ULTRASTRUCTURAL AND CYTOCHEMICAL ANALYSIS OF THE DIFFERENTIATION OF OVARIAN FOLLICULAR CELLS: A Comparative and Embryological Study

by

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A Thesis

Submitted to the School of Graduate Studies

As Partial Fulfilment

for the degree

of

Doctor of Philosophy

Department of Histology and Embryology

University of Ottawa

1973

ACKNOWLEDGMENTS

My sincere thanks are due to Dr. L.F. Bélanger for providing me the necessary laboratory facilities and generously providing with scientific guidance during the course of present work.

I am greatly indebted to my supervisor, Dr. Roberto Narbaitz, for his advice, encouragement and helpful criticism without which this work would not have been possible.

My thanks are also due to Dr. S.S. Jande for his constant help and advice in technical electron microscopical problems. My stay in the Department of Histology and Embryology has been a pleasant and memorable one and this was so, in great measure, due to the kind help and collaboration of the entire staff. Special thanks are due to Mrs. Cécile Bélanger and Mrs. Andrée Boucher for their valuable help and guidance in the histological and histochemical aspects of the work and to the secretary, Mrs. Gail Saunders for solving the administrative problems faced during my stay.

I wish to thank Dr. L.M. Aitkin, Department of Physiology, Monash University, Australia for providing the echidna ovaries and to Miss Christiane Lévesque for typing the manuscript.

Financial assistance of the Medical Research Council of Canada through grant # MA 3872 to Dr. Narbaitz and # MT 799 to Dr. Bélanger , is greatfully acknowledged.

CONTENTS

INTRODUCTION

The present thesis contains the results of various pieces of research. All of these investigations were carried out, however, following one same motive of interest.

1. The relationship between follicular cells and oocytes has occupied the attention of cytologists for many years. In the last few years the electron microscopical study of ovaries from the hen has uncovered a new aspect of interest: the presence of special organelles ('transosomes' or 'lining bodies') which, formed by the follicular cells, would be transferred to the oocytes during oogenesis. Since these organelles appear to be related to yolk formation, the possibility of their being found in other vertebrates laying yolk-rich eggs was considered. A review of the literature showed that they had not been found in the species so far studied. The fine structure of the ovaries from two important groups of vertebrates laying heavy-yolked eggs had not been, however, studied at all (Chelonia and Monotremata). The present thesis includes an electron microscopical study of the growing follicles of a turtle (Pseudemys scripta elegans) and an echidna (Tachyglossus aculeatus).

2. The presence of RNA in the granules of hen

'lining bodies' has been suggested but not proved. The demonstration of this fact is of interest since the presence of RNA would suggest an instructional role of these organelles. As a contribution to the solution of this problem the present thesis includes the results of an electron microscopical study of hen ovaries treated with ribonuclease.

3. Since 'lining bodies' had been shown to exist already at the time of hatching, an electron microscopical study of embryonic chick gonads was carried out in order to establish the age of their appearance. This study showed that the 'lining bodies' were present before initiation of gonadal differentiation and thus, constituted an excellent marker of prospective follicular cells. In addition to attaining its original objective, this aspect of present study supplied important findings relevant to the general problem of gonadal differentiation and in so doing created additional questions. Some of these questions I attained to solve through the electron microscopical study of gonads obtained from hormone-treated embryos or maintained for some time in organotypic culture.

A - FOLLICULAR CELLS AND THEIR RELATIONSHIP WITH OOCYTES IN DIFFERENT VERTEBRATE GROUPS.

In the ovaries of all vertebrates there is a close relationship between follicular cells and oocytes since all the raw materials from the blood must pass through the follicular epithelium in order to reach the oocyte. The follicular epithelium varies in thickness in different vertebrate groups and may be constituted by one or several layers of follicular cells. Although several mechanisms of transport of extrafollicular materials to the oocyte are common to all vertebrate species, there are others which seem to be characteristic of particular groups. Thus, these variations will be reviewed separately for each group.

1. Pisces :

Previous studies on cyclostomes (Lewis and McMillan, 1965; Busson-Mabillot, 1966) and fishes (Jollie and Jollie, 1964; Hurley and Fisher, 1966; Droller and Roth, 1966; Hirose, 1972) have shown that the follicular epithelium in this group is constituted by a single layer of flat follicular cells. The transport of nutritive materials between the follicular cells and the oocyte is facilitated by the presence of microvilli from both types of cells. These microvilli form a complex structure referred to as 'zona radiata' by several authors (Flügel, 1964a; Droller and

Roth, 1966; Hurley and Fisher, 1966; Hirose, 1972). The zona pellucida in this group is organized into several layers(Flugel, 1964b; Hurley and Fisher, 1964; Anderson, 1967) and in somes cases it reaches a very complex organization (Gotting, 1965). Pore canals, which contain oocyte microvilli, have been described in the zona pellucida by these authors.

Droller and Roth (1966) in their ultrastructural studies on the ovarian follicles of the guppy did not find any desmosomes in the follicular epithelium and suggested that nutritive materials may reach the surface of the oocyte crossing the spaces between the follicular cells. The observations of these authors are, however, in contradiction with those of Jollie and Jollie (1964) who found distinct desmosomes interconnecting the follicular cells. Lewis and McMillan (1965) believe that in the sea lamprey the basophilic nurse cells in the follicular epithelium are incorporated into the oocyte. The transfer of organelles from the follicular cells to the oocyte has not been described in this group.

2. Amphibia :

The follicular epithelium in amphibians is also single layered and is constituted by flat cells.Several ultrastructural studies are available on the follicular cell-oocyte relationship in the growing follicles of

amphibians (Wartenberg, 1962,1964; Wischnitzer, 1963,1964; Hope et al.,1963; Kessel and Panje, 1968; Joly and Picheral, 1972). These studies have shown that whereas the plasma membranes of both the follicular cells and oocytes lie closely apposed in the smaller follicles; large number of microvilli, corresponding to the zona radiata of light microscopists, appear in the larger follicles. Thick microvilli arising from the follicular cells penetrate deep into the oocyte cytoplasm (Hope et al., 1963; Wischnitzer, 1963).

Large intercellular spaces between the follicular cells have been reported by Hope et al. (1963), Wartenberg (1964) and Kessel and Panje (1968). These authors suggested that these spaces represented an extracellular route for the passage of nutritive materials to the surface of the oocyte.

The zona pellucida in amphibians is less complex than the one found in fishes. However, two morphologically and histochemically distinct zones can be recognized in it (Wartenberg, 1962; Hope et al., 1963). On this basis, the above mentioned authors suggested a follicular cell and oocyte origin of the material forming the external and internal layer of the zona pellucida respectively.

The ultrastructural changes occuring in the follicular cells during oogenesis were described by Kessel

and Panje (1968). Their study as well as all other available ultrastructural studies on amphibian follicles have failed to show the transfer of any organelles from the follicular cells to the oocyte.

