Study of the development of the pineal gland of the chick.

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A HISTOLOGICAL AND HISTOCHEMICAL STUDY OF THE DEVELOPMENT OF THE PINEAL GLAND OF THE CHICK (*Gallus domesticus*)

by

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ABSTRACT

The pineal gland of the chick is composed of vesicles during much of the incubation period. During incubation, these vesicles increase in volume and number and reach maximum development at the 17-day incubation stage. Following day 17, the vesicular tissue decreases and, by one day posthatching, only a few vesicles remain. All vesicles have disappeared by one month posthatching and the gland has acquired a compact, lobed character. The vesicular cells are secretory in nature and react positively with tests for glycogen, glycoproteins, ribonucleic acid, acid mucopolysaccharides, and neutral lipids. In addition, the specialized apical border of these cells stains intensely for acid lipids. The vesicles contain a secretory product which includes a sulfated mucopolysaccharide component. Based on the intensities of the staining reactions, this secretion is present in greatest amounts during the 15 to 17-day prehatching period. No secretory product could be demonstrated following one day posthatching. It appears, therefore, that the development of the vesicular tissue is related to some secretory role and that this role is culminated during the 15 to 17-day prehatching period. Following day 17, the decrease and eventual disappearance of the vesicles apparently marks the conclusion of this secretory phase for the gland.

INTRODUCTION & LITERATURE

The epiphysis cerebri, or pineal gland, is a small, club-shaped organ on the dorsal surface of the cerebral aqueduct, and was first described by Galen about 2000 years ago. It has since been the subject of innumerable experiments and speculations. However, 300 years ago, the level of advancement was still such that Rene Descartes (in Wurtman and Axelrod, 1965) suggested that this unique, unpaired body, perched in so noble a seat, must be the center of the rational soul. At the end of the 19th century, a German physician, Otto Heubner (in Wurtman and Axelrod, 1965) published the case history of a young boy displaying precocious puberty as the result of a pineal tumor. This gave science its first glimpse at a possible function for the pineal gland, and initiated a large volume of research.

Before 1900, there were several papers on the pineal gland, but they dealt for the most part with the reptilian so-called "third eye", located on the dorsal skull roof, (Klinckowstrom, 1893). From 1900 onwards, when an evolutionary connection was established between the pineal gland of mammals and the third eye of reptiles, researchers began to experiment on fish (Johnston, 1901), amphibia (Holmgren, 1918), and mammals (Izawa, 1925 and Jordan, 1911). The avian pineal gland was not extensively studied in the early literature. Hill (1900) and Cameron (1903) published brief studies of its description. However, these reports were more concerned with its basic anatomical relation-

ships than with its histology and histochemistry. There has, in fact, been so little research in this field that, in the 1965 symposium on the epiphysis cerebri, Dr. W.B. Quay (1965) began a paper on the avian pineal with the words: "In the last twenty years there has been only five articles on the histology and cytology of the avian pineal, and even in most of these, the emphasis is on developmental morphology."

The classical reference to the avian pineal gland is Studnicka's 1905 paper which includes a brief but useful account of its morphology. More recently, there have been several brief papers published. Romieu and Jullien (1942) have reported on its posthatching pattern of development in the chick. Krabbe (1956) has described its development in several species of birds other than the chick, and Spiroff (1958) has published a detailed report of its development in the chick. Again, however, these papers were primarily concerned with a description of the general morphology of the developing gland, and not with the staining affinities of the cells at the different stages of its development.

In the domestic fowl, the pineal gland begins its development at about 48 hours of incubation, first appearing as a small evagination from the dorsal roof of the diencephalon. The early belief was that the epiphysis had a bilateral origin (Hill, 1900; Cameron, 1903) but this has since been proven to be incorrect. The pineal recess is a

single evagination of the third ventricle and the two are connected in the early embryo by the pineal stalk.

This connection to the third ventricle may become much constricted in some species of birds, or it may become severed completely as in the hummingbird (Krabbe, 1955). In the chick, Ward (1946) states that there is a severance of continuity of these two cavities in the eight-day old embryo, while Romieu and Jullien (1942) contend that continuity persists as late as the 2-3 month posthatching stage. This latter observation is supported by Spiroff (1958).

The wall of the pineal gland is initially part of the diencephalon and is histologically similar to it during the early stages of development. Pseudostratified columnar epithelium forms the lining of both organs. However, the lining of the pineal recess differs in two respects from that of the diencephalon. Firstly, the nuclei of the tissue lining the pineal are located in a basal position whereas those of the diencephalon are placed in the apical portion of the cell. Secondly, the ependymal cells of the pineal recess are not ciliated, whereas those lining the diencephalon are (Spiroff, 1958). It is, therefore, possible to distinguish between the two tissues during the early stages of development. According to Spiroff, there is little change histologically in the gland during the first 72 hours of incubation. However, morphologically, it lengthens to twice its original length although there is little increase

in width. Patten's (1945) classical reference on the development of the chick mentions the epiphysis briefly, and states that there is little change in the gland, aside from elongation, until as late as four days or ninety-six hours of incubation.

The mature pineal gland resembles an exocrine gland, having columnar cells arranged in acinar-like "cors secretoria". The origin of these cors secretoria or vesicles of the pineal gland has been the subject of considerable debate. In reptiles, such as the turtle, it has been established that the cavities of these vesicles interconnect and open into the third ventricle (Grignon and Grignon, 1963; and Combescot and Demaret, 1963). However, in birds, there are several different descriptions of the inter-relationships of these vesicles. Romieu and Jullien (1942) described the formation of these vesicles in Gallus as numerous outfoldings of the pineal recess, each with thick walls composed of ependymocytes. Thus, each vesicle opens directly into the pineal recess. Krabbe (1955) studying several species of birds, but not Gallus, and Ward (1946) examining Gallus, claim that the wall of the pineal recess blebs out "solid buttons of cells" which subsequently reorganize to form a cavity which is not connected to the pineal recess. Spiroff (1958) confirms this latter observation of an independent origin of the vesicular cavities and goes a step further to classify them. Those vesicles arising from the

ependymal wall of the pineal recess are referred to as the primary vesicles. Those vesicles developing from the walls of the primary vesicles are secondary vesicles, and those forming along the periphery of the secondary vesicles are called tertiary vesicles.

Initially, large areas of connective tissue are seen in the gland, separating the vesicles. However, by day sixteen of incubation (Spiroff, 1958), the vesicles have begun to increase in size and number and the area of connective tissue is proportionately decreased in amount. The nerve and vascular supply is distributed throughout the gland within these connective tissue septa.

At 96 hours of incubation, there are a number of small capillaries throughout the connective tissue and, at five days, large blood vessels are located within the capsule surrounding the epiphysis. Capillaries are distributed in association with the primary vesicles at six days of incubation, and the primary and secondary vesicles are completely surrounded by a capillary network by the twelfth day of incubation (Spiroff, 1958).

Fine nerve fibres and terminals are also in evidence in the connective tissue surrounding the vesicles. No nerve cell bodies have been described in the body of the gland (Stammer, 1963; Quay, 1965) and it is generally accepted that there are none. However, Quay (1965) does mention cells, in the western sandpiper, which have processes that stain with the Protargol S method, suggesting that

they may be neurons. Also, in the duck, a specialized chemoreceptor-like nerve ending has been described by Stammer (1961) in the stalk region of the pineal gland. This has not been described in the domestic fowl or in any other species and most authors believe that there are no sensory cells in the body of the avian pineal gland (Kappers, 1963). Ependymal cells are present in the pineal recess at the time of its formation, and remain scattered among the cells lining the vesicles. Other neuroglial elements exist, and Quay (1965) has described the presence of astrocytes in the avian pineal gland. The innervation of the avian epiphysis has been described by several authors (Kappers, 1965; Quay, 1965; and Stammer, 1963) and there is little that can be done to contribute further to this field without confining a research project to it exclusively.

Part of the posthatching growth of the gland might be attributed to the invasion of lymphatic tissue, first described by Romieu and Jullien (1942). They observed patches of lymphocytes at the posterior of the gland immediately subjacent to the meninges. A single nodule is first present at the 1-2 month posthatching stage. During the subsequent stages, diffuse lymphatic tissue spreads from this nodule into the glandular parenchyma and eventually, at the four month stage, reaches the centre of the organ. The distribution of vessels throughout the lymphatic tissue and the abundance of mitotic figures within

the lymphocytes suggested to the authors that the lymphocytes differentiated "in situ" and provide a source of lymph cells for the cerebrospinal fluid.

Spiroff (1958) agrees with the sequence of events described above, but first observed lymphocytes distributed along the surface of the pineal body at the 4-12 day posthatching stage. They appeared within the gland at 18 days posthatching being located within the walls of blood vessels. This lymphocyte region is continuous with those at the surface, and Spiroff suggests that the lymphocytes invaded from the surface and did not differentiate "in situ". The lymph nodules in Spiroff's specimens reached their maximum size at three months posthatching and disappeared by 6 months. Quay (1965) has, however, published photomicrographs of one year old roosters with complete nodules present along the posterior border of the gland.

Initially, pineal parenchymal cells are similar to the ependymal cells in structure and arrangement. Other cell types, the hypendymocyte and the pineocyte have been described by Romieu and Jullien (1942). However, in addition to the ependymocyte, Spiroff (1958) has found evidence for the hypendymocyte only. According to Spiroff the ependymocytes are the cells which are "arranged radially about the lumen" and the hypendymocytes are arranged "peripheral to these". According to Spiroff one type of cell is visible in the walls of the follicles between the forty-eight hour and the ten day incubation stages.

Between the tenth and sixteenth days, the two distinct types are present.

Quay (1965) categorizes the cells into three types: 1) those which stain acidophilically and possess small, round nuclei; 2) those with elongate nuclei possessing chromatin granules and several nucleoli and which are radially disposed with reference to the lumen; and 3) those with large, irregularly-shaped nuclei possessing a large single nucleolus. Romieu and Jullien (1942), and Quay (1965) agree that all cell types differentiate from the ependymocytes.

There has been little research on the histochemistry of these cells in the pineal gland of the chick, and the only major work on this subject is the study by Beattie and Glenny (1966). However, these authors confined their study to the one-year old fowl and did not include an investigation of the developmental stages. Quay (1965), in a symposium on the epiphysis, briefly reviews the results of a few cytochemical methods on the pineal gland in several other species of birds. However, in each case, studies of the developing gland were omitted.

Some common histochemical properties have been described for the adult pineal gland in several avians and other vertebrates. Renzoni and Quay (1963) did cytochemical studies on several species of birds and found parenchymal cells possessing cytoplasmic granules which

reacted positively with the chrome alum-hematoxylin method. This was again confirmed by Quay (1965) who described these granules to be present in the basal cytoplasm of the cells lining the vesicles in the cormorant. This staining reaction has also been noted in the salmon (Hafeez and Ford, 1967) and in rats (Quay, 1956).

Beattie and Glenny (1966) studied acid mucopolysaccharides with the metachromatic Toluidene blue and the alcian blue techniques. They were unable to demonstrate metachromasia in the one-year old fowl, although such a reaction has been demonstrated in adult mammals (Mikami, 1951). Also, they found that the vesicles gave a negative reaction with the alcian blue technique although the surrounding connective tissue sheath stained intensely. Similarly, in salmon (Hafeez and Ford, 1967) only the connective tissue sheath stains positively with alcian blue. However, Grignon and Grignon (1963), in the turtle, found inclusions in the apical portion of the cells lining the vesicles which stained positively for acid mucopolysaccharides and phospholipids.

Combescot and Demaret (1955), studying the water turtle, have shown that the secretory product within the vesicular lumina, but not the lining cells, stains positively with the aldehyde fuchsin method for sulphated mucopolysaccharides. Owman (1955) found the cells lining the vesicles of the rat pineal gland to be positive with this technique.

Finally, in the salmon (Hafeez and Ford, 1967), the cells of the pineal gland are aldehyde fuchsin positive.

Beattie and Glenny (1966) stained pineals of the one-year old fowl for glycogen with the alcian blue-periodic acid-Schiff method and found that both the secretory and connective tissue cells revealed a postive reaction. Similarly, Quay (1965), in cormorant pineals, found clusters of periodic acid-Schiff-positive cells. In non-avian species, Hafeez and Ford (1967) found the pineal body of the salmon to be periodic acid-Schiff-positive along the boundaries of the lumina. Mikami (1951), in several domestic mammals, demonstrated glycogen in the parenchymal cells.

Ribonucleic acid (RNA) has been studied in two investigations only and the results differ. Heiniger (1965), using the methyl green-pyronin Y method on pig and human pineal glands, found a low RNA content. Mikami (1951), using the same method, found that pig and goat pineals showed the presence of RNA in large amounts in both the cytoplasm and nucleoli of parenchymal cells.

Alkaline phosphatase has been studied in the pineal glands of several mammals but not in birds. Mikami (1951) has shown that parenchymal cells of the pineal gland in goats and pigs are positive for alkaline phosphatase. However, Bostelmann and Bienengraber (1963) working with one-year old rabbits and Bayerova and Bayer (1967) studying human pineals, were able to demonstrate the presence of

alkaline phosphatase activity in the walls of blood vessels only and not within the pineocytes.

Few studies have been made on the histochemistry of fat in the pineal gland. Prop (1964) found fat droplets consisting of phospholipids and triglycerides in the pineocytes of rats. Grignon and Grignon (1963) found phospholipids in the cells lining the vesicles of the pineal gland of land turtles. No reports are available on the histochemistry of fat in the pineal gland of birds.

The above review indicates that the available descriptions regarding the histology and histochemistry of the avian pineal glands are incomplete, and that such studies of its development have essentially been neglected. The purpose of the present investigation is, therefore, to study the development of the pineal gland in the chick using a histological approach and to supplement this study with a number of histochemical methods. It is believed that such a study, taken in conjunction with those mentioned above, will provide a better understanding of the development and histophysiology of this gland in birds.

MATERIALS AND METHODS

General:

The chicks used in this study were of the White Leghorn genotype. The eggs were obtained from a commercial hatchery and were incubated at 102-104°F. For each fixation technique, three embryos were sacrificed on each day of incubation from the third day to the twentieth, the day of hatching. In addition, pineal glands from birds of several posthatching stages were studied. These stages included birds of the following ages: one day, one week, one month, four months, and one year. These specimens were also obtained from a commercial hatchery.

The pineal gland was first located at day three of incubation. During the early stages, until day 10, it was found to be most efficient to embed and serially section the entire head region of the embryo. To allow the fixing fluids to penetrate adequately, two slits were made in the skull immediately above and behind each eye. From day 11 to day 16 of incubation, the brain only was excised and fixed intact. At day 17, and all subsequent stages, the pineal gland was sufficiently large to be removed with a piece of the skull roof, to which it is attached by a thin strip of connective tissue. That is, the entire upper portion of the cranial roof, with the pineal gland affixed, was excised and prepared for study. This method had the added advantage of providing a larger block of tissue,

facilitating its manipulation in subsequent steps of the techniques.

Histological Techniques

For the study of the developmental histology of the pineal gland, the tissues were fixed either in Bouin's fluid for 24 hours, Carnoy's fixative for 4 hours, or in 10% neutral formalin for 24 hours. Following the appropriate post-fix wash, all tissues were dehydrated through a graded series of alcohols, cleared in toluene, embedded in 56°-58°C paraffin, and sectioned serially at 7 microns.

Several standard staining techniques were applied to examine the basic histology of the developing pineal gland. The hematoxylin and eosin method was used following the description of Lillie (1965). In the application of this technique, the tissues were stained in Erlich's hematoxylin for 2 minutes and subsequently treated with alcoholic eosin Y for 30 seconds.

The alcoholic Toluidine blue O method of Kramer and Windrum, outlined in Pearse (1961), was employed for the study of metachromasia. For this technique, the sections were immersed in a 0.2% Toluidine blue O in 30% ethanol solution for 4 minutes. Following the stain, the sections were rapidly dehydrated through graded alcohols, cleared in xylol, and mounted in permount. It was found that this method gave good staining results, that the metachromatic

reaction persisted after dehydration and clearing, and that the metachromasia was reproducible.

The aldehyde fuchsin technique was used only on tissues fixed in either 10% neutral formalin or Bouin's fixative. This technique was applied following the description of Scott as modified by Gibson (1966). Deparaffinized and hydrated sections were oxidized in a solution of 0.5% potassium permanganate and 0.5% sulphuric acid (equal parts) for two minutes, decolorized in 2% oxalic acid, and stained for one minute in the aldehyde fuchsin stain (prepared according to Halmi, 1952). Subsequent to the aldehyde fuchsin staining, the sections were counterstained for 1.5 minutes in 0.5% phloxine B, rinsed in distilled water, treated with 5% phosphotungstic acid for one minute, washed in tap water, and counterstained in 0.2% fast green FCF for two minutes. All solutions used were aqueous preparations.

The chrome-alum hematoxylin method of Bargmann, outlined in Pearse (1961), was used to stain for neurosecretory substances in the gland. For this technique, the sections, after fixation in Bouin's fluid, were placed in the mordant (3.5% chrome alum in Bouin's fluid) for 24 hours at 37°C, and were subsequently stained for 10 minutes in the hematoxylin solution. The sections were then counterstained in 0.5% aqueous phloxine B for 1.5 minutes.

