes did not fuse or show any appreciable growth towards each other. Most of the processes of the control group did fuse, either epithelially, or mesenchymally.

To elaborate the findings of the previous chapters in vivo, autoradiographic experiments were conducted, employing $^{35}$S-labelled sodium sulphate and $^3$H-thymidine, on 78 embryos of different ages from normal and vitamin A treated mothers and with varying survival times (Chapter VIII).

The results of the normal embryos showed a higher incorporation of $^{35}$S-sulphate in the palatal processes at 16 and 17 days, than at 15 days. In the younger animals comparatively more labelling was observed in the medial part of the palatal processes which was not found in the 17 day old embryos. The palatal processes of vitamin A treated embryos showed considerable more uptake of $^{35}$S-sulphate than the normal ones, and the above mentioned localization effect was recorded here in 16 and 17 day old embryos, instead of the 15 and 16 day old ones. The present findings were discussed in the light of the existing literature on the subject and it was concluded that the role played by the metabolism of sulpho-mucopolysaccharides in the change in position of palatal processes and in the production of cleft palate is still open to question. No decisive remarks could be made regarding this aspect.

The findings of the experiment employing $^3$H-thymidine showed some remarkable features. It was noted that prior to the fusion more cellular proliferation takes place in the medial part, than in the lateral parts of the palatal processes. The labelling of the cells of the palatal processes from the vitamin A treated animals was more intense, and less labelled cells per area were observed. On basis of the present findings and those of others, it was concluded that vitamin A retards the generation cycle of the cells. This disturbance at the cellular level is, in our view, a main factor in the retarded development of the palatal processes of the vitamin A treated animals.
ABRAMOVICH, A and DFVOTO, F C H  
Ectopic maxillo-facial chondrogenesis in fetuses of rats treated with high doses of vitamin A  

ANDERSEN, H and MATTHIESSEN, M  
Histochemistry of the early development of the human central face and nasal cavity with special reference to the movements and fusion of the palatine processes  

ASLING, C W, NSFSON, M M, DOUGHERTY, H L, WRIGHT, H W and EVANS, H M  
The development of cleft palate resulting from maternal pteroylglutamic (Folic) acid deficiency during the latter half of gestation in rats  

BARKER, S A, CRUICKSHANK, C N D and WEBB, T  
The effect of vitamin A, hydrocortisone and estral upon sulphate metabolism in skin  
Exp Cell Res 35: 255-261 (1964)

BARRY, A  
Development of the branchial region of human embryos with special references to the fate of epithelia, in 'Congenital anomalies of the face and associated structures', pp 46-62, ed S Pruzansky (Charles C Thomas, Springfield, Ill 1961)

BAXTER, H and FRASER, F C  
Production of congenital defects in offspring of female mice treated with cortisone  

BENNET, D, BADENHAUSEN, S and DUNN, L C  
The embryological effects of 4 late t-alleles in the mouse, which affect the neural tube and skeleton  
J Morph 105: 105-144 (1959)

BERGERON, J A  
Control staining of autoradiographs  
Stain Technol 33: 221-223 (1958)

BOSTROM, H  
On the sulphate exchange of sulpho-mucopolysaccharides An enzymatic reaction in mesenchymal tissues  

BUNO, W and GOYENA, H  
Effect of cortisone upon growth in vitro of femur of the chick embryo  

BURDI, A R and FAIST, K  
Morphogenesis of the palate in normal human embryos with special emphasis on the mechanisms involved  
Amer J Anat 120: 149-160 (1967)

CANNON, B, FISHER, D and BRITTON, H C  
Medical Progress Plastic surgery, harelip and cleft palate with section on speech therapy  

CHAUDHRY, A P and SIAR, S  
In vitro study of fusion of palatal shelves in AlJax mouse embryos  

CHENG, D W and THOMAS, B H  
Relationship of time therapy to teratogenic in maternal avitaminosis E  
Proc Iowa Acad Sc 60: 290-299 (1953)

CHUNG, A C and HOUCK, J C  
Connective tissue IX Effects of hypervitaminosis A upon connective tissue chemistry  

COHLAN, S Q  
Excessive intake of vitamin A as a cause of congenital anomalies in the rat  
Science 117: 935-936 (1953)

COHLAN, S Q  
Congenital anomalies in the rat produced by excessive intake of vitamin A during pregnancy  

COHLAN, S Q and STONE, S M  
Observations on the effect of experimental endocrine procedures on the teratogenic action of hypervitaminosis A in the rat  

COLEMAN, R D  
Development of the rat palate  
Anat Rec 151: 107-118 (1965)

DAGG, C P  
Sensitive stages for the production of developmental anomalies in mice with 5-Fluorouracil  
Amer J Anat 106: 89-96 (1960)

DEUSCHLE, F M, GEIGER, J F and WARKANY, J  
Analysis of an anomalous oculodentofacial pattern in newborn rats produced by maternal hypervitaminosis A  