3. Reptilia :

Unlike most other sub-mammalian vertebrates the follicular epithelium in lizards and snakes is multilayered at certain stages of oogenesis and is constituted by morphologically different types of cells (Porte and Zahnd, 1968; Betz, 1968; Hubert, 1971a,b): these are the small, medium and large (pyriform) follicular cells. The pyriform cells are derived from the smaller cells (Hubert, 1971a). Differing from lizards and snakes, the follicles from chelonians have a unistratified epithelium and only one type of cells has been described in them (Munson, 1904; Thing, 1918; Altland, 1951).

The ultrastructural aspects of follicular cell-oocyte relationship in reptiles have received little attention. All the available studies at the submicroscopical level in this group are restricted to the analysis of growing follicles in lizards (Ghiara et al. 1968, 1970; Hubert, 1971a,b; Neaves, 1971,1972; Taddei, 1972). Several characteristics in the transport of materials, through the follicular envelope to the developing oocyte appear exclusively in lizards. Thus, true intercellular bridges occur

between the pyriform follicular cells and the oocytes (Ghiara et al., 1968; Hubert, 1971b; Neaves, 1971; Taddei, 1972). The presence of bridges between follicular cells and oocytes has not been described in other vertebrate groups. Ghiara et al. (1968) observed conspicuous bundles of microtubules in the intercellular bridges. Neaves (1971) observed mitochondria, endoplasmic reticulum and Golgi complexes in the apical regions of the pyriform cells indicating a possible synthesis of materials by the follicular cells for transfer to the oocyte. Taddei (1972) has suggested that ribosomes are synthesized in the pyriform cells and then transferred to the oocyte through the cytoplasmic bridges. Pyriform cells regress and degenerate just before the onset of vitellogenesis (Ghiara et al., 1968; Hubert, 1971b; Neaves, 1971). Ghiara et al. (1968) state that the degenerating pyriform cells nourish the oocyte and have referred to them as 'nurse cells'.

Hubert (1971b) believes that the degeneration of pyriform cells leaves large spaces in the follicular epithelium allowing the dark granules present in the theca to pass directly to the perivitelline space, without passing through the follicular cells. Absence of sealing junctions between the follicular cells has also been shown by Neaves (1972) who, by injecting extracellular tracers in the lizard, demonstrated that nutrients may pass through

these large intercellular spaces and reach the oocyte directly.

Ultrastructural studies on lizard ovaries have failed to show the transfer of any organelles from the follicular cells to the oocyte other than those crossing through intercellular bridges.

Similar studies on the ovarian follicles of chelonia are not available in the literature.

4. Aves :

In this group, all the ultrastructural studies related to the growth of the oocytes and to the specializations of the follicular cells in the adult, have been exclusively carried out in the domestic hen.

During oogenesis the size of the oocyte increases from a few microns to about 3.5 cm in diameter. This tremendous increase takes place due to the accumulation of yolk. It has been shown that the yolk precursors are synthesized in the liver and transported through blood to the ovary (see review by Gilbert, 1967). The follicular cells play, however, an important role in yolk deposition into the oocyte. The oocytes of about 20 μ diameter are already surrounded by a layer of flat follicular cells (Greenfield, 1966) and when the oocyte is approximately 1 mm in diameter, short processes extend from the follicular cells into the intercellular space (Bellairs, 1965). In

follicles of 2 mm diameter, a space appears between the oocyte and the follicular cells and is traversed by microvilli from both types of cells (Wyburn et al., 1965a; Bellairs, 1967). The materials from the perivitelline space are taken up by the oocyte through a process of pinocytosis (Bellairs, 1965, 1967; Wyburn et al., 1965a).

Specialized organelles present at the plasma membrane of the follicular cells have been described by several authors. These organelles have been given different names by various authors: 'premitochondria' (Schjeide and McCandless, 1962), 'ring shaped structures' (Ahmen, 1963), 'transosomes' (Press, 1964), 'lining bodies' (Bellairs, 1964, 1965; Paulson and Rosenberg, 1972), 'terminal membranes' (Wyburn et al., 1965a, b) and 'annular desmosomes' (Dahl, 1971).

Schjeide and McCandless (1962) thought that these organelles were involved in the formation of mitochondria and thus called these structures 'premitochondria'. Schjeide et al. (1963a) observed some of these 'premitochondria' included in the yolk spheres in the oocyte and concluded that they may participate in the partial synthesis or organization of yolk proteins. These authors further suggested that the increase in the number of mitochondria in the oocyte was in part due to the transfer of these organelles from the follicular cells to the oocyte.

Ahmen (1963) described 'ring shaped structures' constituted by two membranes and studded with granules 150-250 Å in diameter. She found these structures distributed all over the follicular cells but did not mention if these organelles were incorporated into the oocyte.

Press (1964) suggested the term 'transosomes' to designate these organelles since he observed them passing across the vitelline membrane. According to this author, however, 'transosomes' are never released from the follicular cells: he believes that the vesicles containing these organelles, which were observed by Schjeide et al. (1963a) in the periphery of the oocyte, are oblique sections of terminal evaginations of follicular cells. Press (1964) based his conclusions on the absence of these organelles in the deeper layers of the oocyte. As to the functions of 'transosomes' Press (1964) postulated that they initiate the growth of the follicular cell evaginations since he always observed them at the tip of the extensions. He further suggested that these organelles may also direct the growth of the endoplasmic reticular membranes in the oocyte.

Wyburn et al. (1965a,b) observed the same structures previously described as 'transosomes'. These authors agreed that these organelles remain attached permanently to the plasma membrane of the follicular cells but suggested that they constitute a device which increases the

membrane permeability. They called them 'terminal membranes' because they are always situated at the tip of the projections of follicular cells into the oocyte.

The most descriptive term and which seems most appropriate was given by Bellairs (1964,1965): she named these organelles 'lining bodies' since they appear underlying the plasma membrane and follow its contour. Lining bodies are found between adjacent follicular cells as well as between the follicular cells and the oocytes. Several authors using light microscopic techniques have reported that osmiophilic organelles which were interpreted as Golgi bodies (Brambell, 1926; Guraya, 1957) or mitochondria (Varma, 1954) are transferred from the follicular cells to the oocyte. Bellairs (1965) identified those structures, at the ultrastructural level, as 'lining bodies' and postulated that the engulfment of 'lining bodies' by the oocyte represents a mechanism of delivery of raw materials by the follicular cells.

Schjeide et al. (1966) agree with Bellairs (1965) in that the 'transosomes' are incorporated into the oocyte. They drew an analogy between the close relationship of these organelles with the yolk granules to the intimate relationship of mitochondria and yolk granules as found in amphibians and reptiles by other authors.

Although there are divergencies in the description

of these specialized organelles by different authors it is evident that 'transosomes', 'terminal membranes' or 'lining bodies' do correspond to the same type of organelle. I shall use with preference the term 'lining bodies' in subsequent pages. Different interpretations have also been made concerning the origin and thickness of the membranes and granules of the 'lining bodies'. For a review of these descriptions see Bellairs (1965) and Paulson and Rosenberg (1972).

There are several aspects concerning 'lining bodies' which have not been clarified in the past:

a) The latest works agree in that 'lining bodies' are incorporated into the oocyte (Bellairs, 1964,1965; Schjeide et al., 1966; Paulson and Rosenberg, 1972). Thereafter, the fate of these organelles is not clearly understood.

b) The origin and fate of the different membranes, which constitute the lining bodies, remains controversial.

c) The mechanism of formation of 'lining bodies' at the plasma membrane of follicular cells as well as the origin of the dark granules attached to their inner membrane have been debated.

and d) The chemical nature of these organelles is not known. In the present study an attempt was made to try to solve some of these questions.