Histochemical Techniques:

The periodic acid-Schiff reagent technique (PAS) of McManus, as outlined by Pearse (1961), was used for the demonstration of neutral mucopolysaccharides, glycoproteins, and mucoproteins. The tissues were fixed in Baker's formalin and embedded either in paraffin and sectioned at 7 microns, or in gelatin and sectioned at 10 microns with the freezing microtome. In both cases the sections were oxidized in 5% aqueous periodic acid and stained with the Schiff reagent. It was found that ten minutes in the staining solution was adequate. No counterstain was used. As a control, alternate sections were treated with 0.1% diastase in 0.02 M phosphate buffer, then stained as above.

The alcian blue method of Steedman, as outlined in Pearse (1961), was used for the demonstration of acid mucopolysaccharides in addition to the aldehyde fuchsin and metachromatic methods outlined above. These tissues were fixed in 10% neutral formalin, Carnoy's fluid, or Bouin's fluid. The sections were stained in the alcian blue solution for two hours, rather than the prescribed 30 minutes, and counterstained in 1% aqueous neutral red for 5 minutes rather than the suggested 30 seconds.

The carmine method of Best, described in Pearse (1961), was used for the localization of glycogen. These tissues were fixed in 10% neutral formalin, Carnoy's fluid, or Bouin's fluid, and embedded in paraffin. The sections were

placed in 1% celloidin in a solution of absolute alcohol and ether (equal parts) for 2 minutes. These sections were then dried in air, dehydrated, stained in Erlich's hematoxylin for 5 minutes, and stained in the carmine solution for twenty minutes.

The methyl green-pyronin Y method of Brachet, described in Pearse (1961), was used to demonstrate ribonucleic acid (RNA). Tissues for this technique were fixed either in Carnoy's fluid for 4 hours or in 10% neutral formalin for 24 hours. Tissues were stained in the methyl green-pyronin Y solution for 16 hours, dehydrated rapidly in acetone, and mounted in permount. To confirm the identification of RNA, control sections were incubated in bovine pancreatic ribonuclease (1 mg/ml) for one hour at 37°C. No differences were observed between tissues fixed in Carnoy's and those fixed in 10% neutral formalin.

The alkaline phosphatase technique of Gomori (in Thompson, 1966) was applied to tissues fixed in 80% alcohol at 2°C for 24 hours. The sections were incubated in the substrate medium (ph 9.4) for 30 minutes at 37°C. The substrate medium used consisted of 25 ml 2% sodium glycerophosphate, 25 ml 2% sodium barbitol, 50 ml distilled water, 5 ml 2% calcium chloride, 2 ml 2% magnesium sulphate, and a few drops chloroform. To remove preformed salts, which are also demonstrated with this technique, the sections were treated with citrate buffer (pH 4.5) for 15 minutes

before incubation in the substrate medium. To confirm the identification of alkaline phosphatase, companion sections were prepared by an identical procedure except that the sodium glycerophosphate was omitted from the incubation medium. Following the demonstration of the phosphatase, the tissues were counter-stained in nuclear fast red for 5 minutes.

Sudan black B in 70% alcohol, following the method of McManus (in Pearse, 1961), was used to demonstrate lipids. These tissues were fixed in Baker's formalin and embedded in paraffin. The sections were stained for 30 minutes at room temperature in the Sudan solution and 1% aqueous neutral red was applied for 5 minutes as a counterstain.

Sudan black B in propylene glycol, following the technique of Chiffelle and Putt (in Pearse, 1961), was also used for lipids. These tissues were fixed in Baker's formalin, and both paraffin and gelatin embedding methods were applied. In the latter case, tissues were sectioned at 10 microns on the freezing microtome. Staining in the Sudan black B solution was optimum at 7 minutes and no counterstain was found to be necessary.

Sudan black B in acetone, from the technique of Berenbaum (in Pearse, 1961), was applied for locating bound lipids in the tissue. Both Baker's formalin and Carnoy's fixed tissues were used, followed by paraffin embedding. It was found that staining in 2% Sudan black B at 37^{o}C

for 18 hours produced the best results.

The Nile blue sulphate technique of Cain (in Pearse, 1961) was used to distinguish acidic and neutral lipids. Baker's formalin fixed tissues were embedded in gelatin and sectioned at 10 microns with a freezing microtome. These sections were treated in a 1% aqueous solution of Nile blue sulphate 60°C for 5 minutes. A second group of sections was treated as above and then restained in a 0.02% aqueous solution of Nile blue sulphate at 60°C for an additional 5 minutes.

Oil red O was used, following the method of Lillie as outlined in Pearse (1961) to stain for neutral lipids. Baker's formalin fixed tissues were embedded in gelatin and sectioned at 10 microns with the freezing microtome. These sections were stained in the Oil red O solution for 10 minutes, and counterstained in dilute Erlich's hematoxylin for a few seconds.

Sudan III and IV, according to the method of Kay and Whitehead in Pearse (1961), was used to stain neutral fats. Baker's formalin fixed tissues were gelatin embedded, frozen-sectioned, and stained in the Sudan III and IV solution for 45 seconds. These sections were counter-stained in dilute Erlich's hematoxylin for a few seconds.

All paraffin sections were mounted in permount, except the Sudan black B techniques for which glycerin jelly was used. All gelatin sections were mounted in

glycerin jelly.

In recording the results of the above staining reactions, a description of the localization of the stain is given. In addition, in some instances, an estimation of the relative amounts of the substance under study is given. This estimation is based on a visual comparison of the staining intensities and is illustrated in graph form. Since, for each technique, all sections were treated in an identical manner, it is assumed that the intensity of the stain gives evidence of the amount of the substance present.

OBSERVATIONS

Histology:

In the chick, the epiphysis cerebri is formed as an evagination (figure 4) of the roof of the diencephalon at the two day incubation stage. As the cavity of the pineal recess is continuous with the third ventricle, the cells of the evagination, when first formed, are similar to those of the lining of the diencephalon (Spiroff, 1958). There is little change until the fourth day of incubation, other than one of elongation. For this reason the present observations begin at day three of incubation.

At day three, the cells lining the evagination are, as also described by Spiroff (1958), ependymal in nature. Their nuclei are large, contain two large nucleoli, and are basally placed in the cell. At day four, the ependymal cells are, based upon the intensity of their staining, very active. Their cytoplasm, not usually stained except by special techniques, is strongly basophilic. In figure 5, it is seen that these cells form a thick, stratified layer of ependymal-like cells. Romieu and Jullien (1942) and Spiroff (1958) refer to these cells as ependymocytes.

On the fifth day of incubation, a second type of cell is distinguishable. The nuclei of this type are small and spherical and have only one nucleolus. They are not ependymal in nature and are designated the pineocytes. The pineocytes are distributed in small clusters peripheral to the ependymocytes (figure 6). The cells in these clusters

become arranged radially around a central lumen, and thus form the primary vesicles. These pineocytes are abundant by the end of day five.

At day six (figure 7), the two cell types are clearly distinguishable and the iron haematoxylin technique demonstrates numerous mitotic figures in both types. The pineocytes are distributed as described above. The ependymocytes primarily surround the pineal recess although a few are scattered within the clusters of pineocytes. The cell clusters, at this stage, have advanced peripherally and the connective tissue surrounding the area is organized to form the early capsule of the developing gland.

At day seven, many of the clusters (figure 8) possess a definitive central cavity. These are the primary vesicles and the cells lining them, the pineocytes, are columnar with basally placed, round nuclei. The basal cytoplasm of these cells stains more basophilically than the apical portion. A few ependymocytes can be distinguished scattered within the vesicles. By this stage, the mesenchyme has invaded the area between the vesicles and has enclosed them. This layer of mesenchyme forms the connective tissue stroma of the gland which, as indicated by aldehyde-fuchsin staining, contains some elastic tissue. Also, by the seventh day of incubation, arterioles and venules are distributed through the connective tissue which encapsulates the gland and capillaries are present around the primary vesicles.

On the eighth day of incubation, many vesicles are separated from the original evagination. New vesicles are present along the boundary of the pineal recess having been formed by the multiplication of the ependymocytes lining it, and also along the boundary of the vesicles already present (figures 8 and 9). These latter are referred to as the secondary and tertiary vesicles, according to their designation by Spiroff (1958).

By the ninth day of incubation, many vesicles are separated from the original pineal recess. As each vesicle becomes separated, it is surrounded by a sheath of connective tissue containing capillaries. By day ten, the development of the connective tissue is well-advanced and forms definite layers separating the vesicles and encapsulating the gland (figure 10).

The number and size of the vesicles within the pineal gland varies during the different stages of the incubation period. This variation is illustrated in the graph given in figure 1. This graph shows the percentage of gland area which is occupied by vesicles. These percentages were determined for each of the major stages of development studied. For each stage, the percentage was calculated for six sections, two sections from each of three specimens, and the points plotted are the mean and standard deviation of the six values. Only true vesicles, that is those possessing a lumen, were included in measuring the area of the vesicles. These measurements were made by projecting the section onto

graph paper and outlining the gland and the vesicles. This graph shows that vesicles are present at day seven and that the percentage of vesicle area decreased during the seven to eleven day incubation period. In fact, this percentage decrease does not represent a reduction in the number or size of the vesicles. Rather, the connective tissue stroma develops and expands during this period increasing the total gland and separating the existing vesicles (compare figures 8 and 33). This stage is followed by a period of very rapid increase in vesicle area which reaches a peak at the seventeen day incubation stage (figure 11). Following the seventeenth day, the total area of vesicles decreases rapidly, although they remain temporarily numerous in the region of the pineal recess. There are only a few vesicles at the one week posthatching stage (figures 12 and 13) andnone are present at the one month or later posthatching stages examined (figures 14 and 15).

The pineal recess, when it is first formed, is attached to the third ventricle by means of the pineal stalk (figure 16). This attachment persists until the one week posthatching stage. The lining of this recess, as noted above, is composed of ependymal-like cells. During the prehatching period from day five onwards, this ependymocyte lining remains unchanged. Vesicles, during this period of proliferation, form from cell clusters along its periphery (figures 7 and 17). Thus, from day eleven to day fourteen prehatching, there is little change in the structure of the

gland, other than an increase in size. This increase in size is attributable to the growth in numbers and volume of the vesicles. This, in turn, causes a change in the general histological character of the gland.

After day thirteen, there is a noticeable compacting of the gland, resulting from the enlargement of vesicles and their gradual filling with secretory material. There is no significant overall increase in the size of the gland however, and the space for vesicular expansion is provided by the crowding out of connective tissue from the spaces between the vesicles. This "compacting" of the gland is most evident between the fourteenth and seventeenth days of incubation (compare figures 11 and 18).

At day seventeen, the gland is quite compacted and composed of vesicles. Most of the vesicles are large although there are small ones along the anterior ventral border of the gland, near the pineal recess. As noted, the vesicles begin to decrease in number at day eighteen and, by one day posthatching, there is a significant reduction in the number of vesicles while those present do not usually contain secretory material (figure 19). At the one week posthatching stage (figures 13 and 20), the vesicles are generally misshapen and contain cells and cell debris within their lumina. The lumina disappear during this stage, and the vesicles become solid massess of cells.

Following day nineteen of incubation, with the decrease

in vesicle number, there is a corresponding re-invasion of connective tissue into the gland. This is particularly apparent at the one-day and one week posthatching stages (figures 19 and 20). At one year, the main mass of the gland is composed of connective tissue (figures 21 and 22).

At day sixteen of incubation, a third cell type can be distinguished for the first time (figure 25). At this stage it appears within the small areas of connective tissue still existing within the gland. These cells are small and spherical, and possess a small basophilic nucleus. Their origin is difficult to determine because of the small proportion of connective tissue in the gland at this stage and because of their close proximity to the vesicles. They begin to invade the vesicles at day nineteen and, at one-day posthatching, have filled a number of vesicles obliterating their lumina. These cells establish themselves in the posthatching stages as the dominant cell type of the gland, and they comprise most of the parenchyma of the one-month posthatching pineal gland. It it likely, therefore, that they are responsible for the elaboration of the secretory product in the chick pineal gland during the posthatching stages.

At one month posthatching, there are no vesicles present. At the posterior end of the gland, a prominant lymph node is present within the connective tissue capsule (figures 14 and 23). There is no indication of the formation of this node at the one-week posthatching stage.

The cells of the node stain intensely basophilically and several germinal centres can be clearly discerned in the body of the node. However, this entire node with its complement of nodules has disappeared by the four-month posthatching stage, leaving only a small patch of lymphocytes at the posterior end of the gland (figure 15). This patch is highly vascularized, as is the node with its germinal centres. Because of the extent of vascularization associated with the node (figure 14) and the numbers of lymphocytes and germinal centres, it is believed that the node is very active.

The connective tissue capsule distributes large blood vessels to the gland and, as early as the seventh day of incubation, the vascularization of the capsule is apparent. The body of the gland contains only small blood vessels and numerous capillaries. The gland becomes highly vascularized by the tenth day of incubation (figure 10), and this vascularization is not affected by the subsequent decrease in connective tissue stroma caused by the rapid expansion of the vesicles. However, with the process of filling in of the vesicles in the early posthatching stages, the connective tissue re-invades the parenchyma and conducts large blood vessels into the body of the gland. These connective tissue stroma divide the gland into lobules composed of solid masses of cells. This division into lobules is distinct in the gland at the one-month posthatching stage

(figures 14 and 23). However, with further development, the size of the lobules decreases. At four months, (figure 32) connective tissue can be seen to be further invading the parenchyma of the gland, and at one year, the lobules are small and the shape of the gland is distorted as a result of the formation of large areas of connective tissue (figures 21 and 22).

At day ten of the incubation period (figure 24), the vesicles first contain a secretory product. This secretion first appears in some of the larger vesicles only but, gradually, it appears in the smaller ones also. The rate of secretion based on the amount and the intensity of the luminal staining, is at its highest level between the fifteenth and seventeenth days of incubation. This observation is also supported by the histochemical reaction (figure 2) of the secretory product described below. By one day posthatching, the lumina are empty and no secretory product can be demonstrated.

As previously described, the cells lining the vesicles are of two types. The first is the ependymal-like cell which is scattered in small numbers in the vesicles, and which is designated the ependymocyte, following the nomenclature of Spiroff (1958). The second type, first distinguishable at the five-day incubation stage is the pineocyte. The nuclei of the ependymocytes are more oval than those of the pineocytes and are arranged perpendicular to the basement membrane (figure 25). The cytoplasm of the

ependymocytes is faintly basophilic and, unlike that of the pineocyte, stains with fast green FCF. This is particularly visible during the later prehatching period, providing an excellent diagnostic characteristic. The nuclei of the pineocytes are spherical and basally placed. The cytoplasm of those pineocytes which line vesicles containing secretory material is granular. This cytoplasm exhibits a gradation in staining properties from a basophilic basal portion to an acidophilic distal portion. The acidophilic reaction varies in intensity during the incubation period. At day seven the acidophilia is faint but, during the subsequent stages, it increases in intensity and during the fifteenth to seventeenth day period, it exhibits a strong reaction. The cytoplasmic granules of the pineocytes, particularly during the fifteenth to seventeenth days of incubation, also give a positive reaction with the aldehyde-fuchsin method and exhibit Toluidene blue metachromasia. Following day seventeen, there is a decrease in the acidophilic reaction of these cells and only a weak reaction is present at day one posthatching. No reaction could be demonstrated at the one-week stage. This decrease parallels the reduction in the amount of secretory product within the lumina during this period. Additional reactions of the pineocytes with histochemical methods are described below.

Histochemistry:

The luminal contents give a positive reaction with the

alcian blue (figure 27), the aldehyde-fuchsin, the periodic acid-Schiff reagent (figures 11 and 31), and the metachromatic Toluidene blue (figure 30) techniques. These four groups of observations are similar and are illustrated together in the graph in figure 2. This graph shows that the secretory product first appears in the lumen at day ten, that it increases rapidly in staining intensity, and is at its peak staining intensity during the fifteenth to seventeenth days of incubation. During the eighteenth and nineteenth days of the incubation period, the staining intensity of the luminal contents decreases abruptly. This would suggest that the secretory material is released most rapidly during the fifteenth, sixteenth, and seventeenth days of incubation.

The periodic acid-Schiff reagent (PAS), the aldehyde-fuchsin, and the metachromatic Toluidene blue methods also stain the cytoplasmic granules within the apical portion of the pineocytes. These granules could not be demonstrated with the alcian blue method. In addition, the cells of nearly all vesicles possess an apical border which stains with the periodic acid-Schiff reagent, the aldehyde-fuchsin, and the metachromatic Toluidene blue methods(figures 24, 26, 27, and 28). This border is of approximately equal width with each of the stains used to demonstrate it. The intensity of the staining reaction of the border does not vary during the incubation period, and the border is found in both empty vesicles and those containing secretory product. It is suggested that this staining demonstrates

the presence of a specialization of the free surface of the cells to facilitate secretion.

The Best's carmine method and the periodic acid-Schiff reagent technique for glycogen reveal a positive reaction in the pineocytes. The graph in figure 3 illustrates the intensity of staining with the carmine technique at the different stages of incubation. This graph shows that the period of most intense staining, suggesting the presence of the greatest quantities of glycogen, occurs during the fifteenth to seventeenth days of incubation. It should be noted that this glycogen reaction occurred only in the apical cytoplasm, and was not observed in the basal regions of the cells (figure 34).