DZIEWIATKOWSKI, D D  *Rate of excretion of radioactive sulphur and its concentration in some tissues of rat after intraperitoneal administration of labeled sodium sulphate*  J biol Chem 178: 197–202 (1949)

DZIEWIATKOWSKI, D D  *Effect of thyroxine and thiouracil on $^{35}$S deposition in articular cartilage*  J biol Chem 189: 717–727 (1951)


FELL, H B  *The application of organ culture to medical and biological research*  in 'Tissue Culture', pp 17–26, ed C V Ramakrishna (Dr W Junk Publishers, the Hague 1965)


FITCH, N  *A mutation in mice producing dwarfism, brachycephaly, cleft palate and micromelia*  J Morph 109: 141–150 (1961a)


FOGH-ANDERSEN, P  *The inheritance of harelip and cleft palate*, pp 1–266 (Nyt Nordisk Forlag-Arnold Busck, Copenhagen 1942)

FOGH-ANDERSEN, P  *Harelip and cleft palate 1000 patients submitted to operation*  Acta chir scand 94: 213–242 (1946)


FRASER, F C  *Some experimental and clinical studies on the causes of congenital clefts of the palate and of the lip*  Acta paediat 77: 151–156 (1960)


FRASER, F C and FAINSTAT, T D  *Causes of congenital defects*  review Amer J Dis Child 82: 593–603 (1951)


FROMMER, J  *The lack of evidence for elastic fiber involvement in the closure of the palatal shelves*  Anat Rec 160: 471 (1968)

GAIIARD, P J  *De invloed van vitamine-D op de beenvorming in vitro*  Ned T Geneeskr 76: 4175–4176 (1932)


GIROUD, A and MARTINET, M  Hypervitaminosis 'A' et anomalies chez le foetus de rat Int Z Vitaminforsch 26: 10-18 (1955)

GIROUD, A and MARTINET, M  Teratogenese par hautes dose de vitamine A en fonction des stades de developpement Arch Anat micr Morph exp 45: 77-98 (1956)


GREENSPAN, F S, HOUGHTON, G and DEMING, Q B  A comparison of the effects of cortisone administered intraperitoneally and subcutaneously in the rat Endocrinology 52: 638-645 (1953)

GUNBERG, D L  Some effects of exogenous hydrocortisone on pregnancy in the rat Anat Rec 129: 133-153 (1957)

HALE, F  Relation of vitamin A to anophthalmos in pigs Amer J Ophthal 18: 1087-1093 (1935)

HARDY, M H  Response in embryonic mouse skin to excess vitamin A in organotypic cultures from the trunk, upper lip and lower jaw Exp Cell Res 46: 367-384 (1967)


HASPIN, D  Some effects of nitrogen mustard on development of external body form in foetal rat Anat Rec 102: 493-511 (1948)

HEIBERG, E, KALTER, H AND FRASER, F C  Production of cleft palate in offspring of mice treated with ACTH during pregnancy Biol Neonat 1: 33-37 (1959)

HUMPHREY, T  The dynamic mechanism of palatal shelf elevation in human fetuses Anat Rec 16: 369 (1968)


JACOBS, R M  Histochemical study of morphogenesis and teratogenesis of the palate in mouse embryo Anat Rec 149: 691-697 (1964a)

JACOBS, R M  Effects of cortisone on water binding capacity of embryonic palate in mice J oral Ther Pharm 1: 161-164 (1964b)

JACOBS, R M  Preliminary survey of the effects of cortisone upon palate formation, litter size and fetal weight in CD-1 strain of mice J Dent Res 43: 715 (1964c)


KALTER, H  Inheritance of susceptibility to teratogenic action of cortisone in mice Genetics 39: 185-196 (1954)