5. Mammalia :

The follicular epithelium in marsupials and placental mammals changes greatly with the growth and differentiation of the oocyte. In the primary follicles the follicular epithelium is constituted by a single layer of flat follicular cells which becomes cuboidal and multilayered in the secondary and tertiary follicles (see review by Brambell, 1956). In monotremes, however, the follicular epithelium remains single-layered throughout the development of the oocyte (as in platypus, Garde, 1930) or may become two cell deep in late stages of development of the oocyte (as in echidna, Flynn and Hill, 1939).

Apart from being the intermediary between the oocyte and the general circulation, the follicular cells in mammals appear to be related to the production of liquor folliculi and, at certain stages differentiate to produce steroid hormones.

Important ultrastructural studies on the relationship between follicular cells and growing oocytes have been done in eutherian species. Among them I shall mention those in the rat (Franchi, 1960; Odor, 1969; Bjorkman, 1962), rabbit (Trujillo-Cenoz and Sotelo, 1959; Zamboni and Mastroianni, 1966), guinea-pig (Anderson and Beams, 1960; Adams and Hertig, 1964), hamster (Weakley, 1966), rhesus (Hope, 1965) and man (Baca and Zamboni, 1967). These studies have

supplied details on the morphological expressions of transfer of substances from the follicular cells to the oocyte; the transfer of whole organelles, however, has not been reported in these species.

Unique among mammals, in the monotremes, large amounts of yolk are laid down in the oocytes and the size of the ovulated egg reaches well over 3.5 mm in diameter (Flynn and Hill, 1939). Ultrastructural studies on the follicles of this last group are completely lacking.

B - THE DIFFERENTIATION OF FOLLICULAR CELLS IN THE CHICK OVARY.

Follicles appear in the chick ovary on the fourth day after hatching (Greenfield, 1966). It would thus seem logical to begin analyzing the degree of differentiation of the follicular cells at this age. However, as shown by Greenfield (1966) and Narbaitz (1971) several aspects of differentiation of these cells occur even before that time. In addition, the origin of the cells which will eventually form the follicular cells can be traced back to the coelomic epithelium already present in the primitive undifferentiated gonad. It is thus appropriate to review the literature on the differentiation of the gonad beginning at the early stages of development.

1. Normal development of the gonad :

The gonadal primordia in the chick embryo can be first recognized on the third day of incubation and appear as a pair of ridges on the median surface of the mesonephri (Swift, 1915; Bishop-Calame, 1966). Each gonad at this age is constituted by a layer of coelomic epithelium, which is referred to as the germinal epithelium or cortex, overlying a dense mass of mesenchymal tissue known as the medulla. The germinal epithelium consists of a large number of epithelial cells which are cuboidal or columnar in shape.

Distributed among these, are the large primordial germ cells (p.g.c.). It has been shown that the p.g.c. have an extragonadal origin and are derived from the anterior germinal crescent (Swift, 1915; Goldsmith, 1935; Meyer, 1964; Clawson and Domm, 1969; Dubois and Croisille, 1970). The p.g.c. penetrate actively into the vascular network during its formation (Dubois, 1969) and reach the genital ridge colonizing progressively the band of coelomic epithelium between the 22nd and 28th somites (Bishop-Calame, 1966; Dubois and Croisille, 1970).

By the 4th and 5th day of incubation the gonads are recognizable macroscopically. This great increase in volume is attributed to the development of the stroma (Swift, 1915; Bishop-Calame, 1966). On the fifth day of incubation the germinal epithelium appears multilayered and contains numerous p.g.c. A further differentiation of the genital ridge is marked by the formation of sexual cords. These cords arise from the coelomic epithelium and begin to appear by the middle of the 5th day of incubation (Swift, 1915,1916). The cords continue to form until about the 7th day. This sex cord formation is accomplished by a localized multiplication of the cells of the germinal epithelium and Swift (1915) observed that the basement membrane of the germinal epithelium was at first continuous around the budding cord. Around 6½-days the cords begin to

separate from the epithelium and shortly after the formation of cords of first proliferation or primary sex cords ceases (Swift, 1916).

Histological sex differentiation occurs on the seventh day (Swift, 1915) and is completed on the eighth day. Thus, in the genetically male embryos the germinal epithelium becomes flat remaining somewhat thicker in the left than in the right gonads until the 11th day (Laulanie, 1886). The primary sex cord in males persist forming the seminiferous tubules.

In genetically female embryos after the 6th day of incubation there is a great difference between the development of left and right gonads. Whereas the left cortex continues to grow and form secondary sex cords (Pfluger's cords), no such proliferation occurs on the right side (Willier, 1939). The formation of secondary sex cords in the left ovaries is preceded by a multiplication of the germ cells which in the germinal epithelium, transform into oogonia (Swift, 1915). Before the time of hatching all oogonia stop dividing mitotically and enter meiosis thus becomming oocytes (Hughes, 1963). During this period, the epithelial cells of the cortex continue to surround the oogonia or oocytes: after hatching they will transform into follicular cells. The primary sex cords in the female disappear as such; while portions of them acquire a lumen

and form the medullary lacunae (Brode, 1928; Willier, 1939), other portions form islands of vacuolated cells which are known to be estrogen producers (medullary interstitial cells). Germ cells in the primary sex cords persist for some time. While these changes occur in the left ovary, in the right one the cortex is reduced to a thin layer of epithelial cells (although occasional islands of thick cortex may also be present, Wolff and Pinot, 1961). The medullary cords, however, follow the same evolution as those in the left ovaries.

2. Contributions from experimental embryology :

Our understanding of the mechanisms of gonadal differentiation has been increased by the contributions of experimental embryologists. The hormonal theory of sex differentiation enunciated by Lillie (1916,1917) for mammals and extended to amphibians by Burns (1925) and Witschi (1927) was later confirmed also for birds. Thus, several works appeared simultaneously describing the sex reversal produced by injections of estrogenic hormones in the chick embryo (Wolff and Ginglinger, 1935; Wollf, 1936; Willier et al.,1935,1937; Dantchakoff, 1935,1936).All of these studies showed that the injections of estrogens to genetically male chick embryos before the 7th day produced characteristic changes: while the left gonad is transformed into

an ovary or an ovotestis resulting from the proliferation of its cortex and the formation of lacunae in the medulla, the right gonad becomes atrophic.

The results obtained using female hormones were later complemented by other experiments consisting in grafting gonads from 6-11 day old embryos into undifferentiated hosts of around 50 hours of incubation. Ovarian grafts transformed the male hosts into intersexes by producing the transformation of the left gonad into an ovary or an ovotestis; the right gonad remained usually slender or atrophic (Wollf, 1947). These results were later confirmed by Huijbers (1951) who grafted several ovaries onto the chorioallantoic membrane of a host embryo to produce complete intersexuality.