Several methods were employed for the localization of fats in the cells of the pineal gland. Sudan black B, in three techniques, was used to locate lipids generally. These techniques demonstrate a staining reaction in the pineocytes that is localized in the cytoplasm distal to the nucleus. The Sudan III and IV and the Oil red O methods produce identical staining reactions. With each technique the staining reaction is most intense at the fifteenth day of incubation and declines rapidly thereafter. By one-month posthatching, there is no staining in the pineal gland with these techniques. The Nile blue sulphate method for acidic lipids exhibits a very weak reaction in the connective tissue, and no reaction in the cytoplasm of either the pineocytes or the ependymocytes (figure 9). However, the

luminal border of the pineocytes (figure 9) shows an intense staining reaction with this technique.

The chrome alum haematoxylin method failed to produce any reaction in the chick pineal gland at any of the stages studied. Also, the Gomori method for the identification of alkaline phosphatase in the pineal gland produced negative results for all the stages studied, other than staining the nuclei which is presumably artifactual (figure 29).

Finally the methyl green-pyronin Y method demonstrates pyroninophilia in the cells of the vesicles, (Figure 33). During the early stages of incubation this reaction is moderate in intensity and is distributed through the entire cytoplasm. During the fifteenth, sixteenth, and seventeenth days prehatching the pyroninophilia is much more intense in the apical cytoplasm than in the basal portion. On the eighteenth day and during subsequent stages only the apical cytoplasm exhibits an appreciable reaction. Treatment of the sections with ribonuclease eliminates the reaction within the basal cytoplasm but does not influence the intensity of the pyronin reaction in the apical regions, indicating that the apical pyroninophilia is not caused by the presence of ribonucleic acid.

DISCUSSION

The histological development of the pineal gland as reported in this investigation follows basically the same pattern as that which was described by Spiroff (1958), with certain modifications and additions where the present observations enlarge upon those previously recorded. The evagination of the pineal recess from the third ventricle occurs at day two of incubation and, until day four of incubation, there is little change other than one of elongation of the gland. The present study begins at day three of incubation.

At day three the cells lining the evagination are ependymal in nature. At day four, for the first time, the cytoplasm of these cells stains intensely basophilically, indicating initiation of activity at this stage. The iron haematoxylin technique demonstrates the presence of numerous mitotic figures in these cells. By day five, two distinct cells types can be distinguished, and these have been designated the ependymocyte and the pineocyte. Based on their staining affinities, the ependymocyte appears to have a primarily supportive function. The pineocyte is so named because of its probable function as the secretory cell of this gland.

Generally, the pineal recess is lined by ependymocytes and the pineocytes arrange themselves in peripherally-placed clusters. Renzoni and Quay (1963) have described cells in the pineal stalk of pigeons, similar to the pineocytes of

the present study, which they call parenchymal cells. They reported that these cells contain apical cytoplasmic granules which stain with the chrome alum haematoxylin method. However, in the present study, the pineocytes did not exhibit a positive reaction with this technique. Pineocytes were not numerous in the pineal stalk at any of the stages examined. Furthermore, the pineal stalk did not contain any demonstrable secretory product at any stage of development examined.

At the end of day five, clusters of pineocytes are present along the periphery of the pineal recess. By day seven, the cells within these clusters, as well as some ependymocytes are organized in a radial fashion surrounding a central lumen. Thus the cell clusters are converted into the primary vesicles. Spiroff (1958) has also reported this sequence of events in his observations of the development of the chick pineal gland.

Micheletti and Duncan (1966) noted two distinct cell types in the pineal gland of the cat which they term pinealocytes and astrocytes. The pattern then, is the same as that here described for the chick; that is, a supportive neuroglial element, and a definitive secretory cell. Krabbe (1955) observed the development of the pineal gland in several species of birds, including the owl and the swan, and describes the formation of "buttons" of cells peripheral to the original evagination. He does not, however, describe their histology. Presumably, these "buttons" correspond to

the "clusters" of the present study.

Fine strands of connective tissue appear around the gland as early as day five and the stroma of connective tissue is distinct between the vesicles at day seven. A capsule is present at day eight and large blood vessels, though present as early as day seven, are readily apparent in the capsule by day ten. At the end of day five, a few capillaries are present around the first-formed or primary vesicles and, by the end of day ten, each separated vesicle is surrounded by a complete network of capillaries. These findings are essentially in agreement with those of Spiroff (1958).

By the eighth day of incubation, many new vesicles are formed and the first-formed vesicles have separated from the pineal recess. Secondary and tertiary vesicles are present, forming along the periphery of the primary vesicles. The present study shows that the area of the vesicles increases rapidly and is greatest on the seventeenth day of incubation. After the seventeenth day, the amount of vesicular tissue decreases and, by one day posthatching, only a few vesicles remain. The area of the vesicles continues to decrease and, by one week posthatching, those that remain are in a distorted and fragmented state. By one month posthatching, the vesicles have entirely disappeared. As the vesicles store the secretory product elaborated by the pineocytes lining them, it appears that the rapid increase in area up to day seventeen is related to some secretory event. After

day seventeen, their decrease in number and subsequent disappearance apparently marks the culmination of one type of secretory phase for the gland. The fact that after one month posthatching the character of the gland changes so markedly indicates that the nature of secretion and probably the secretory product itself may also change in posthatching stages. It would seem, therefore, that the function of the pineal gland changes with development from the embryonic to adult stages. Owman (1963) has shown that there is a secretion by the pineal gland in the foetal rat and that this secretion has a definite function in the regulation of certain events in the ileum of the embryo. Other authors have suggested a variety of other functions for the pineal gland of different animals. It is possible then that the prehatching secretion by the chick pineal gland might be related to the development and functioning of other tissues or organs. Also, with the disappearnce of the vesicles, it appears that a new role, which might be altogether different, develops in the pineal gland during the posthatching stages.

This posthatching change in the structure of the gland appears to be brought about in two ways. The first means is by the simple breakdown of the cells lining the vesicles. At one-week posthatching the vesicles appear distorted in shape and contain debris within their lumina. The cells also undergo a change at this stage. The pineocytes that remain have lost the acidophilia noted in

these cells in the prehatching stages. Spiroff (1958) has also noted the change from the vesicular to the compact structure of the gland in the chick, although he doesn't specifically state at what point or how this alteration occurs. He reports that the pineal gland becomes progressively more compacted as development continues. "In older specimens, the typical vesicular appearance disappears, thus resulting in a compact parenchyma." Ward (1946) has found however, that the sixteen day prehatching pineal gland assumes the compact condition of the adult bird. In contrast, the report of Beattie and Glenny (1966) on the pineal gland of one-year old fowl describes a vesicular structure and Romieu and Jullien (1942) have found the vesicular condition to persist as late as seven months posthatching. On the other hand, it is the present finding that all vesicles are completely obliterated by one-month posthatching.

In other vertebrates, Grignon and Grignon (1963) have found, in the turtle, that the vesicular condition exists in the adult. In the rat, Owman (1964) has found that the embryonic pineal gland is a saccular structure. Quay and Levine (1957) have described a postnatal proliferative stage of the rat pineal gland which extends for about two weeks after birth, and that the rat adult pineal gland appears much more compact than that of the embryo.

It was suggested by Desogus (in Spiroff, 1958) that the adult pineal body exhibits an "active" (vesicular)

condition in birds engaged in egg or sperm production, and a compact condition in those that are "inactive", or not so engaged. Spiroff (1958) found no evidence that this fluctuation between the two conditions occurs in the adult. The four month old birds used in the present study had begun to lay at the time of extraction of the pineal glands. There was, however, no evidence of an "active" vesicular condition. Rather, the picture presented appears to be that of a more progressive and complete change in the structure of the gland.

Quay (1965) has described three separate variations in the arrangement of the pineocytes in birds: (1) the saccular arrangement, (2) the follicular arrangement and (3) the solid lobular arrangement. While these variations may exist in different species of avians, the present evidence suggests that in Gallus, two of these three types occur at separate stages of the life cycle. The follicular arrangement occurs during the prehatching period and appears to be associated with a major secretory event during this stage. With hatching, the gland becomes progressively more compacted to form the solid lobular arrangement of pineocytes.

The second method of change in the structure of the gland appears to be the invasion of new cells into the body of the gland. This occurs rapidly immediately after hatching, but these cells can be seen for the first time as early as day 16 prehatching. They become more numerous as the gland becomes

compacted and they may originate from connective tissue cells or from one of the cell types lining the vesicles. As development continues they invade the vesicles and often obliterate the lumina. At the one-week posthatching stage they are abundant in and around the distorted vesicles of the gland. By one-month posthatching, they form the main body of the gland and the pineocytes of the prehatching gland are no longer in evidence. During the same period that these cells are proliferating in the gland, the connective tissue re-invades the gland, conducting large blood vessels with it into the body of the gland. Thus, at one-month posthatching, the gland is divided into distinct lobules by the connective tissue stroma, and is composed primarily of this third cell type. It is likely then, that this cell is the definitive secretory cell in the adult gland.

The elaboration of the secretion by the pineal gland is first in evidence at day ten of incubation. The secretory product is accumulated in the lumen of the vesicles. Based on the amount and staining intensity of the secretory product, the investigation shows that secretory substance accumulates rapidly after day ten and reaches peak storage amounts during the fifteenth to seventeenth days of incubation. Following day seventeen, the amount of stored secretion decreases and none can be demonstrated following the one-day posthatching stage. These observations again

support the hypothesis that the pineal gland has a major secretory role during the prehatching stages, and that, following day seventeen prehatching, this function rapidly decreases in importance and terminates with hatching. Further, this might suggest that the secretion of the pineal gland during this period is essential to some phase of development occurring elsewhere in the embryo. Romieu and Jullien (1942) have described a secretory function of the vesicles, comparing them to the thyroid in structure. Owman (1963) has suggested an embryonic function for the pineal gland of the rat. He has connected this function to the normal development of the ileum in the embryo. However, others (Thieblot, Berthelay, and Blaise, 1966; Houssay, Pazo and Epper, 1966; Jouan, 1965) have established quite different functions for the adult rat pineal gland. These functions include effects on the sexual development, on the metabolism of other glands and related cycles in the rat. Further, only one type of secretory cell has been described for this gland, and the possibility that the same cell is responsible for the elaboration of two very different secretory products is remote. It is more probable that there are two distinct phases in the life of the pineocyte, and that the secretion it produces changes with the differentiation of the original cell or the production of a new cell type and the maturation of the gland into a different type of secretory body.

At one-month posthatching a large lymph node is present

at the posterior border of the gland. This node is well developed with several large germinal centres. By four months posthatching, this node is less extensive and is reduced to a small patch of diffuse lymphatic tissue. At one-year, there is no lymphatic tissue present anywhere in the gland. Quay (1965) has described the presence of a several lymph nodules during the second and third months of the posthatching period. Romieu and Jullien (1942) described the formation of patches of lymphocytes along the posterior border of the gland at the end of the one-month posthatching stage, and that these subsequently invaded the body of the gland. The present study found that the lymphocytes did not, at any stage examined, actually significantly invade the body of the gland.

Histochemistry:

The luminal contents reacted positively with the periodic acid-Schiff reagent technique (PAS). This reaction also occurs in the apical cytoplasm. Owman (1955) found the cells of the rat pineal gland also reacted positively with this method. In the fowl, Beattie and Glenny (1966) found that most of the gland of one-year old fowl was positive with the PAS method. Quay (1965), working with cormorants, found that clusters of cells within the gland stained positively with PAS.

The alcian blue method also produces an intense staining of the luminal contents, but gives no reaction

within the apical cytoplasm. Beattie and Glenny (1966) and Hafeez and Ford (1967) reported a negative reaction to this stain by the pineal glands of the one-year old fowl and the salmon respectively. However, Grignon and Grignon (1963) found apical inclusions in the cells which stained with this method in the land turtle. The connective tissue sheath in this, as in previous investigations, stained intensely with this technique.

The metachromatic Toluidene blue method produced reactions in the lumen contents that correspond to the PAS reaction in intensity. The apical cytoplasm also exhibited positive metachromasia. Beattie and Glenny (1966) found no metachromasia in one-year old fowl. Mikami (1951) described this reaction in both the apical cytoplasm and in the lumen of several domestic animals.

The luminal contents stain positively with the aldehyde-fuchsin technique. Combescot and Demaret (1963) found a gel secretion in the pineal gland of the turtle that stained with the aldehyde-fuchsin method. Owman (1963) described aldehyde-fuchsin positive cells that seemed to be of a secretory nature in the pineal gland of the rat.

The techniques described above (figure 2) indicate that the secretory product of the chick pineal gland during the prehatching stages includes glycoprotein and/or mucoprotein as well as sulphated mucopolysaccharide components. Each of these staining reactions is at peak

intensity during the fifteenth to seventeenth days of incubation. This would again support the hypothesis that a major secretory event occurs during this period.

The apical cytoplasm stains with the PAS, the metachromatic Toluidene blue, and the aldehyde-fuchsin methods, suggesting that the precursors of the secretion are located here. Further, the digestion with diastase as a control for glycogen, though it decreased the staining intensity, did not remove all the PAS staining in this region. This would confirm that some form of glycoprotein or mucoprotein precursor is located in this region. The apical staining decreases rapidly after seventeen days prehatching, and has almost completely disappeared at one day posthatching.

The carmine method for the localization of glycogen stained the apical cytoplasm of the pineocytes. Mikami (1951) also found glycogen staining within the parenchymal cells of the pineal glands of several domestic animals. Charlton (1967) found glycogen in pineal cells of Xenopus laevis d., an amphibian.

The pyronin method for RNA revealed a moderate reaction in the cells lining the vesicles. This reaction was distributed through the cytoplasm of these cells generally and, in the latter prehatching stages was most intense in the apical regions of the cells. Treatment of the sections with ribonuclease eliminated the pyronin reaction from the basal part of the cell, confirming the localization of RNA.

Ribonuclease treatment, however, did not reduce the pyroninophilia of the apical cytoplasm, suggesting that this reaction is caused by substances other than RNA. This apical pyroninophilia is possibly caused by the acid mucopolysaccharide component of the secretory product as discussed above. It is known that cells and tissues rich in acid mucopolysaccharides, such as mast cells and cartilage and bone matrices, stain with pyronin. Mikami (1951), studying pig and goat pineal glands, found large amounts of RNA present in the cells. Charlton (1967) describes a region rich in RNA in the cells of the toad, Xenopus. Heiniger (1965) found a very low RNA content in pig and human pineal glands.

The presence of RNA indicates the pineocytes are producing a protein substance, and would confirm the hypothesis that the secretory product is a glycoprotein or mucoprotein. There is evidence that Alcian blue staining can be blocked by a protein component associated with the acid mucopolysaccharide. Also, one type of sulphated acid mucin can stain metachromatically but produce little if any reaction with Alcian blue (Spicer, 1960). However, the presence of aldehyde-fuchsin, PAS and metachromatic reactions in the apical cytoplasm indicate the precursor contains an acid mucopolysaccharide (Halmi and Davies, 1953). Grignon and Grignon (1963) have described apical inclusions in the cells of the epiphysis of reptiles which they suggest is

the acid mucopolysaccharide precursor of the secretory product. Beattie and Glenny (1966) have also reported the presence of cytoplasmic basophilia, suggesting ribonucleoprotein, in the avian pineal gland with the methylene blue method.

Alkaline phosphatase has been found to be present in the pineal parenchymal cells of pigs and goats by Mikami (1951). However, other investigators studying one-year old rabbits have found no reaction within the pinealocytes (Bostelmann and Bienengraber, 1963). Further, Bayerova and Bayer (1967) could not demonstrate any alkaline phosphatase activity in the pinealocytes of humans. The present study gave no indication of phosphatase activity in any part of the pineal gland of Gallus, either during prehatching or posthatching stages. No previous work on the presence of alkaline phosphatase in the pineal glands of other avians is reported in the literature.

Sudan III and IV and Oil red O techniques for fats produced consistent staining in the apical cytoplasm of the cell. These two methods test for neutral lipids. Sudan black B techniques further substantiated these findings. Anderson (1968) has shown the presence of large lipid inclusions in the pinealocytes of sheep and cows. No acidic lipids or phospholipids were found in the gland in the present study. However, Grignon and Grignon (1963) found phospholipid inclusions in the apical region of

pinealocytes of land turtles. Also, Prop (1964) found phospholipids in rat pineocytes, as well as triglycerides.

There has been no previous record of a specialized border for secretion in the pineal gland of chicks. Spiroff (1958) stated that there is no ciliated border. However, the present study has demonstrated a definite border along the cells lining the vesicles which stains intensely with the PAS, Alcian blue, and aldehyde-fuchsin techniques. This border is first demonstrable at the seven day incubation stage, when there is as yet no secretion which can be stained. It is believed that this staining represents a border, possibly formed of microvilli, which is specialized to assist in the secretory mechanism of the cell. Anderson (1968) found a specialized border in an electron microscope study in the pineocytes of sheep and cows. Hafeez and Ford (1967) record the presence of such a specialized border in the salmon and also note that it stains positively with the periodic acid-Schiff method.

This study has provided further information on avian pineal histology and histochemistry and related these to possible pineal function. Nevertheless, it is evident from the literature and from the findings of the present study, that there is still much controversy as to the nature of the pineal gland, the reactions occurring within its cells, and even its probable function.