KALFR, H  Factors influencing the frequency of cortisone induced cleft palate in mice J exp. Zool 134: 449-468 (1957)
KALTER, H The teratogenic effects of hypervitaminosis A upon the face and mouth of inbred mice Ann N Y Acad Sci 85: 42-55 (1960)
KAMEI, T Embryological and histochemical studies on artificially induced cleft palate in mice Acta anat Nippon 37: 140-158 (1962)
KOCHHAR, D M and JOHNSON, E M Morphologic and autoradiographic studies of cleft palate induced in rat embryos by maternal hyper vitamminosis A J Embryol exp Morph 14: 223-238 (1965)
LANGMAN, J and WELCH, G W Excess vitamin A and development of the cerebral cortex J comp Neurol 131: 15-26 (1967)
LARSSON, K S Studies on the closure of the secondary palate III Autoradiographic and histochemical studies in the normal mouse embryo Acta morph neerl scand 4: 349 367 (1962a)
LARSSON, K S 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 (1962b)
LASNITZKI, I Influence of hypervitaminosis A on effect of 20-methylcholanthrene on mouse prostrate glands grown in vitro Brit J Cancer 9: 434-441 (1955a)
LATOSH, E A Development of palate in normal murine embryo and influence of various agents on the conditions of palatine plates at different stages of embryonic development Acta chir Scand 10: 1-6 (1968)
LAYTON, L L Effect of cortisone upon chondroitin sulphate synthesis by animal tissues Proc Soc exp Biol med 76: 596-598 (1951)
LAZZARO, C Sul meccanismo di chiusura de palato secondario Monit Zool Ital 51: 249-273 (1940)
LOEY, H Developmental changes in the palate of normal and cortisone treated strong A mice Anat Rec 142: 375-390 (1962)
MAC MOHAN, B and MCKEOWN, T Incidence of harelip and cleft palate related to birth rank and maternal age Amer J hum Genet 5: 176 183 (1953)
MARÍN-PADILLA, M Mesodermal alterations induced by hypervitaminosis A J Embryol exp Morph 15: 261-269 (1966)
MILEN, J W and WOOLLAM, D H M Influence of cortisone on teratogenic effects of hypervitaminosis A Brit med J 2: 196-197 (1957)
MILEN, J W and WOOLLAM, D H M Effect of vitamin B complex on the teratogenic activity of hypervitaminosis A Nature 182: 940 (1958)
MURPHY, M L and KARNOFSKY, D A Effect of azaserine and other growth-inhibiting agents on fetal development of the rat Cancer 9: 955-962 (1956)
MYERS, G S and PETRAKIS, N L and LEE, M Cultivation of embryonic rat palate in defined and semi-defined media Arch Oral Biol 12: 565-567 (1967a)
MYERS, G S and PETRAKIS, N L and LEE, M Effect of 6-aminonicotinamide and of added vitamin A on fusion of embryonic rat palates in vitro J Nutr 93: 252-262 (1967b)

NISHIMURA, H and KUGINUKI, N Congenital malformations induced by ethylurethan in mouse embryos Okajimas fol ant 31: 1-10 (1958)
NISHIMURA, H and SHIKATA, A The maldevelopment of the foetuses of mice treated with gonadotropic hormone before the conception Okajimas fol med 31: 195-202 (1958)
PATTEN, B M The normal development of the facial region in 'Congenital anomalies of the face and associated structures' pp 11-45, ed S Pruzansky (Charles C Thomas, Springfield, Ill 1961)
PELC, S R and FELL, H B The effect of excess vitamin A on the uptake of labelled compounds of embryonic skin in organ culture Exp Cell Res 19 99 113 (1960)
PETER, K Die Entwicklung des Saugetier Gaumens Ergeb Anat Entw Gesch 25: 448-454 (1924)
PINSKY, L and DIGEORGE, A M Cleft palate in the mouse A teratogenic index of glucocorticoid potency Science 147: 402-403 (1965)
PINSKY, L and FRASER, F C Production of skeletal malformations in the offspring of pregnant mice treated with 6-aminonicotinamide Biol Neonat 1: 106-112 (1959)
PONS-TORTELLA, Von E Über die Bildungsweise des sekundaren Gaumens Anat Anz 84: 13-17 (1937)
POURTOIS, M Onset of the acquired potentiality for fusion in the palatal shelves of rats J Embryol exp Morph 16: 171-182 (1966)
POURTOIS, M La fusion des cretes palatines et son alteration par quelques agents teratogenes in vitro Arch Biol (Liege) 79: 1-75 (1968)
REED, S C and SNELL, G D Harelip, a new mutation in the house mouse Anat Rec 51: 43-50 (1931)

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THESSES

I

In Vitro experiments have opened new horizons in teratological studies.

II

Experimental teratology is a valuable means of learning more about the origin of malformations and the mechanism of drug teratogenicity. Extrapolation from experimental teratology to man is unwarranted unless supported by evidence in man.

Fraser, F. C., in "Congenital malformations", 277-287, 1964)

III

The teratogenicity of a drug depends on the susceptibility of the species, the dose, the mode of administration and the stage of embryonic development.

IV

Malformations found with experimental cleft palate in laboratory animals require more attention regarding their influence in the etiology of cleft palate.

V

Transseptal fibers frequently play a role in the relapse of treated orthodontic cases.
VI

Most orthodontic cephalometric analyses are restricted to skeletal measurements; a combination with a soft tissue analysis gives better possibilities in orthodontic diagnosis and treatment planning.

VII

By using a Kloehn headgear as much attention should be paid to transversal, sagittal and vertical developmental changes, as to the inter-relationship of the upper and lower jaws.

VIII

Fluoridation of drinking water is now the only feasible answer to most of the dental problems.

IX

Research is an indispensable tool in graduate education.

X

Help to developing countries is mostly ill planned and badly constituted.

RAVINDRA NANDA

Nijmegen, 20 June 1969