These results by showing that grafted embryonic gonads could imitate the action of pure estrogenic hormones suggested that embryonic ovaries do secrete estrogens. The fact that estrogens are secreted by the embryonic ovary was much later confirmed by biochemical techniques (Gallien and LeFoulgoc, 1957; Weniger and Zeiss, 1971).

Mintz and Wolff (1952,1954) repeated the previously mentioned grafting experiments but using as a graft only the medullary tissue. Since feminization of the male hosts was also obtained in this case these authors concluded that the medulla was the site of production of female

hormone.

On the basis of these and other results, the following mechanism for sex differentiation in birds has been suggested (Wolff, 1950; also see reviews by Wolff, 1960, 1965): In genetically female birds the medullary zone produces estrogenic hormones which stimulates the proliferation of the cortex and hence the differentiation of an ovary; in genetically male embryos the lack of estrogenic stimulation allows the cortex to undergo atrophy while the sex cords remain and transform into seminiferous tubules.

Difficulties arise, however, when one tries to explain on this basis the behaviour of the right female gonads. Since Mintz and Wolff (1952,1954) have shown that the grafts of right gonads are capable of feminizing male embryos it is clear that the failure of the right gonads to differentiate cannot be attributed to lack of estrogenic stimulation.Wolff and Wolff (1948) showed that if female hormones are injected before the germinal epithelium has started to regress this epithelium can be stimulated to proliferate also in the right gonad. These results were later repeated and extended by Wolff and Pinot (1961).Thus, the failure of the right cortex to develop must be attributed either to an inherent deficient capacity of the right cortex to respond to estrogenic stimulation or to an inadequate timing between the beginning of estrogen secretion

and the end of the period of cortical sensitivity to such a stimulation (Wolff and Pinot, 1961).

Whichever the mechanisms responsible for the gonadal asymmetry may be, several arguments have been put forward to explain its original cause. Swift (1914), Witschi (1935), Venzke (1954) and Limborgh (1958, 1960, 1968) concluded from their studies on chick and duck embryos that it was due to an unequal distribution of primordial germ cells (p.g.c.): p.g.c. were, according to them, more numerous in the left than in the right gonad. Simon (1960a), however, while studying chick gonads from 25-32 somite stages did not find asymmetrical distribution. Stanley and Witschi (1940) believe that the p.g.c. are at first symmetrically distributed in the gonadal primordia but that later on, some of the germ cells move from the right to the left gonad. Limborgh (1957) disproved this hypothesis by dividing duck embryos medially, thus destroying all vascular and other connections between the left and the right side, and still finding asymmetrical distribution of germ cells.

The application of organ culture techniques has confirmed the results of in vivo experiments and has supplied additional information. Wolff and Haffen (1951, 1952a,b,c,d) successfully cultured gonads from 7-day old duck embryos in a hormone free medium consisting of an agar

solution in physiological saline and containing extract from chick embryos which were cultured for 9 days. They were able to obtain typical development of the gonads similar to the one occuring in'vivo'. The results were somewhat different with gonads from chick embryos. In this case the medullary zone of both the left and the right gonads did not form typical lacunae and the primary sex cords persisted as such (Wolff and Haffen, 1952c).

When chick gonads from different sexes were linked in culture, the female left or right gonads had a marked feminizing action on left genetically male gonads (Weniger, 1958a,b).

Perhaps the most important contribution of organ culture techniques has been to demonstrate that gonads can differentiate in a totally synthetic medium (Stenger-Haffen, 1957). This demonstrated beyond doubt that pituitary hormones play no role in sex differentiation as had been previously suggested by classical hypophysectomy experiments of Fugo (1940).

3. Cytological findings :

While classical experimental embryologists, on analyzing the gonadal differentiation, stressed the role of whole tissues such as the cortex and the medulla, the modern embryologists have realized that these are complex

entities and that the role of each cellular type should be studied in particular.

a) The primordial germ cells (p.g.c.) : Simon's experiments with in vitro parabiosis of chick blastoderms (Simon, 1960a) have finally confirmed that the p.g.c. originate in the germinal crescent situated extraembryonically and migrate to the gonad through the blood stream (Dubois, 1969). Dubois (1964, 1965, 1966) and Cuminge and Dubois (1967) have supplied evidence indicating that the germ cells are attracted to the germinal epithelium by a chemotactic phenomenon and that this is initiated by a merocrine secretion of exportable proteins at the germinal epithelium (Cuminge and Dubois, 1969a, 1971). Dubois and Cuminge (1967) analyzed the ultrastructure of the p.g.c. and did not find any contractile filaments or similar structures that would account for the movement of these cells. The above mentioned authors did not find any characteristic difference between the submicroscopical cytology of the p.g.c. in the anterior germinal crescent and that of p.g.c. localized in the gonad. According to Dubois and Cuminge (1968) the lipids which are present in large quantities in the p.g.c. persist in the spermatogonia at later ages but disappear from the oogonia.

Since gonads can differentiate in the absence of p.g.c. (Reynaud, 1969; Simon, 1960b) it appears that they

do not represent the initial stimulus for their differentiation. Genetically male germ cells can develop into oocytes if situated in the cortex of sex iverted gonads (Wolff and Ginglinger, 1935; Wolff, 1936; Haffen, 1965). Similarly, genetically female germ cells, develop into spermatogonia if located in the medulla of the masculinized right gonads in female pullets to which the left ovary has been removed (Domm, 1927). The above mentioned facts indicate that the differentiation of germ cells towards spermatogonia or oogonia depends on the influence of the tissues that surround them.

b) Steroid producing cells : The experiments by Mintz and Wolff (1952, 1954) indicated that estrogenic secretion was localized in the medullary zone of the ovaries. Histochemical techniques for lipids (Scheib, 1959; Narbaitz and Sabatini, 1963a,b) confirmed this by localizing the lipid rich interstitial cells. These results were also confirmed by Narbaitz and Kolodny (1964) and Chieffi et al. (1964) using a histochemical technique for 3- β-hydroxysteroid dehydrogenase. The electronmicroscopical characteristics of steroid producing cells in the chick embryonic gonad were later analyzed by Narbaitz and Adler (1966a). These authors confirmed that the estrogen producing cells in the ovary are formed from the primary sex cords.

c) The seminiferous cord cells : Histochemical (Scheib and Haffen, 1968, 1969) and electronmicroscopical (Narbaitz and Adler, 1966a; Scheib, 1970) findings tend to indicate that the cells forming the seminiferous cords in the newly differentiated embryonic testis have steroid secreting characteristics. On this basis, Scheib (1970) has insisted in Benoit's (1923, 1929) idea in the sense that some of these cells migrate to form the interstitial or Leydig cells. Narbaitz and Adler (1966a) consider that there is not enough proof to warrant this idea.

It is only during sexual maturation that the cord cells will transform into Sertoli cells.

d) The epithelial cells of the cortex or 'pre-follicular' cells : The youngest germinal epithelium studied at the ultrastructural level is from a 32-35 somite (at the beginning of the third day) embryo (Cuminge and Dubois, 1969a,b). According to these authors the epithelium at this age has a syncytial aspect since distinct plasma membranes are present only at the apical poles of the cells, the basal portions being represented in a fragmentary manner (Cuminge and Dubois, 1971).