SUMMARY

1. The embryonic avian pineal gland is composed of two main cell types, the ependymocyte or supportive cell, and the pineocyte, or secretory cell.

2. The avian pineal gland has two different secretion stages during its life cycle, one in the embryo, and one in the adult. The first type involves the formation of vesicles, and the second probably is a result of direct secretion from each cell into the bloodstream.

3. In the late embryonic stages, a third cell type appears and predominates in the posthatching stages. It is postulated that this cell is responsible for the elaboration of secretory product in the posthatching stages of the life cycle.

4. The secretion was in granules located apically in the cell, then released into the vesicular lumen.

5. The secretory product was found to contain a glycoprotein or mucoprotein as well as a sulphated mucopolysaccharide component.

6. Neutral lipid inclusions were located in the cell apical to the nucleus. Glycogen was also observed in this position.

7. Lymphocytes occurred as a well-formed node at one month posthatching, but these were dispersed to form a small diffuse patch by four months, and were absent at one year.

8. The structure of the gland, while vesicular in the embryo, has become filled in and solid lobular in the adult. By one month posthatching, all traces of vesicles have disappeared.

9. There is a peak in staining intensity at 17 days prehatching suggesting a secretory role at this stage. Thereafter, the secretory product disappears from both the apical cytoplasm and the vesicular lumen.

10. A specialized apical border of the pineocytes is postulated as a result of the staining of this border with histochemical techniques.

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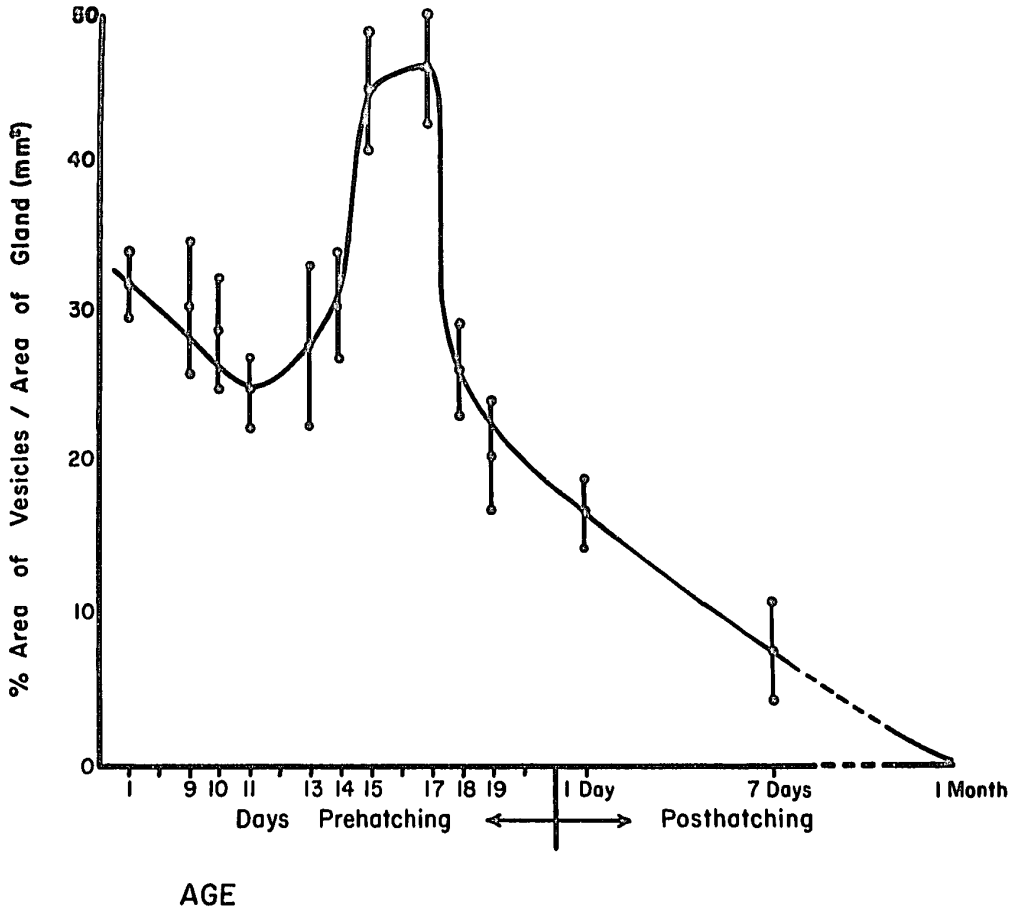
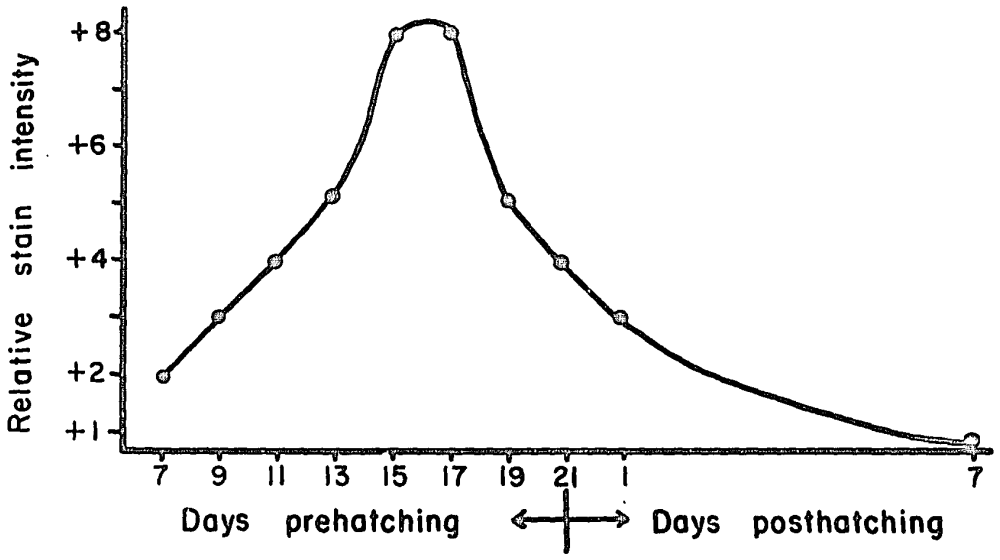
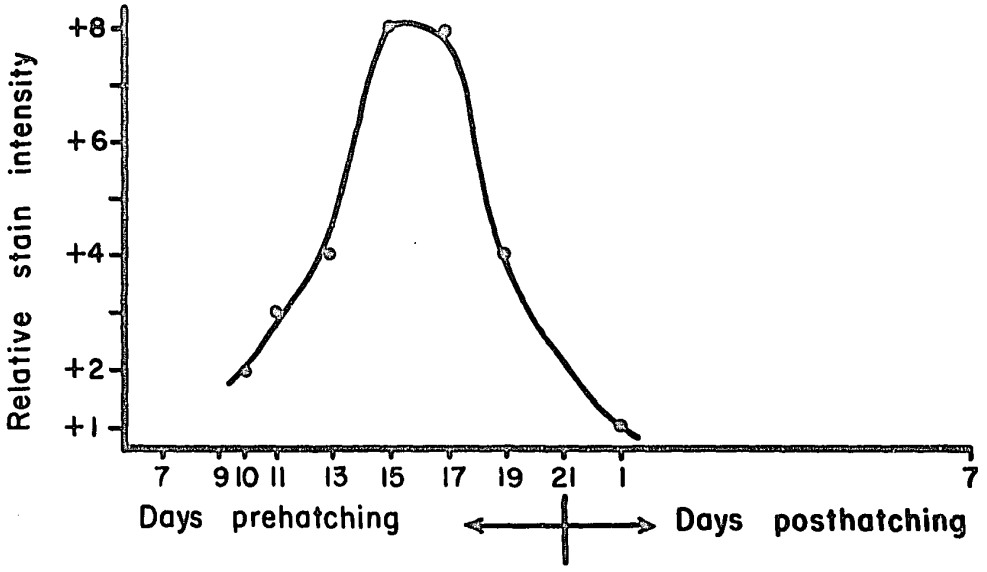
Figure 1. Percentage of gland area occupied by vesicles.


% Area of Vesicles / Area of Gland (mm²)
50
40
30
20
10
0
1
9 10 11
13 14 15
17 18 19
1 Day
7 Days
1 Month
Days Prehatching
Posthatching
AGE


Relative stain intensity
+8
+6
+4
+2
+1
7
9
11
13
15
17
19
21
1
7
Days prehatching
Days posthatching


Relative stain intensity
+8
+6
+4
+2
+1
7
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10
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13
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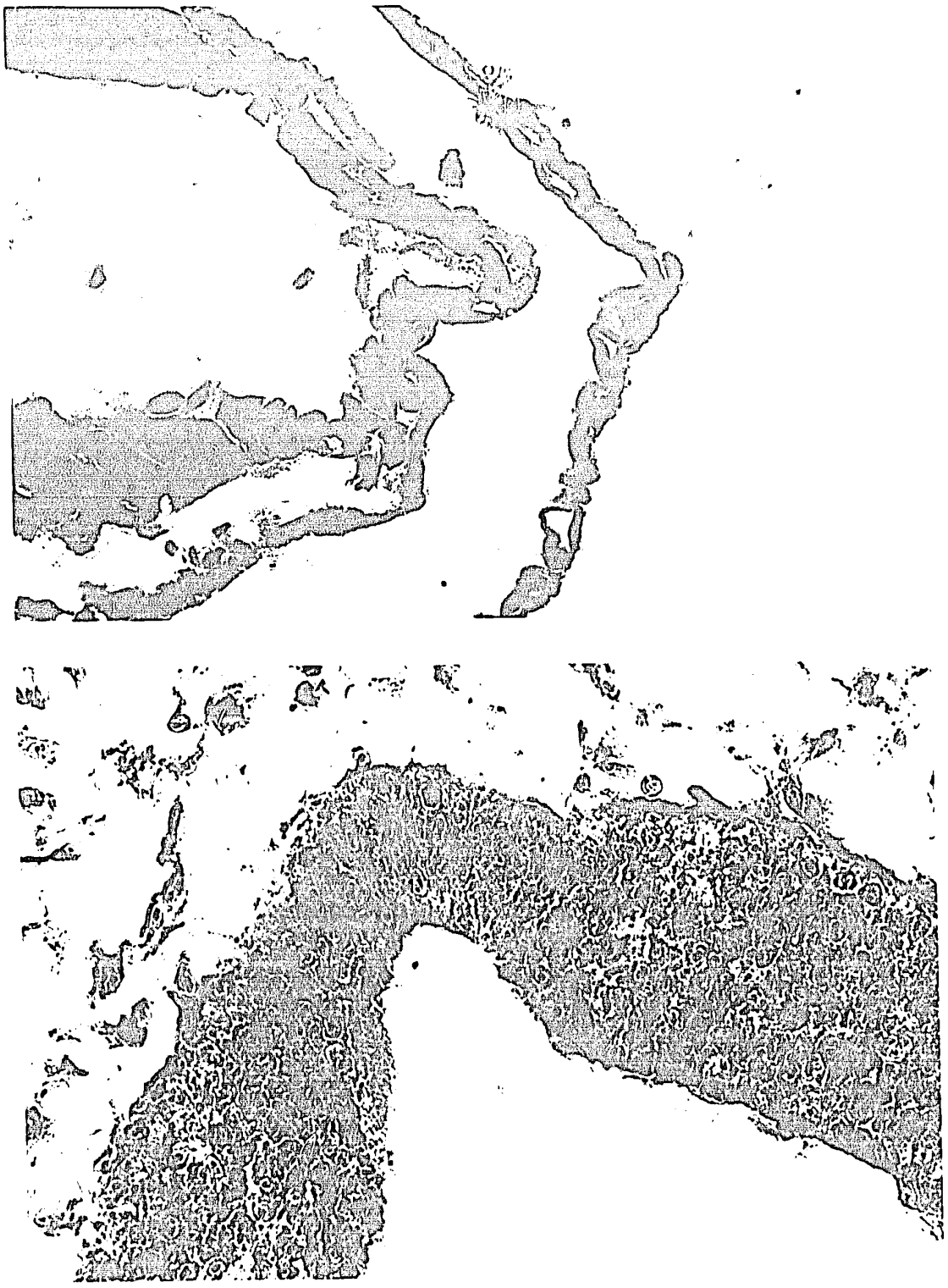
17
19
21
1
7
Days prehatching
Days posthatching



Splice page

51 - Figure 1

Figure 1. Percentage of gland area occupied by vesicles.

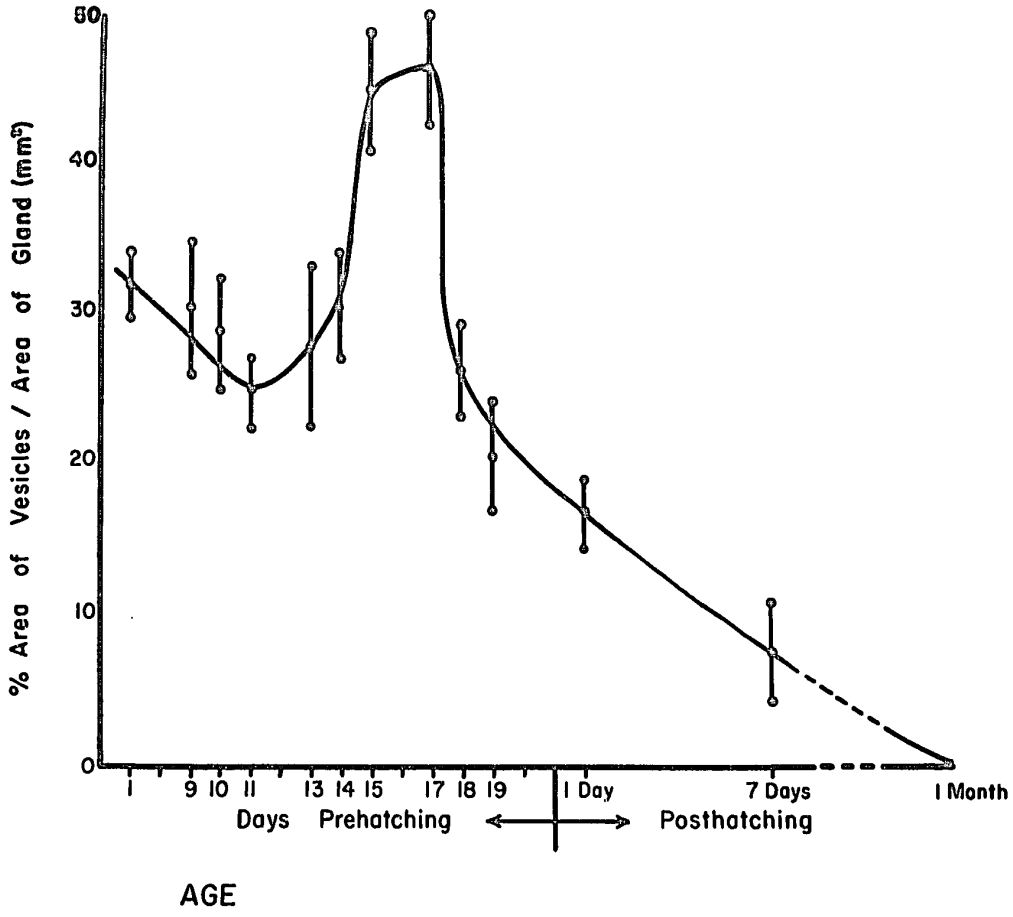

% Area of Vesicles / Area of Gland (mm²)
50
40
30
20
10
0
1
9
10
11
13
14
15
17
18
19
1 Day
7 Days
1 Month
Days Prehatching
Posthatching
AGE

Figure 2. Staining Intensity of luminal secretory product.

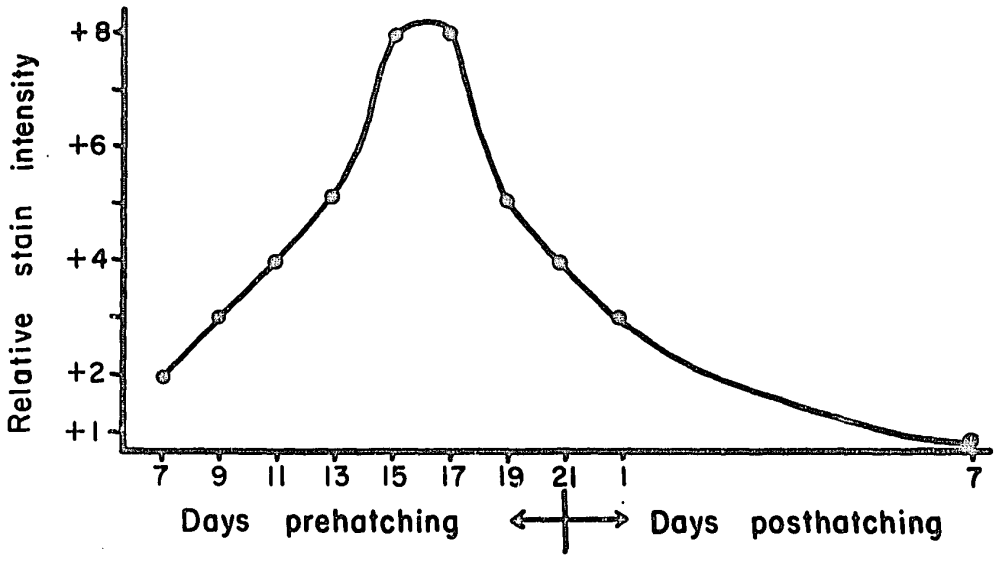

Relative stain intensity
+8
+6
+4
+2
+1
7
9
11
13
15
17
19
21
1
7
Days prehatching
Days posthatching

Figure 3. Staining Intensity of Apical Cytoplasm.

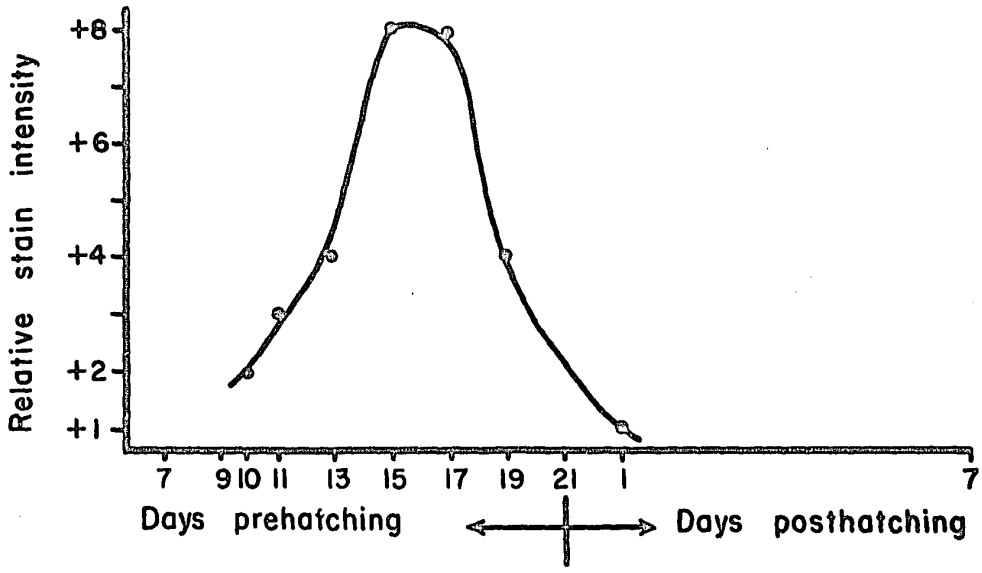

Relative stain intensity
+8
+6
+4
+2
+1
7
9
10
11
13
15
17
19
21
1
7
Days prehatching
Days posthatching

Figure 4. Day 3. X125 (Haematoxylin-Eosin)
Shows evagination of pineal recess from diencephalon roof.

Figure 5. Day 4. X125 (Aldehyde-fuchsin)
Cells are beginning to differentiate from those of the diencephalon.

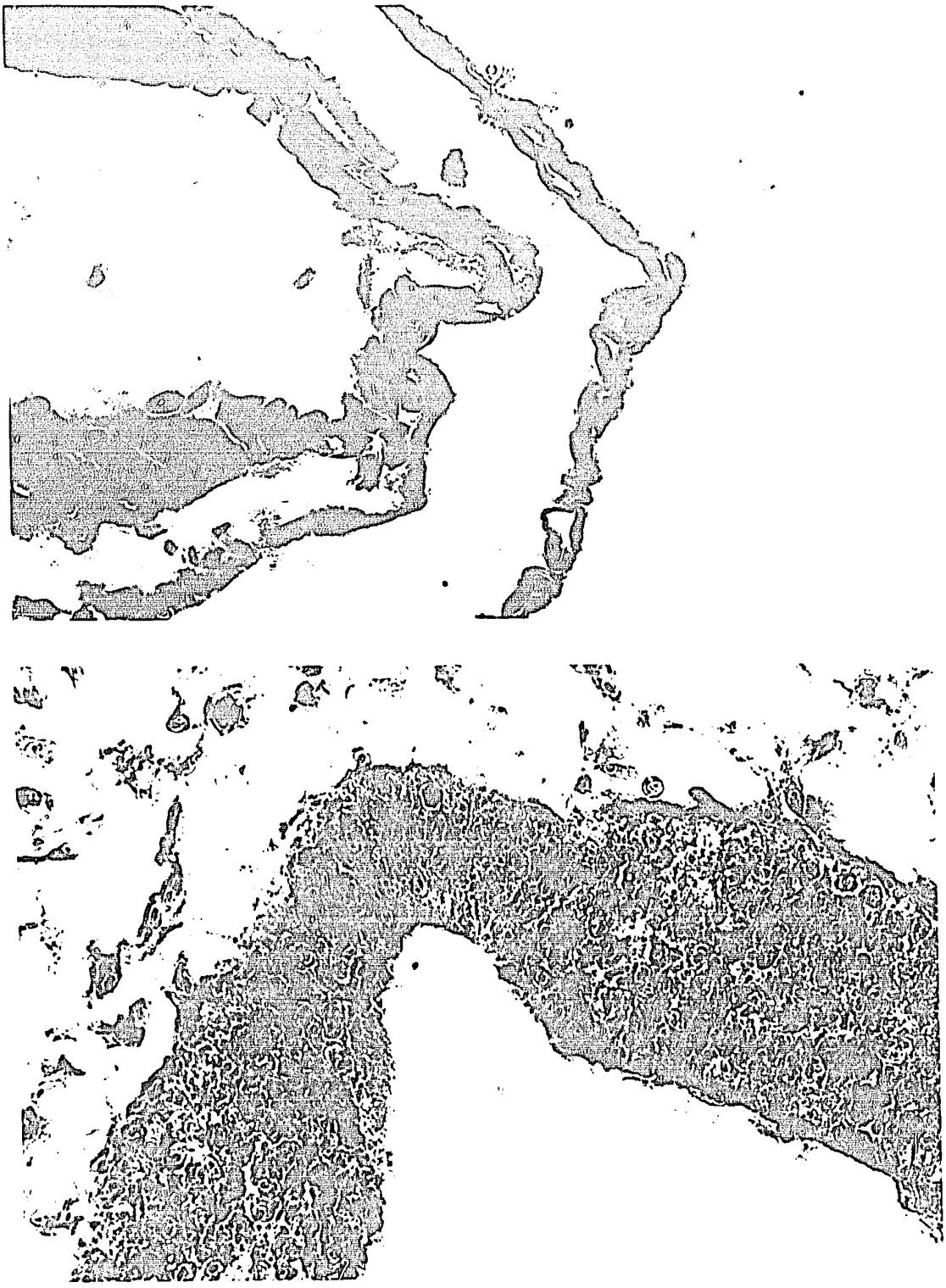
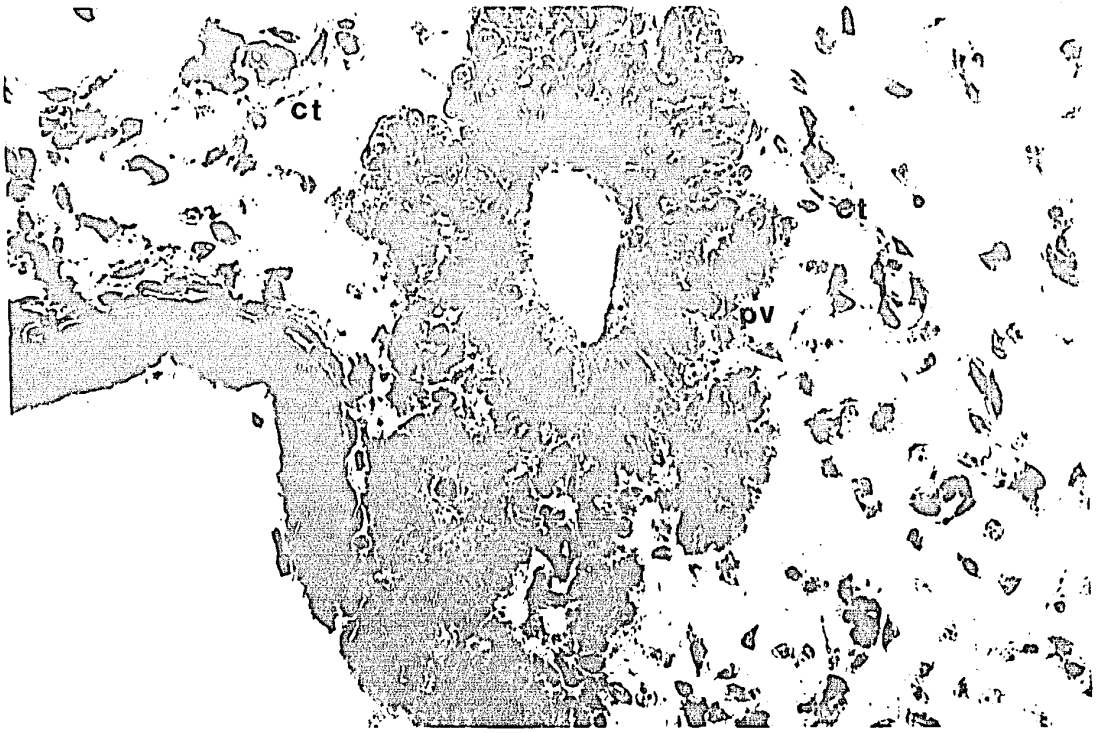


Figure 6. Day 5. X125 (Haematoxylin-Eosin)
Pineal recess with primary vesicles (pv) forming peripherally. Note connective tissue (CT) collecting around site.

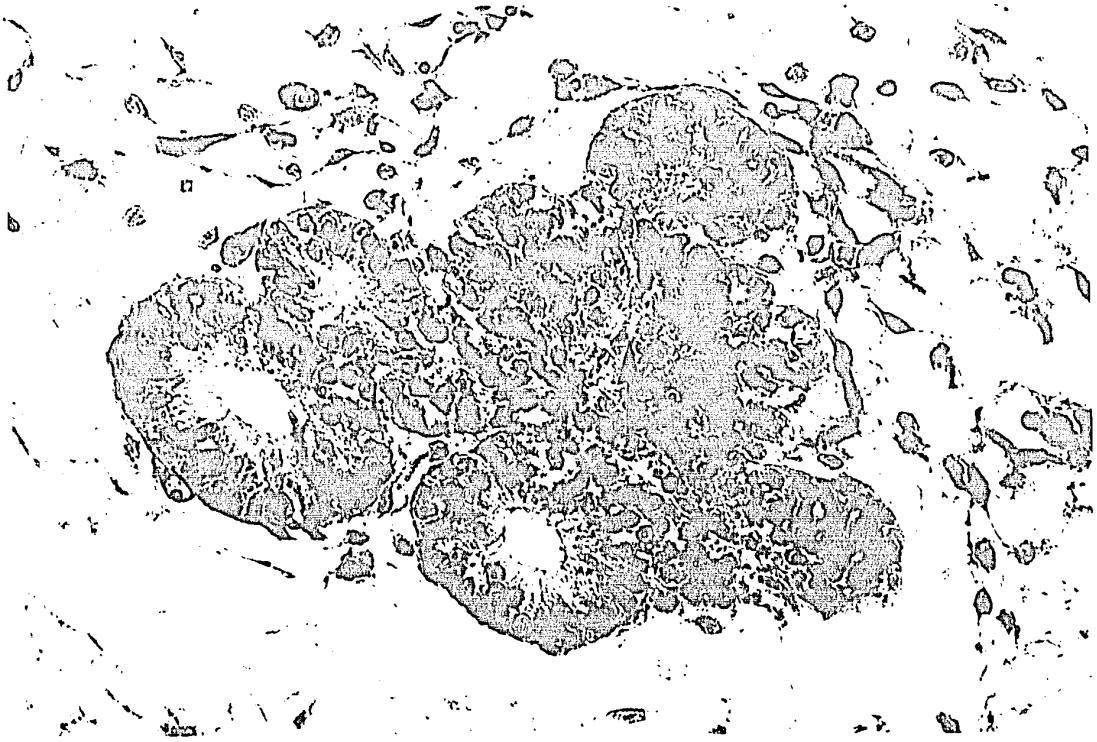
Figure 7. Day 6. X160 (Iron Haematoxylin)
Mitoses in cells are visible. Secondary vesicles are breaking away and arranging to form their own lumina.


ct
ct
pv


ct
ct
s

Figure 8. Day 7. X100 (Haematoxylin-Eosin)
Several new vesicles have formed. The pineal recess is designated by an arrow.

Figure 9. Day 8. X40 (Nile Blue Sulphate)
Note dark inner border of lumina (arrows). Connective tissue capsule is quite well developed here. Vesicles are separated.



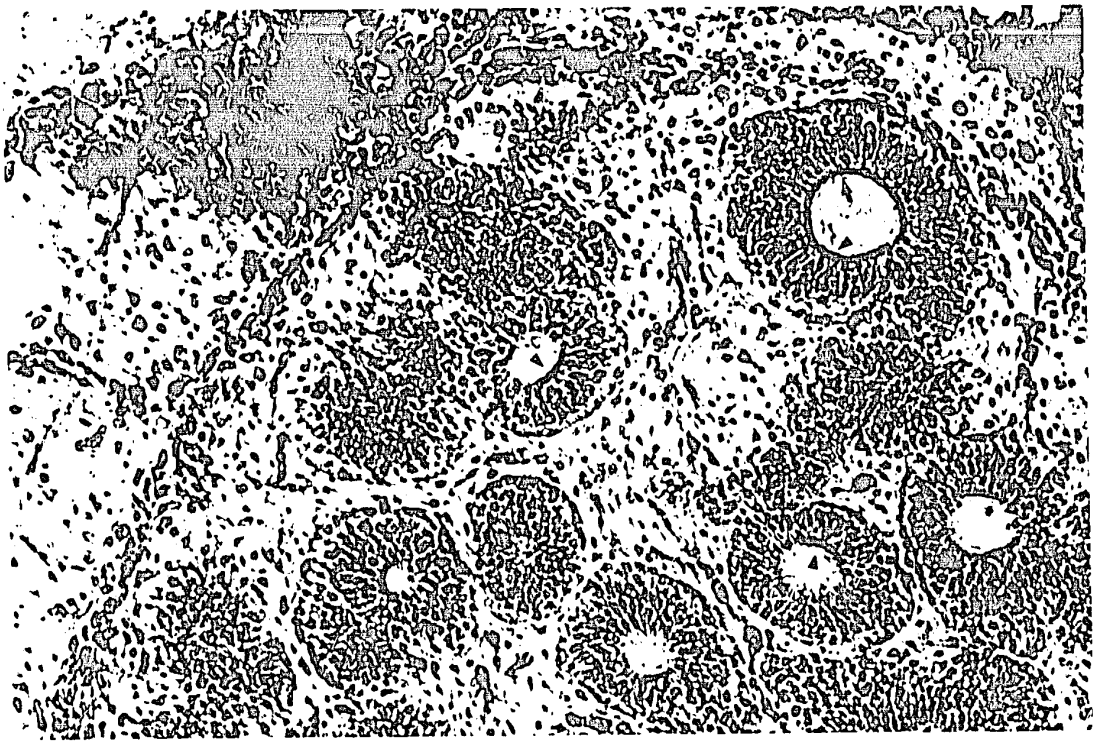
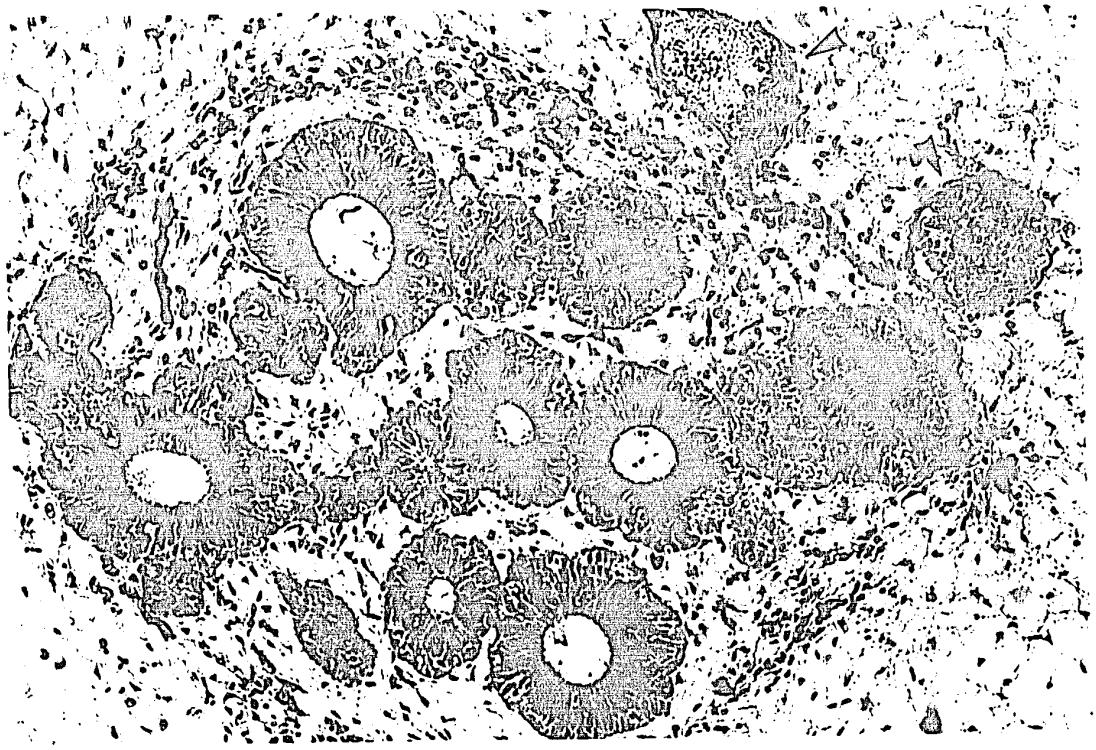


Figure 10. Day 10. X40 (Haematoxylin-Eosin)
The capsule is well developed, and there are large areas of loose connective tissue inside. Note large blood vessels (arrows) outside the gland.