A regular basement membrane is, however, present in the four day gonads (Cuminge and Dubois, 1971). On the third and the fourth day short bleb-like cytoplasmic projections arise from the germinal epithelium (De Simone-

Santoro, 1969). Cuminge and Dubois (1969, 1971) termed these structures 'boursouflures' and claimed that they represent a secretory process from the germinal epithelium and that this secretion plays a role in the chemotactic attraction of the germ cells towards the gonadal area. These short projections or 'boursouflures' disappear at about the sixth day of incubation (De Simone-Santoro, 1969; Cuminge and Dubois, 1969).

Studies on the cytology of the pre-follicular cells after hatching are available (Greenfield, 1966; Narbaitz, 1971). These studies have shown that several cytological characteristics of the mature follicular cells such as the dense cytoplasm and the production of 'lining bodies' are already present before the formation of follicles which usually begins four days after hatching (Greenfield, 1966). A gap in our knowledge on pre-follicular cell differentiation exists since no study is available bridging the descriptions of Cuminge and Dubois (1969a,b, 1971) which reached the age of six days of incubation and those of Greenfield (1966) and Narbaitz (1971) which begin with the newly hatched chicks.

MATERIAL AND METHODS

1. ANIMALS USED :

a) Turtle : Seven female turtles of the species Pseudemys scripta elegans with a carapace length of 7"-8" were used in the present study. The animals were obtained from a commercial source and had been originally collected from the field in early spring. They were killed by decapitation as soon as they reached the laboratory. The plastron was sawed off, thus exposing the viscera. Both ovaries were dissected out and immersed in the fixative.

b) Echidna : Two adult females of the species Tachyglossus aculeatus, each weighing approximately 3.5 kilograms, were used. The animals were obtained at Monash University, Australia, in December. The ovaries of the echidnas were fixed by perfusion and were shipped by air mail to this laboratory.

c) Chick embryos : Eggs from white Leghorn hens were obtained from a commercial source. The eggs were incubated at 38° C and 60% relative humidity in a forced air incubator. The embryos were dissected on 4th, 5th, 6th, 7th, 8th, 10th, 12th and 14th day of incubation. The undifferentiated gonads, ovaries and testis from the left and the right sides of the embryos were fixed separately and at least ten embryos from each group were used. No effort

was made to establish the sex of gonads from embryos six days old or younger.

d) Post-hatched chicks : Left ovaries from newly hatched white Leghorn chicks were used for experiments involving organotypic culture.

e) Hens : Ovaries from three month old white Leghorn hens were fixed for ultrastructural study and ribonuclease digestion experiments.

2. TECHNIQUES FOR ELECTRON MICROSCOPY :

a) Fixation : The tissues were fixed in Karnovsky's fixative (Karnovsky, 1965) which was prepared as follows : Two grams of paraformaldelyde were dissolved in 25 ml of distilled water by heating it to 60-70° C with constant stirring. One or two drops of IN NaOH were added until the solution cleared. The solution was then cooled and 5 ml of 50% glutaraldehyde were added and finally the volume made upto 50 ml by adding 0.2M phosphate buffer at pH 7.4. The fixative was diluted to one half strength with 0.1M phosphate buffer.

Whole ovaries of the turtle, hen and the echidna were immersed in the fixative immediately after dissection. The individual follicles were then dissected out and immersed in the fresh fixative. While the gonads from younger chick embryos were fixed as a whole; those from embryos 8

days of age or older were cut into small pieces prior to fixation. The fixation was carried out at 4° C for 6-24 hours. The tissues were washed overnight in 0.1 M phosphate buffer containing 0.2 M sucrose and post-fixed in 1% solution of osmium tetroxide in 0.1 M phosphate buffer at 4° C.

b) Dehydration and Embedding : Dehydration was carried out in graded ethanol and in propylene oxide. The duration of time in each grade is listed below :

30%, 50%, 70% and 90% ethanol	10 minutes each
Absolute ethanol	3 changes of 15 mts. each
Propylene oxide	3 changes of 10 mts. each

The tissues were then transferred to a 1:1 mixture of propylene oxide and araldite. Araldite was used as the embedding medium and was prepared according to the following formula obtained from Coulter (1967):

Araldite resin 502	20 grams
Dodecenyl succinic anhydride (DDSA)	13.6 grams
Tri (Dimethyl aminoMethyl) Phenol DMP-30	0.46 ml

The tissues were transferred to the above plastic mixture for 24 hours for imperagnation of the plastic. Fresh plastic was prepared the next day and poured into gelatin capsules which had been previously dried. The tissues were then transferred from the specimen tubes to the capsules. The polymerization of the plastic was carried out by placing the capsules in an oven at 35° C, 45° C, 60° C and 80° C

progressively. Capsules were kept at each temperature for 12-24 hours until sufficiently hard.

c) Sectionning : Thick and thin sections were cut using an LKB Ultratome-III. One micron thick section were obtained for orientation. Thin sections ranging from 600-850 Å were cut using both the glass as well as the diamond knives.

Copper grids with 100, 150 and 200 meshes were used to pick up the sections. The sections, while still in the trough of the cutting knife, were stretched with xylene vapours.

d) Staining : One micron thick sections were stained with a 0.5% solution of toluidine blue in 1% sodium borate.

The thin sections were stained, first, with a saturated solution of uranyl acetate in 50% ethanol, for 30-40 minutes at room temperature. The staining was carried out by floating the grids on the staining solution; the section side of the grid facing the solution. The sections were washed by rinsing the grids in several changes of distilled water.

In a second step the sections were stained with a solution of lead citrate (Reynolds, 1963). Lead citrate was prepared by mixing 1.33 gms of lead nitrate and 1.76 gms of sodium citrate in 30 ml of distilled water; the

contents were shook vigourously for one minute and allowed to stand for 30 minutes with intermittent shaking. Eight ml of IN NaOH were then added and volume made upto 50 ml by adding distilled water. The stain was diluted 1:25 times with distilled water.

The thin sections were stained for varying periods from 5-12 minutes, washed in distilled water, dried and studied with Philips EM 300 electron microscope.

3. ENZYME DIGESTION EXPERIMENTS :

a) Ribonuclease digestion: For RNAse digestion experiments, pieces of left ovaries from 3 month old hens were fixed either in Karnovsky's fixative as described previously or in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer. In either case the fixation was carried out for 6 hours at 4° C and the tissues were then washed with 0.1 M phosphate buffer for 24 hours.

The tissues were embedded in glycol methacrylate (a water soluble plastic) according to the procedure recommended by Leduc and Bernhard (1967). Thus, no post-fixation in osmium teroxide was done in this case. After washing, the tissues were dehydrated as follows :

i.	80% GMA monomer (2-hydroxymethyl methacrylate) and 20% distilled water	20 mts.
ii.	97% GMA plus 3% distilled water	20 mts.
iii.	Unprepolymerized embedding mixture	20 mts.

The embedding mixture was prepared as follows :

97% GMA plus 3% distilled water	7 parts
2% Luperco in butylmethachrylate	3 parts

Before embedding, this mixture was partially polymerized by heating over an open Bunsen burner flame with constant stirring till it became viscous. Impregnation was carried out for 24 hours in the pre-polymer. The tissues were then transferred to previously dried gelatin capsules and filled with the plastic.