Figure 11. Day 17. X63 (Periodic acid-Schiff)
Note staining of secretory product in the lumen and apical cytoplasm.



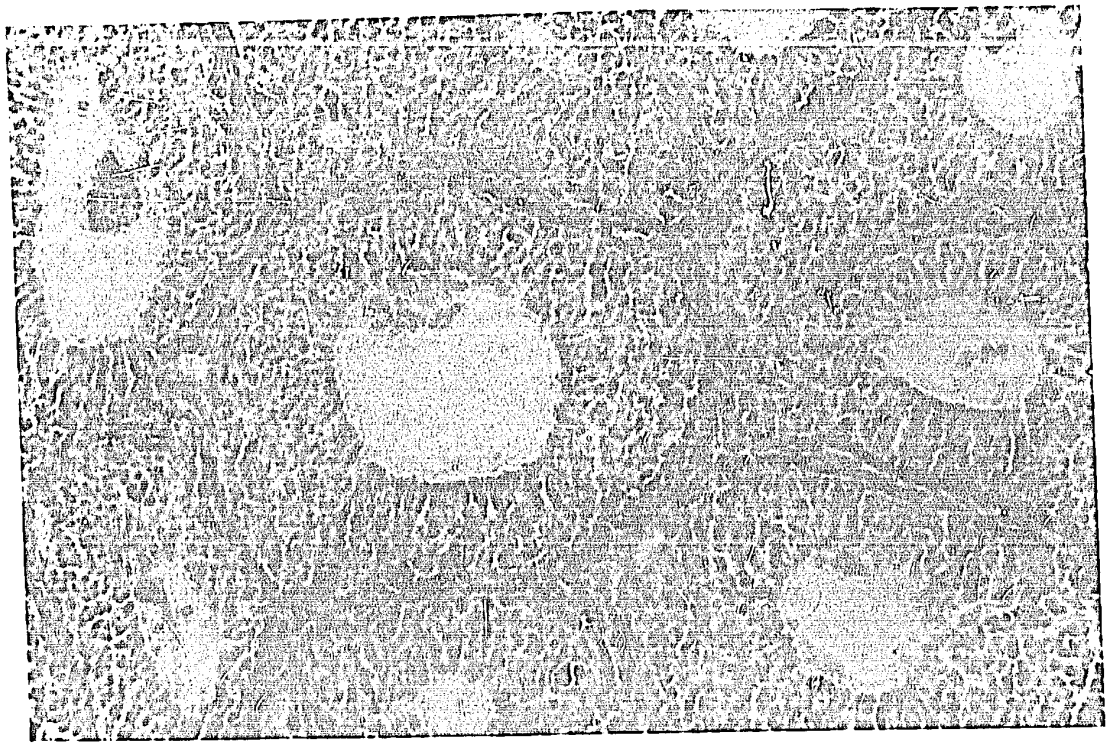
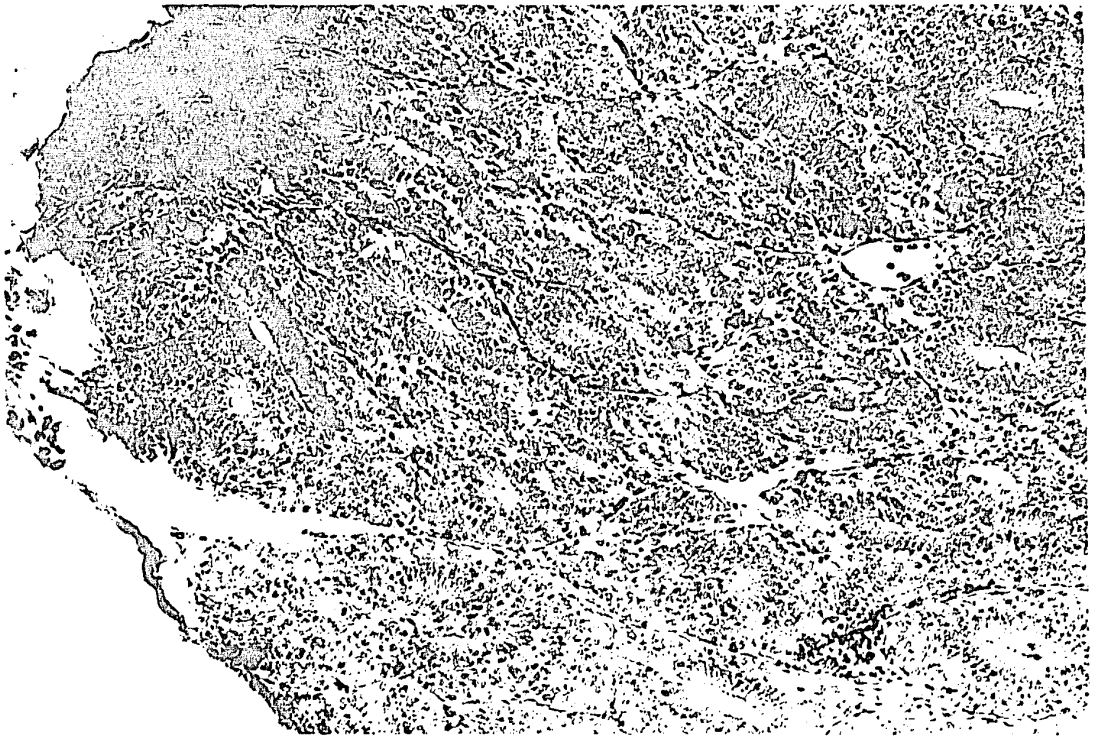


Figure 12. One week posthatching. X40. (Iron Haematoxylin)

Note amount of connective tissue present. Note too large blood vessels in the body of the gland. (BV)

Figure 13. One week posthatching. X63. (Iron haematoxylin)

Vesicles are distorted and contain cell debris.



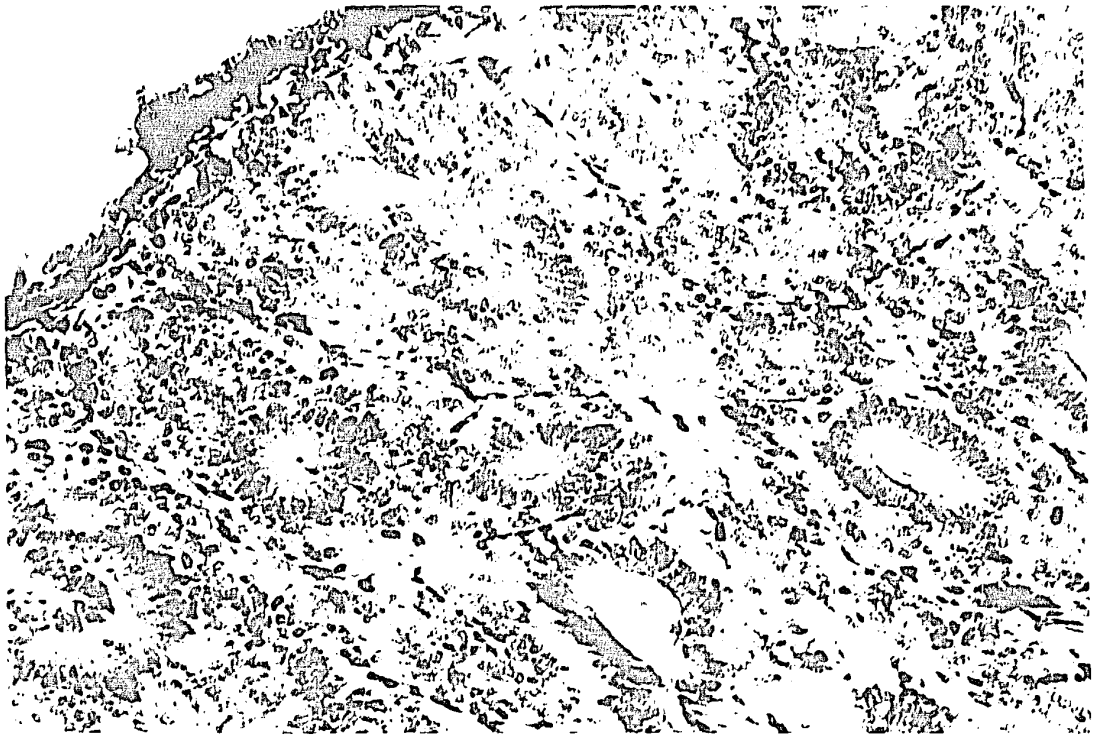


Figure 14. One month posthatching. X40.
(Haematoxylin-Eosin)
The cells are different from the cells of the prehatching stages. Note too the lymph cells of the node.

Figure 15. Four months posthatching. X100.
(Haematoxylin-Eosin)
Only small lymph area remains.

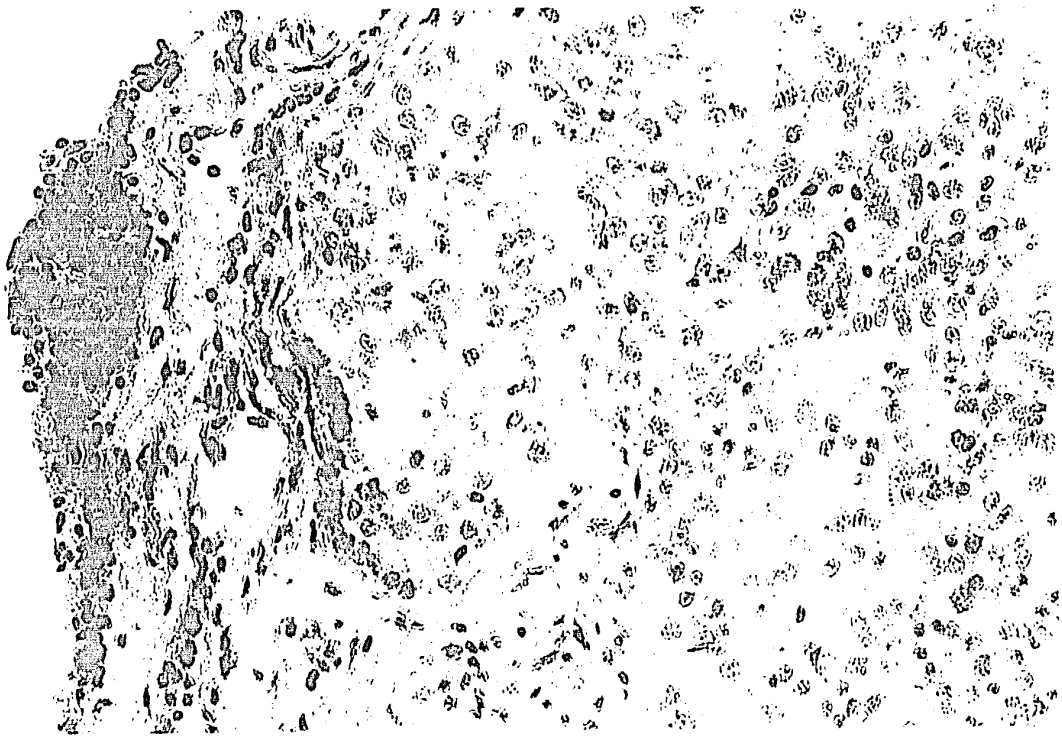


Figure 16. Day 5. X10. (Sudan Black B)
The pineal gland on top of the diencephalon (arrow).

Figure 17. Day 10. X40. (Toluidene Blue O)
Note separated vesicles and well developed capsule.

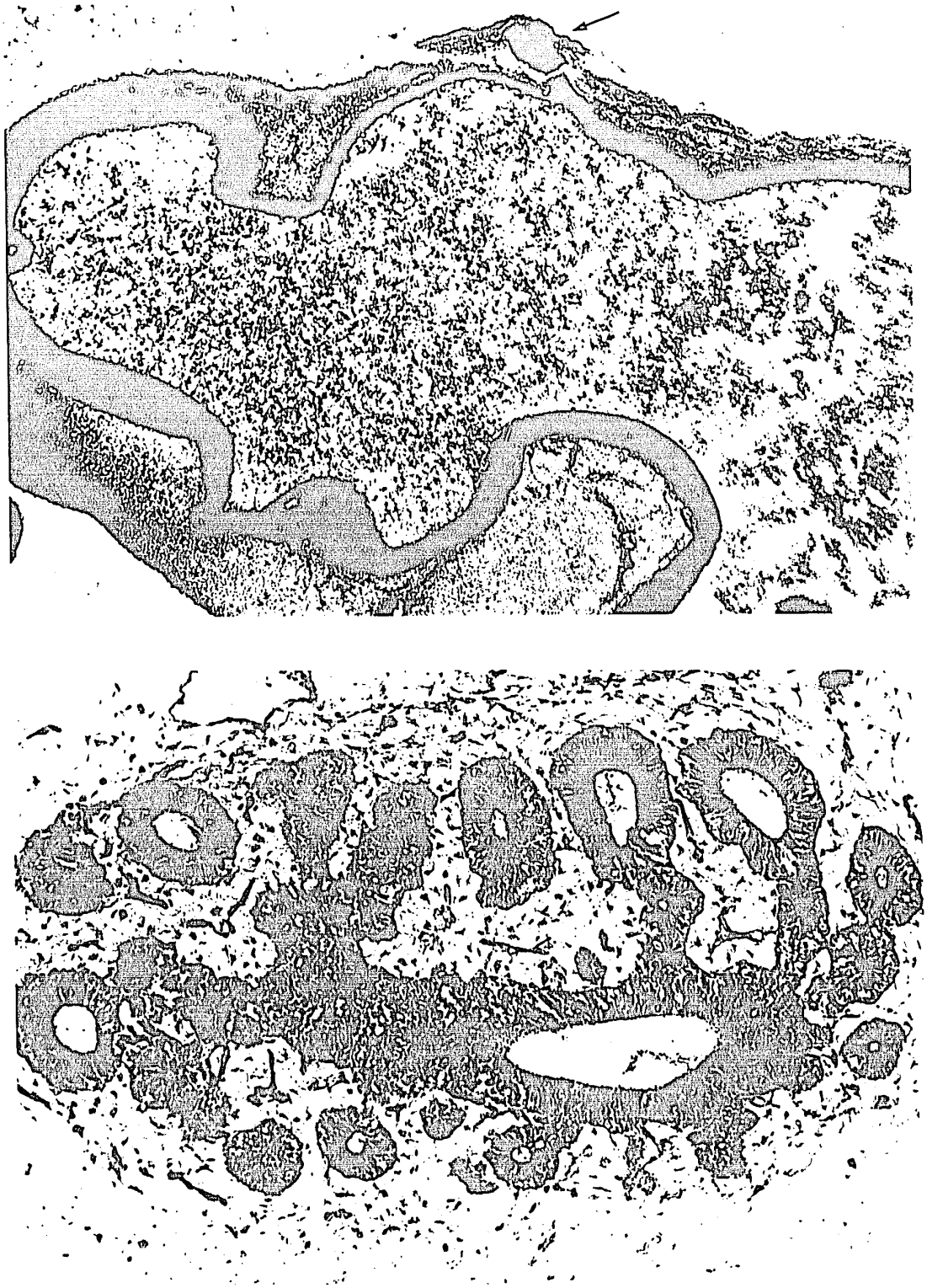
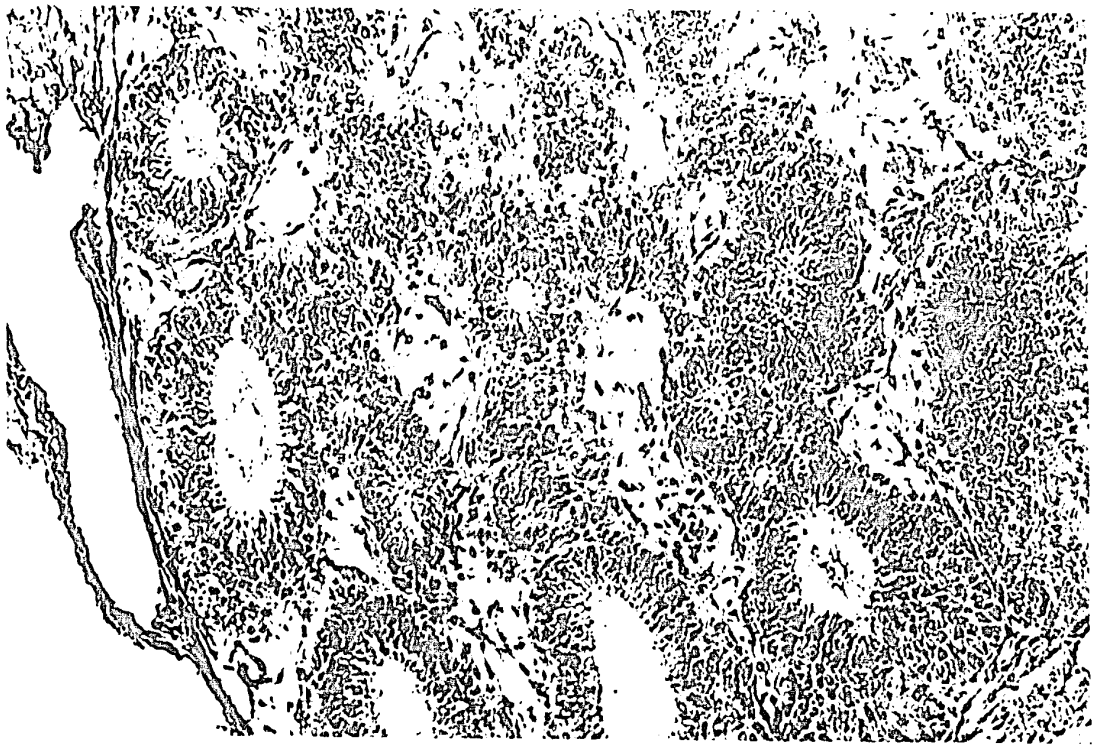


Figure 18. Day 14. X50. (Periodic acid-Schiff)
Apical Cytoplasm secretory product in lumina are PAS-positive. There are still large areas of connective tissue with the gland.

Figure 19. One day posthatching. X16.
(Gomori Alkaline phosphatase)
Note small number of vesicles and that they are empty.



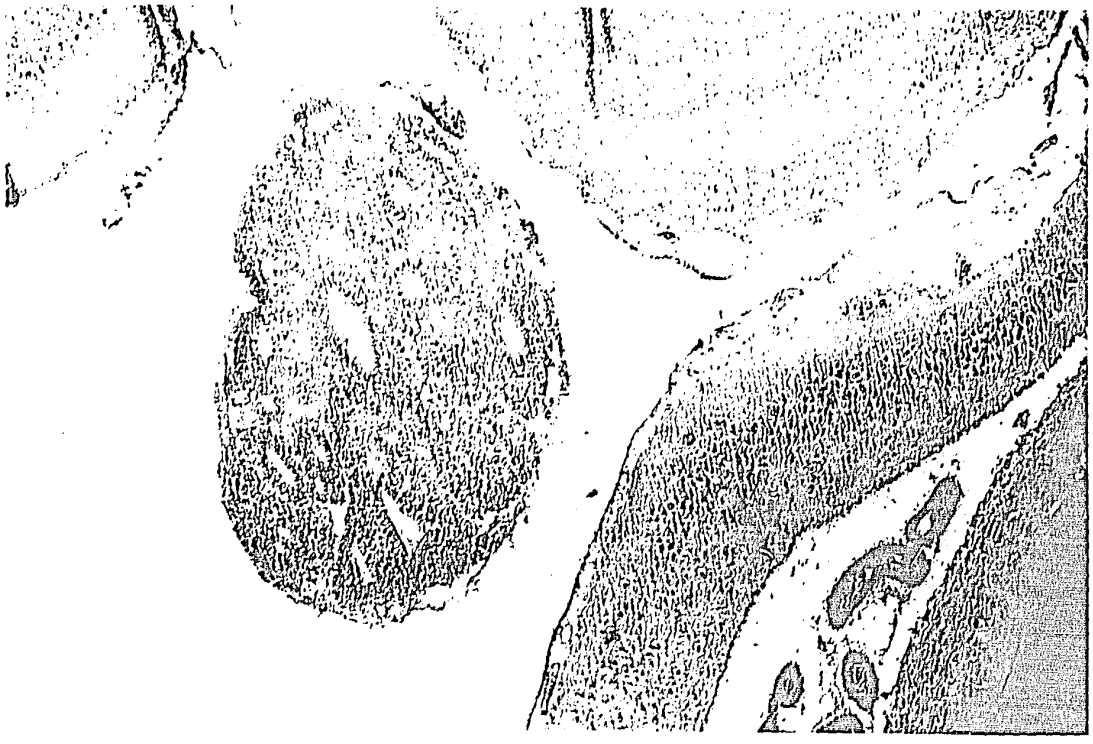


Figure 20. One week posthatching. X100.
(Iron haematoxylin)
Cell debris is found in lumina vesicles are distorted. Connective tissue stroma are invading the gland.

Figure 21. One year posthatching. X16.
(Haematoxylin-Eosin)
Large amounts of connective tissue have invaded and changed the shape of the gland.

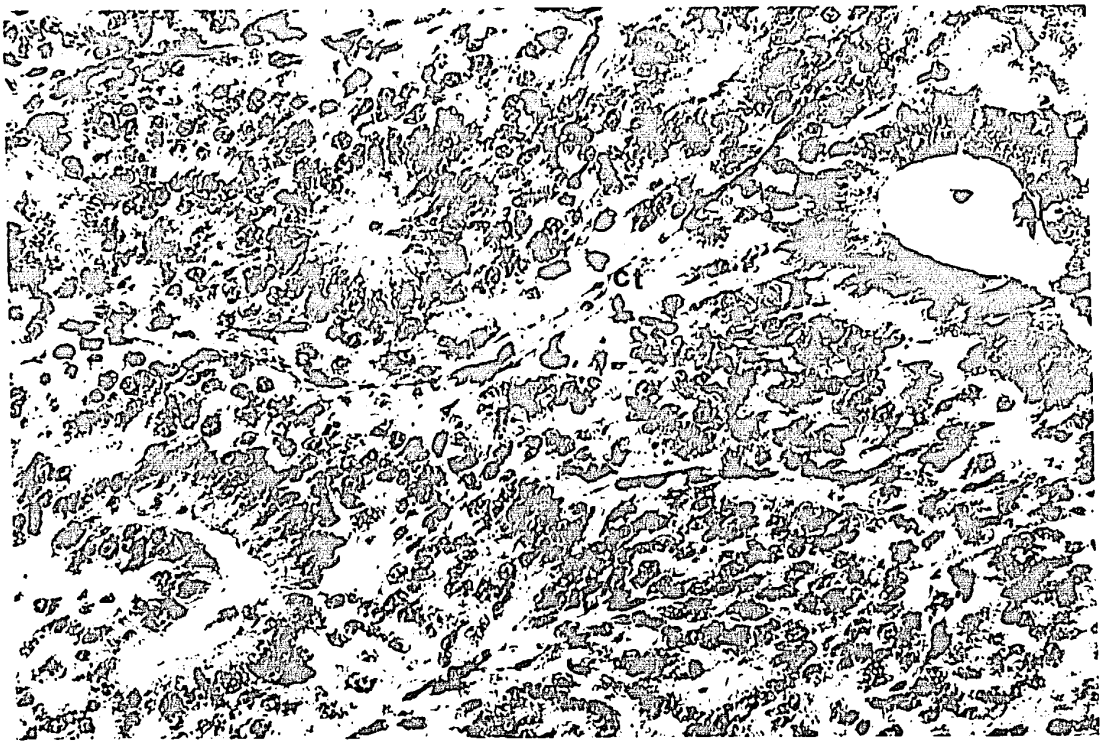

Cf

Figure 22. One year posthatching. X160.
(Aldehyde-fuchsin)
An enlargement of Figure 21. Connective tissue and large blood vessels in gland tissue.

Figure 23. One month posthatching. X125
(Haematoxylin-Eosin)
A large lymph node is seen at the posterior end of the gland. The glandular structure is now solid, not vesicular.

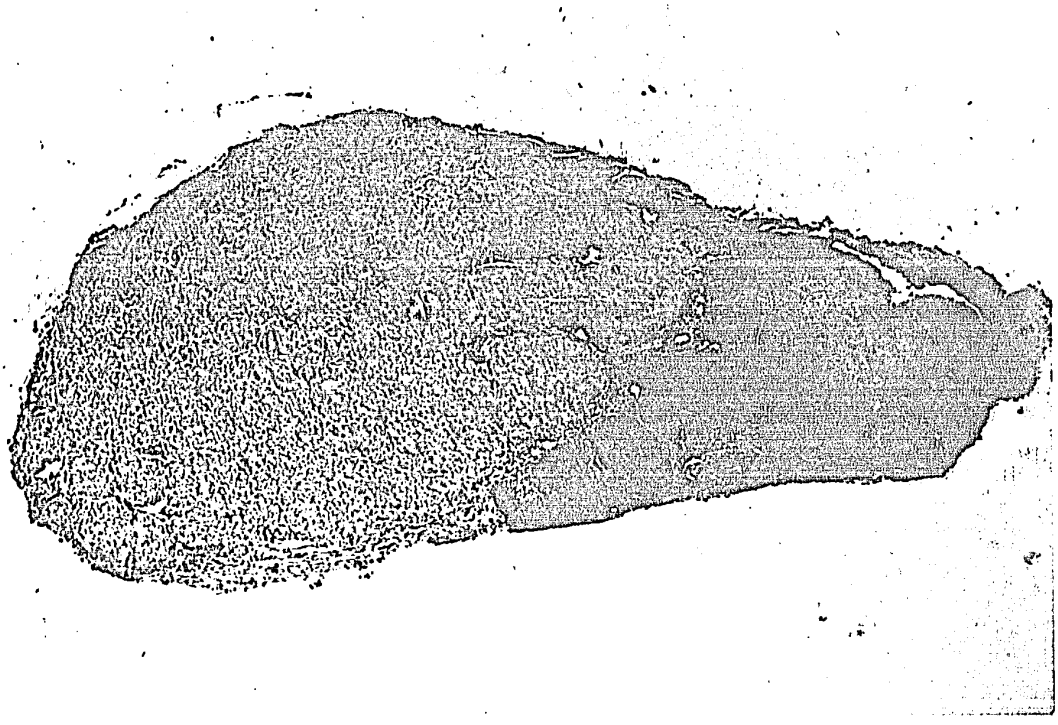
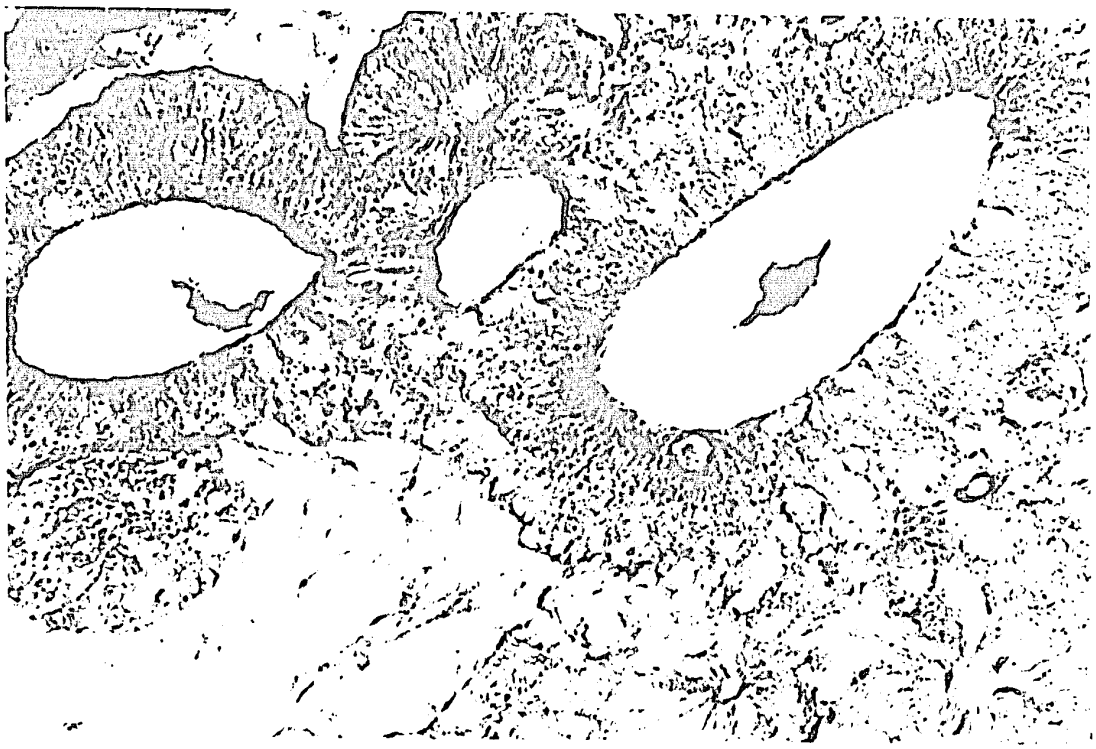


Figure 24. Day 10. X125. (Toluidene blue 0)
Metachromasia of the secretory product.
The apical border has also stained metachromatically.

Figure 25. Day 17. X160. (Haematoxylin-eosin)
Notice the three distinct cell types.



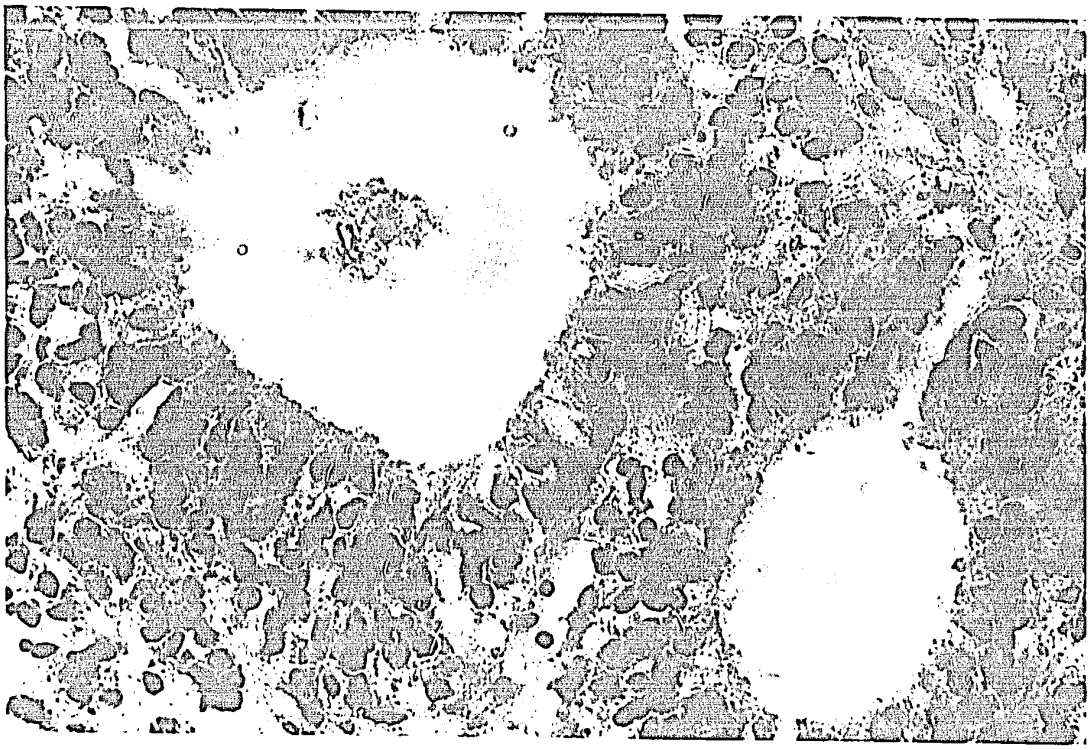
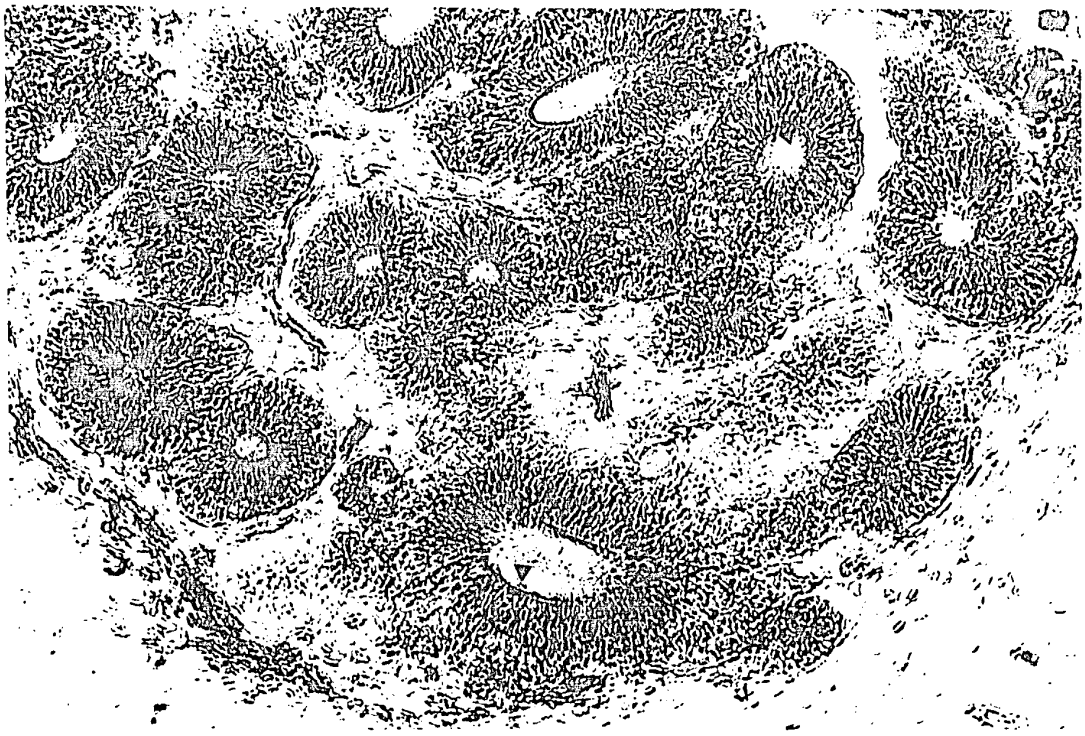


Figure 26. Day 14. X40. (Periodic acid-Schiff)
Apical border is stained.