The final polymerization was done by using ultraviolet light from an ordinary sun lamp. The plastic was sufficiently hard after 24 hours.

Thin sections were transferred from the trough of the cutting knife to small dishes for incubation with ribonuclease. Crystalline ribonuclease (type I-A, protease free, Sigma) was dissolved at a concentration of 1 mg/ml in distilled water, adjusted to pH 6.8 with 0.1N NaOH and incubated for 2-6 hours at 37° C. Control sections were incubated for the same period in distilled water alone. Carbon-coated grids were used to pick up thin sections. The thin sections were stained in a similar manner as described previously.

b) Amylase digestion : Pieces of turtle ovaries were fixed in half strength of Karnovsky's fixative and washed with sucrose buffer. The tissues were then frozen and sec-

tioned with a cryostat. The 16μ thick sections thus obtained were treated with 0.1% amylase (Sigma type III-A -amylase) in 0.02M phosphate buffer (pH 6) for 2 hours at 37° C. Control sections were incubated in buffer alone. Treated and control sections were then washed in the phosphate buffer and post-fixed in 1% osmium tetroxide for 2 hours. The dehydration and embedding in Araldite was done as described before.

4. ORGAN CULTURE TECHNIQUES :

Newly hatched white Leghorn chicks were used in these experiments. Left ovaries were carefully dissected out and immersed in Hank's balanced salt solution. The ovaries were then cut into small pieces not larger than one cubic millimeter and care was taken to include significant portions of the cortex in each explant. The explants were cultured on a solid medium which was prepared as follows :

Medium T.C. 199 with Hank's base 2X conc.	5 ml
Agar 2% in bidistilled water	5 ml
Penicillin	500 units

The contents of the medium were mixed with melted agar and transferred to the sterile plastic culture dishes (obtained from GIBCO). The medium was allowed to solidify and explants were then transferred to the culture dishes. The incubation was carried out at 38° C for 5 and 10 days. The explants when kept longer than 5 days were transferred

to fresh media. The cultured explants were fixed in Karnovsky's fixative and processed for electron microscopical study as described before.

5. ESTRADIOL INJECTIONS :

White Leghorn chick embryos were used in these experiments. The eggs were injected at 48 and 96 hours of incubation into their air chamber with a single injection of 0.1 mg estradiol propionate in oil solution (CIBA). For this purpose, a small hole was made in the air chamber and after the injection, the hole was closed with scoth tape to prevent the loss of moisture from the embryo during incubation. In the eggs, used as controls, a similar hole was made and sealed.

The embryos which had received injection on the second day of incubation were sacrificed on 4th and 5th day; while those injected on the fourth day, were sacrificed on 6th, 8th, 10th, 12th and 14th day of incubation. Left and right gonads from all ages were fixed separately and processed for ultrastructural studies. Gonads from at least 10 embryos were fixed from both the experimental and the control groups at each age.

PART-I COMPARATIVE STUDIES

A- ULTRASTRUCTURE OF THE GROWING FOLLICLES OF THE TURTLE, PSEUDEMYS SCRIPTA ELEGANS.

Numerous follicles of various sizes up to 10 mm in diameter were present in all the ovaries examined. The large follicles protruded from the surface of the ovary or even hung from short stalks. The examination of thick sections revealed that in addition to the numerous small follicles, the ovaries contained a large number of germ cells. Only one layer of follicular cells was present in all follicles of different sizes; their shape and characteristics and the width and organization of the perivitelline space varied according to the size of the follicle. In order to facilitate the description of submicroscopical details, the follicles were divided into four groups according to their diameter. When measuring the diameter of each follicle both the oocyte and the follicular envelope were included. All measurements were approximate.

1- Follicles 50-400μ in diameter:

In the follicles at this stage the follicular envelope was constituted by a layer of flattened follicular cells (Fig. 1). The plasma membrane of these cells lied close to that of oocytes and was separated by intercellular space 150 Å wide (Figs. 2 and 3). True desmosomes were never

observed between follicular cells and oocytes although at certain places the plasma membranes of both cells were closely apposed. Occasionally thickenings of the plasma membranes of follicular cells were observed (Fig. 2). Desmosomes were, however, found joining adjacent follicular cells at this as well as at later stages (Fig. 4).

A definite perivitelline space was absent at this stage. Microvilli were not usually present although isolated patches of them could be observed in the largest follicles of this group (see arrows in Fig. 3).

The follicular epithelium rested upon a thick basement membrane separating it from the cells of the theca interna. The follicular cells contained elongated nuclei oriented parallel to the basement membrane; few mitochondria, well developed Golgi complexes and few profiles of endoplasmic reticulum were also observed (Fig. 3). Fine microfilaments with a approximate diameter of 90 Å were distributed throughout the cytoplasm. Very dense granules morphologically similar to those which are characteristic of glycogen β-particles were found all over the cytoplasm of both the follicular cells and the oocytes (Fig. 3). Similar granules could also be observed in the theca interna cells and the endothelial cells of blood capillaries.

2- Follicles 0.5-1 mm in diameter:

The most important change with respect to the follicles in the preceding group was the appearance of a perivitelline space (Fig. 4). This space started to appear at the junctions of the follicular cells and later became continuous thus surrounding the whole oocyte. The space was occupied by numerous short microvilli originating both from the oocyte and the follicular cells (Fig. 4). Follicular cells at this stage contained large amounts of glycogen-like granules. Mitochondria were also more numerous and had a very dense matrix; an increase of the rough endoplasmic reticulum was usually observed.

Numerous glycogen-like granules were now found free in the perivitelline space among the microvilli (Fig. 4). These granules appeared to originate in the follicular cells and to reach the perivitelline space by a process of apocrine-like secretion: the granules contained in a membranous structure of the follicular cells were pinched off and released into the perivitelline space (Fig. 5). Secreted portions of the cytoplasm appeared to release the dense granules and then remained for sometime in the perivitelline space (See arrow in Fig. 4).

Other elements constantly found in the perivitelline space were horse-shoe shaped organelles constituted by two thick membranes separated by a material of lesser density (Fig. 6). These organelles initially appeared as

forming part of the plasma membranes of the follicular cells, became secondarily detached from them and acquiring an annular shape, remained free in the perivitelline space (Fig. 7). The name 'transosomes' applied to organelles found to be secreted by follicular cells in bird ovaries (Press, 1964; Bellairs, 1965) appears adequate to designate these structures. Free transosomes were fairly uniform in size (0.09-0.13μ). In many cases both the glycogen-like granules and the transosomes were present in the developing yolk granules in the peripheral cytoplasm of the oocyte (Figs. 8 and 9).

3- Follicles 1.5-4.5 mm in diameter:

At this stage the follicular cells as well as the perivitelline space reached their maximum size. The follicular cells were cuboidal or low columnar in shape (Fig. 10). The nuclei also became more rounded in shape although indented. Some clear cells started to appear in the follicular envelope and could be distinguished both with the optical (Fig. 11) and the electron microscope (Fig. 12). The cytoplasmic characteristics of these follicular cells appeared to be similar to those of regular follicular cells. The only apparent difference being the lower density of their cytoplasmic matrix.