Figure 27. Day 17. X50. (Alcian blue)
The lumeninal contents are positive and a
dark apical border is seen.



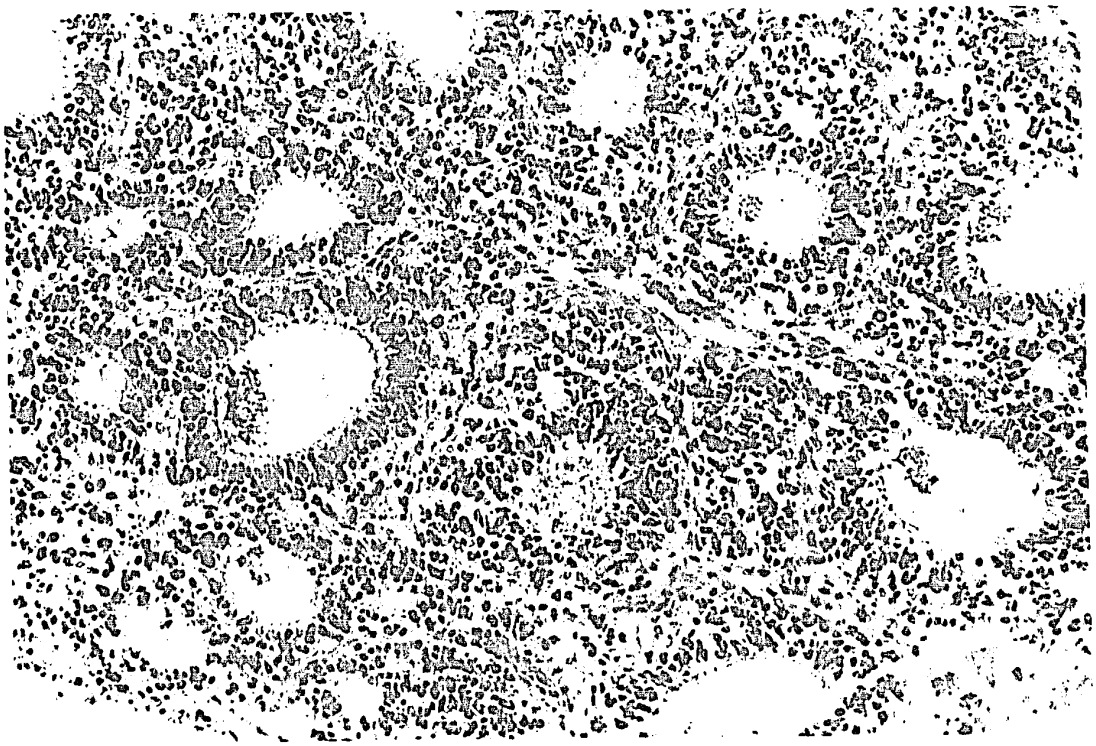
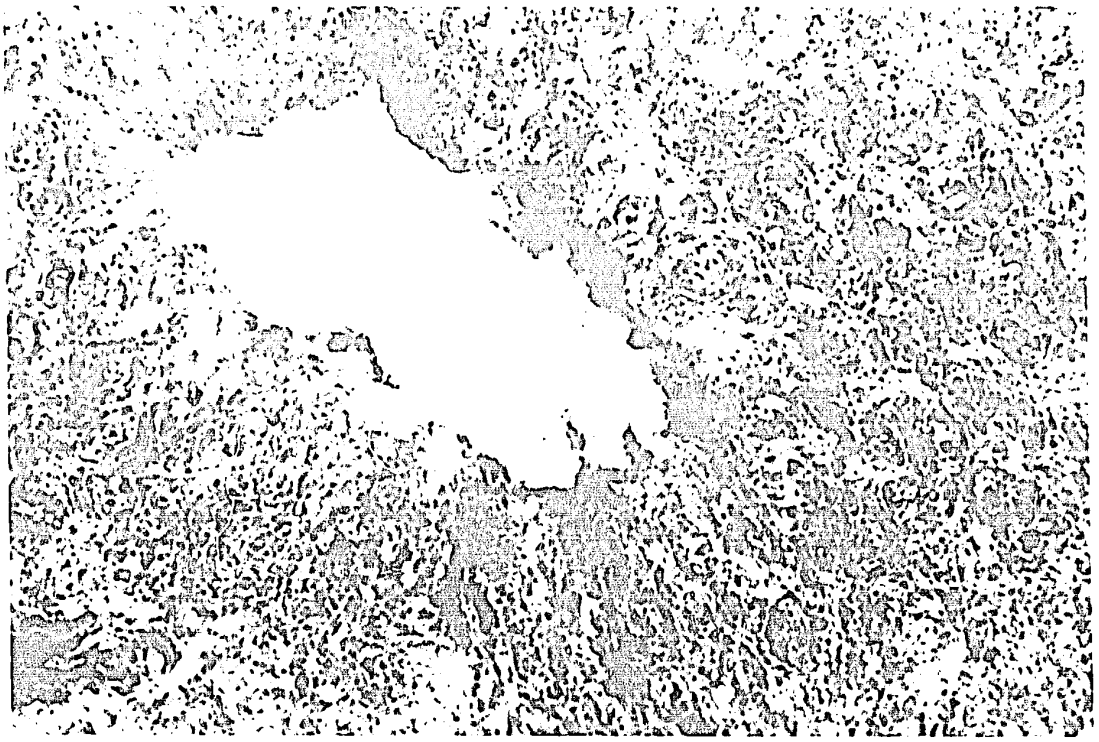


Figure 28. Day 15. X400 and oil emmersion. (Aldehyde-fuchsin)
Note distinctly darker and uneven luminal border.

Figure 29. Day 16. X40. (Gomori's alkaline phosphatase)
Gland is compacting at one end and vesicles are being pushed close together.



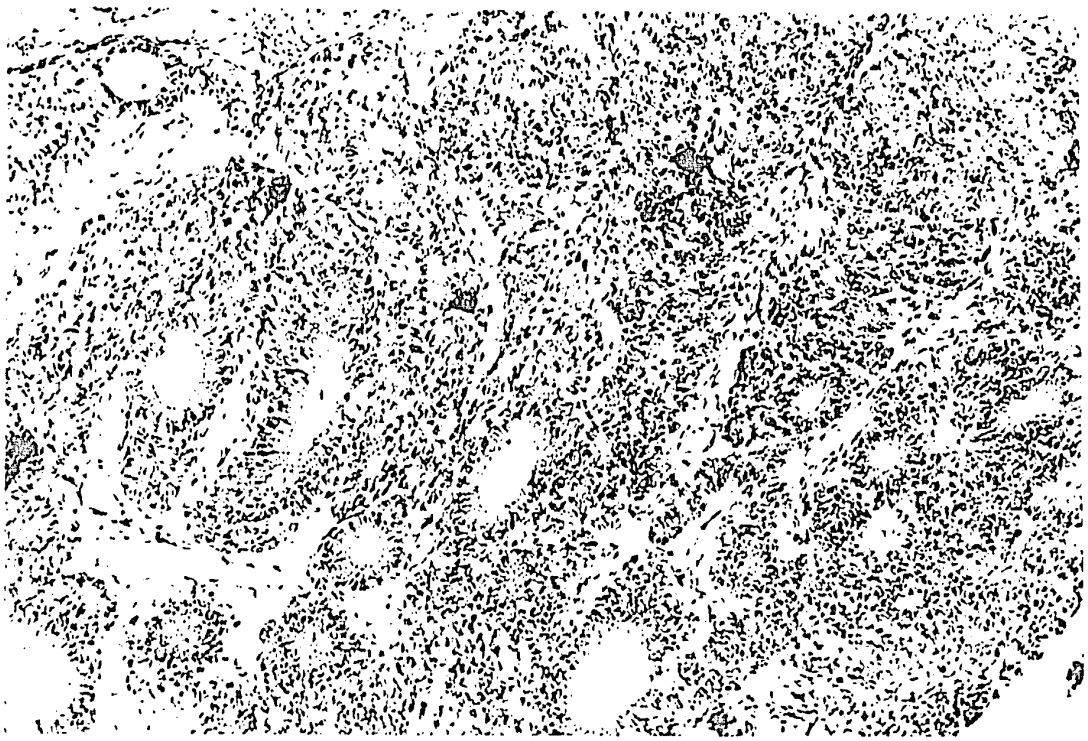


Figure 30. Day 15. X63. (Toluidene blue 0)
Metachromasia is seen in the lumen and in the apical cytoplasm.

Figure 31. Day 19. X63. (Periodic acid-Schiff)
Positive-staining secretion in centre of one lumen. This is the only case where secretion was found at this stage. Note the compacting of the gland and the positive reaction in the connective tissue.

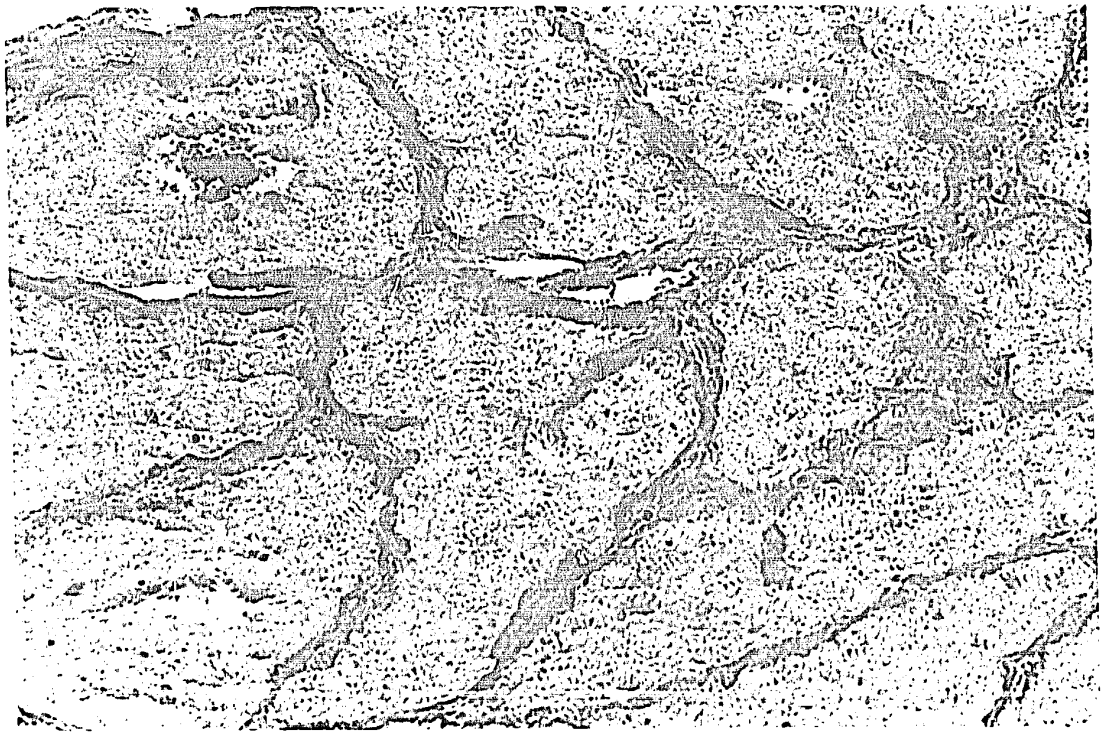
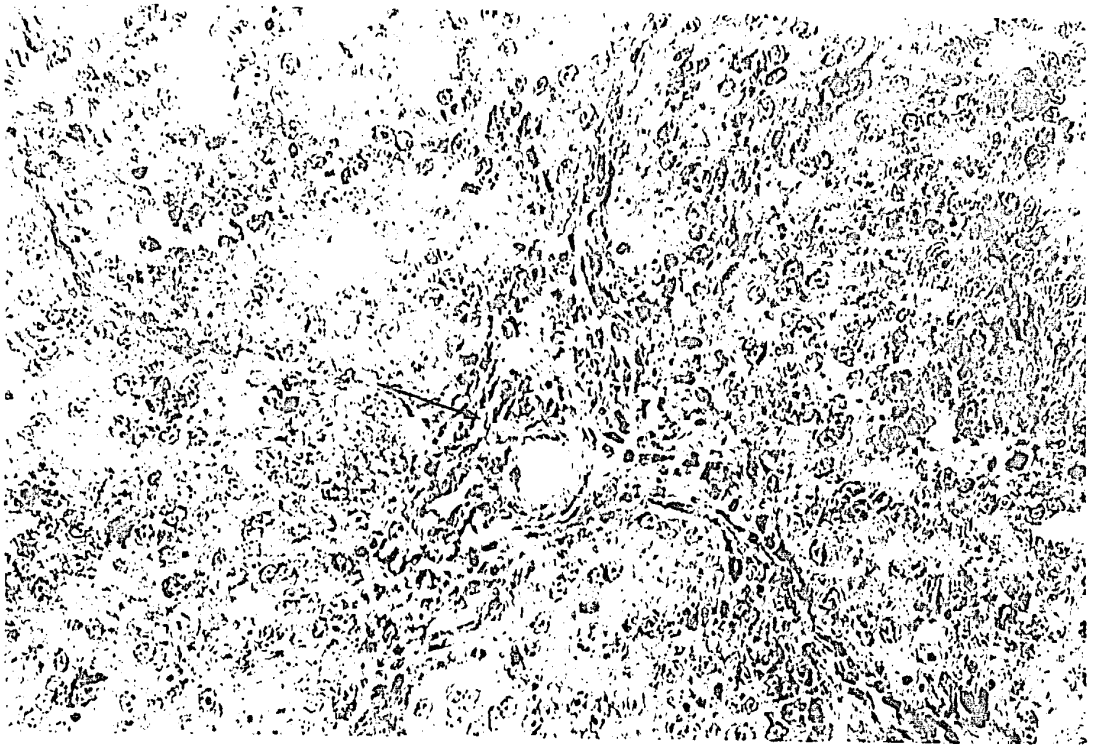


Figure 32. Four months posthatching. X100.
(Aldehyde-fuchsin)
Connective tissue invades the gland bringing large blood vessels.

Figure 33. Day 11. X16. (Methyl green-pyronin Y)
The number of vesicles has greatly increased.



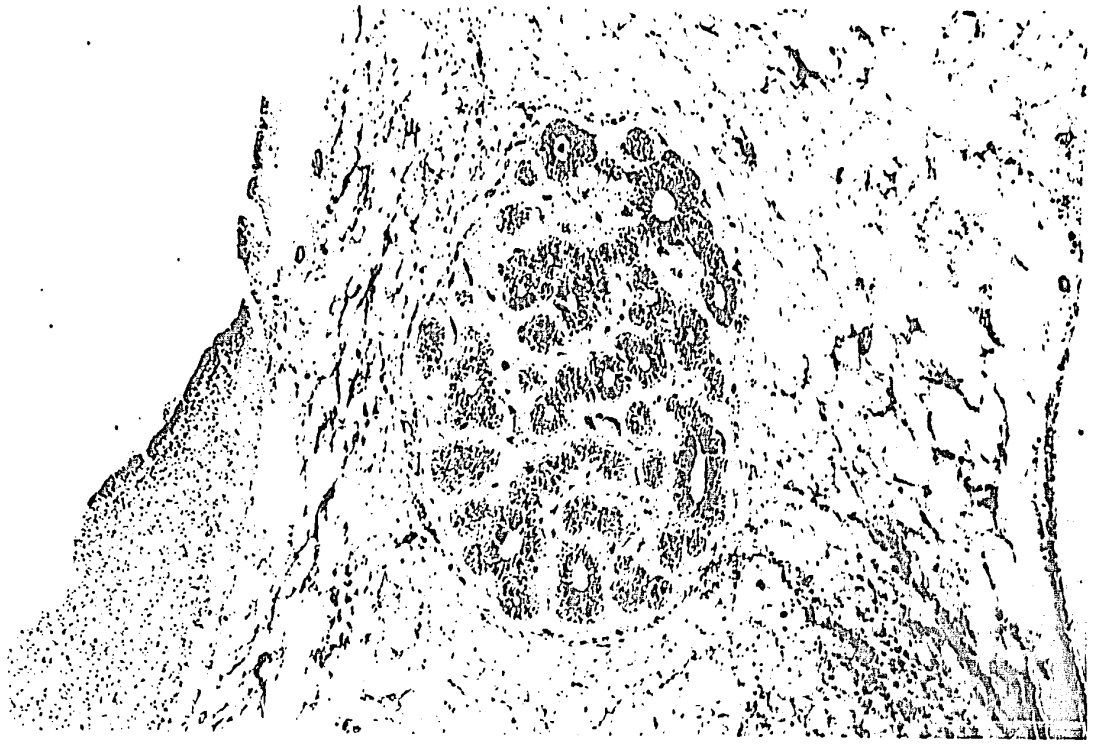


Figure 34. Apical cytoplasm has reacted positively with Best's carmine indicating the presence of glycogen.