The width of the perivitelline space increased from 3μ in the smaller follicles of this group to 21μ in

the large ones.

This increase in width was due in part to the growth and branching of the microvilli and in part to the accumulation of the material of the zona pellucida (Figs. 10 and 13). In the smaller follicles of this group the thick cytoplasmic processes from the follicular cells branched to form microvilli; some of them reached the oocyte (Fig. 10). In the larger follicles, however, while the microvilli from the follicular cells grew very little, those arising from the oocyte branched profusely and became closely packed (Fig.13). The stems of these microvilli contained fine microfilaments ending in the peripheral cytoplasm of the oocyte. Both the microvillar stems and the peripheral zones of the cytoplasm of the oocyte were full of pinocytic vesicles (Fig. 16).

The zona pellucida began to be formed in 1.5 mm follicles; and appeared as a filamentous material. This material was first deposited among the microvilli, especially in the outer zone near the follicular cells (Fig. 10). As the follicles grew, the amount of filamentous material increased untill it constituted a very thick layer. Small amounts of the same type of material were found in the superficial layer of the oocyte (Fig. 10).

Figure 11 shows a photomicrograph of a portion of a 3 mm follicle. The layers separating the oocyte from

the follicular cells correspond to those described by Thing (1918). The electronmicroscopical study has shown that: 1- the outer zone (a in Figs. 11 and 13) is mainly represented by accumulations of the filamentous material (Fig. 14); 2- the darker intermediate zone (b in Figs. 11 and 13) corresponds to the numerous ramifications of the oocyte microvilli and contains also filamentous material and 'transosomes' (Fig. 15); 3- the clear zone immediately surrounding the oocyte (c in Figs. 11 and 13) corresponds to the stems of the microvilli arising from the oocyte (Fig. 16). Glycogen-like granules were no longer observed in the perivitelline space at this stage.

4- Follicles more than 5 mm in diameter:

At this stage the oocytes appeared to grow at a much quicker pace than the enveloping structures and as a result of this the follicular cells appeared each time flatter (Fig. 17) and the perivitelline space became very much reduced in height (only 3 μ in 10 mm follicles).

In the 5 mm follicles ramifications of the oocyte microvilli were extremely reduced, although microvillar stems were still present (Fig. 18). The peripheral cytoplasm of the oocytes was full of pinocytotic vesicles and few scattered glycogen-like granules (Fig. 19). The outer zone of the perivitelline space was devoid of any microvilli

and was occupied by the zona pellucida material (Fig. 18).

As development progressed, the microvilli were fewer and smaller. In the 10 mm follicles, the microvilli were represented by small projections only (Fig. 20). The oocyte at this stage was full with yolk granules and the cytoplasmic organelles were restricted to a thin rim of cytoplasm towards the perivitelline space.

The follicular cells, however, continued to show signs of synthetic activity; the rough endoplasmic reticulum was abundant, the Golgi complex was very extended and glycogen-like granules were always present in large amounts (Fig. 20). At this stage, the follicular cells were not apposed so closely and the intercellular spaces between them were larger and a continuous passage appeared from the basement membrane to the perivitelline space. The desmosomes or tight junctions were absent at these locations (Fig. 20).

Diastase-treated tissues :

When sections from material treated with amylase before embedding were examined, it was found that the dense granules previously identified as glycogen-like, had completely disappeared both from the follicular cells and the oocytes (Fig. 21), empty spaces appeared in the places originally occupied by them (Arrows in Fig. 21). In the follicular cells, due to the extraction of the glycogen granules, the distribution of microfilaments

in the cytoplasm is clearly shown in Figure 21. The glycogen granules were always present in the sections from control material incubated in buffer alone (Fig. 22).

Fig. 1. Follicle 0.4 mm in diameter. A single layer of follicular cells (F) surrounds the oocyte (O). T theca. X 600.

Fig. 2. Thickenings of the plasma membrane of a follicular cell (F) are shown at arrows. O oocyte. X 51000.

Fig. 3. Same follicle as in Fig. 1. Microvilli are present only in isolated patches (arrows). BM basement membrane, Go Golgi complex, Gl glycogen-like granules, O oocyte, T theca. X 15000.

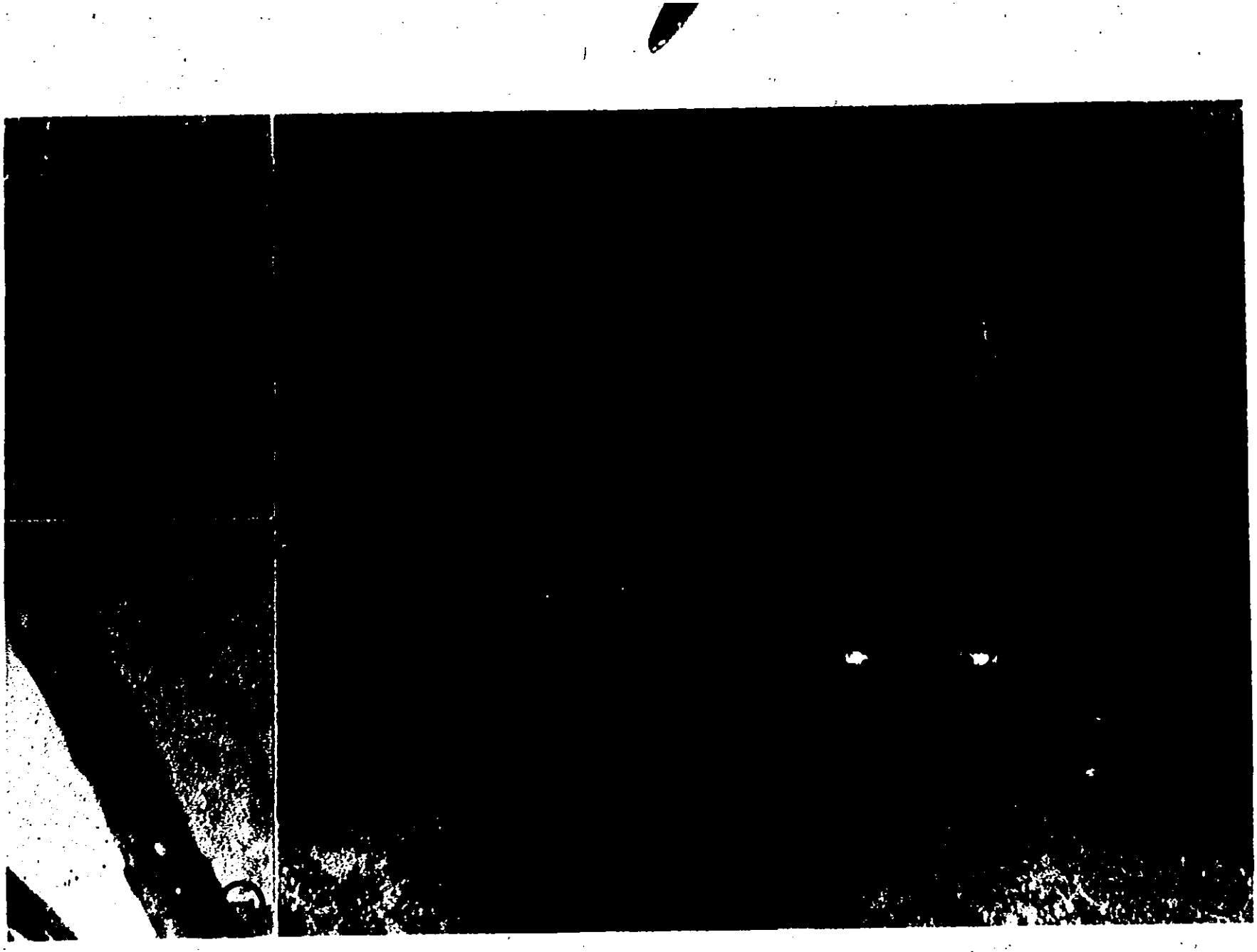


Fig. 4. Follicle 0.5 mm in diameter. Glycogen-like granules are seen in the perivitelline space (PS). The arrow is indicating a membranous structure probably originated by an apocrine type of secretion. BM basement membrane, D desmosome, O oocyte. X 14000.

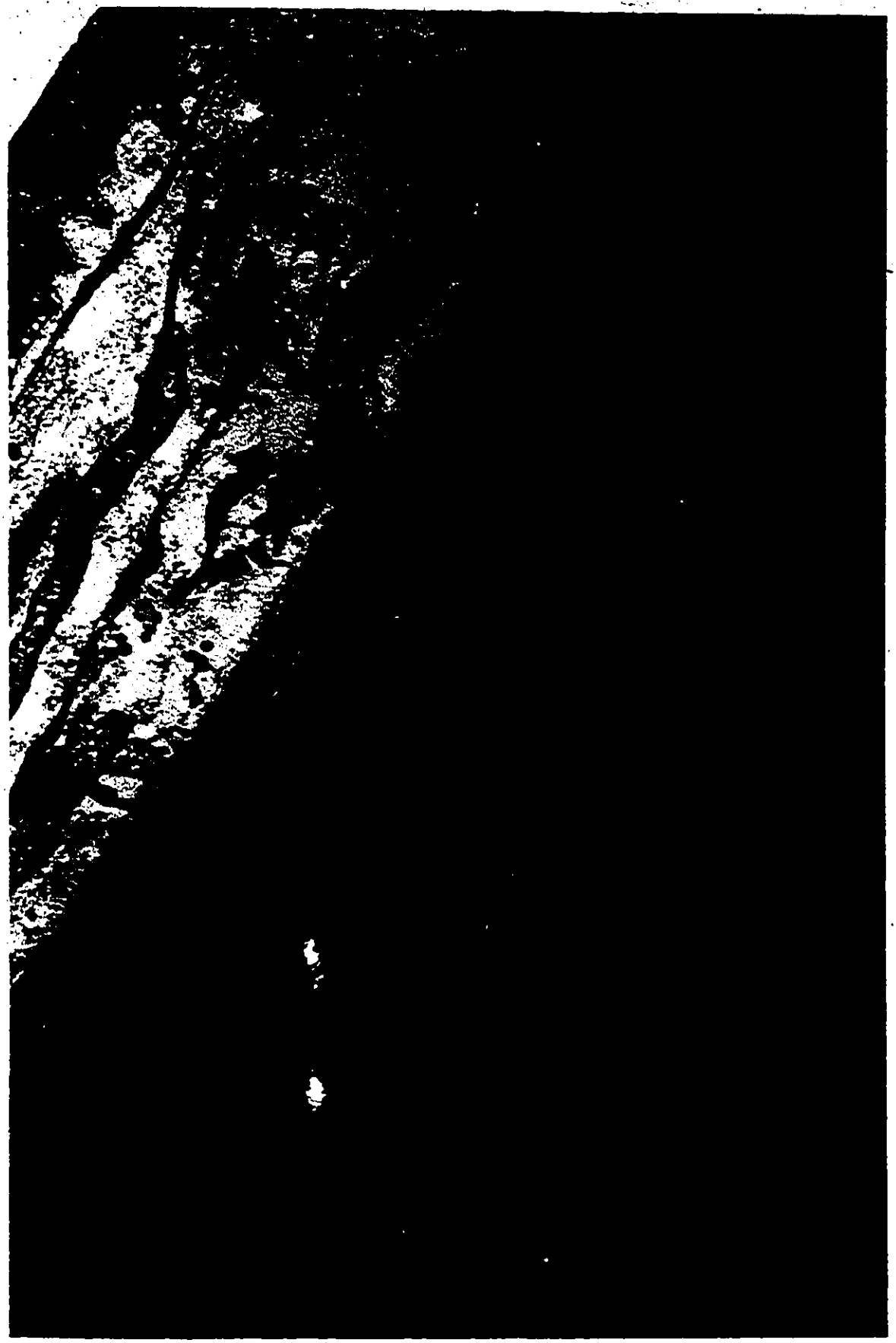


Fig. 5. Follicle 0.5 mm in diameter. At two sites (arrowheads) portions of follicular cell (F) appear to be pinching off. PS perivitelline space. X 26000.

Fig. 6. Same follicle. Transosomes (arrows) can be seen arising from the follicular cell (F) or free in the perivitelline space. X 80000.

Fig. 7. Portion of another follicle in stage 2 is shown. Numerous free transosomes can be observed in the perivitelline space. Two of these organelles (arrows) can be seen arising at the follicular cell (F) plasma membrane. BM basement membrane, o oocyte. X 27000.

Fig. 8. Shows a transosome (arrow) incorporated into a vesicle in the oocyte. X 51000.

Fig. 9. Shows a transosome (arrow) incorporated into a yolk granule. X 53000.

Fig. 10. Follicle 1.5 mm in diameter. Filamentous material (arrows) has started to appear. Transosomes (arrowheads) can also be seen in the perivitelline space. BM basement membrane, o oocyte, T theca. X 8300.

Fig. 11. Follicle 3 mm in diameter. Dark and clear cells as well as different zones in the space separating the follicular cells (F) from the oocyte (O) are shown. Areas marked a, b and c correspond to similarly marked regions in Fig. 13. T theca. X 600.

Fig. 12. Dark and clear follicular cells are shown. BM basement membrane, o oocyte. X 9500.

11

Fig. 13. Same follicle as in Fig. 11. Areas a, b and c correspond to areas similarly marked in Fig. 11 and to the areas shown, respectively, in Figs. 14, 15 and 16. BM basement membrane, F follicular cell, o oocyte, T theca. X 6000.

Fig. 14. Higher magnification of the zone a in Fig. 13. It shows the presence of a filamentous material. X 25000.

Fig. 15. Higher magnification of the zone b in Fig. 13. Microvilli, filamentous material and transosomes (arrows) can be seen. X 20000.

Fig. 16. Higher magnification of the zone c in Fig. 13. Stems of oocyte microvilli full of coated vesicles are shown. X 32000.

Fig. 17. Follicle 5 mm in diameter. Follicular cells (F) have decreased in height than the previous stage. Yolk granules can be seen in the oocyte (o). T theca. X 600.

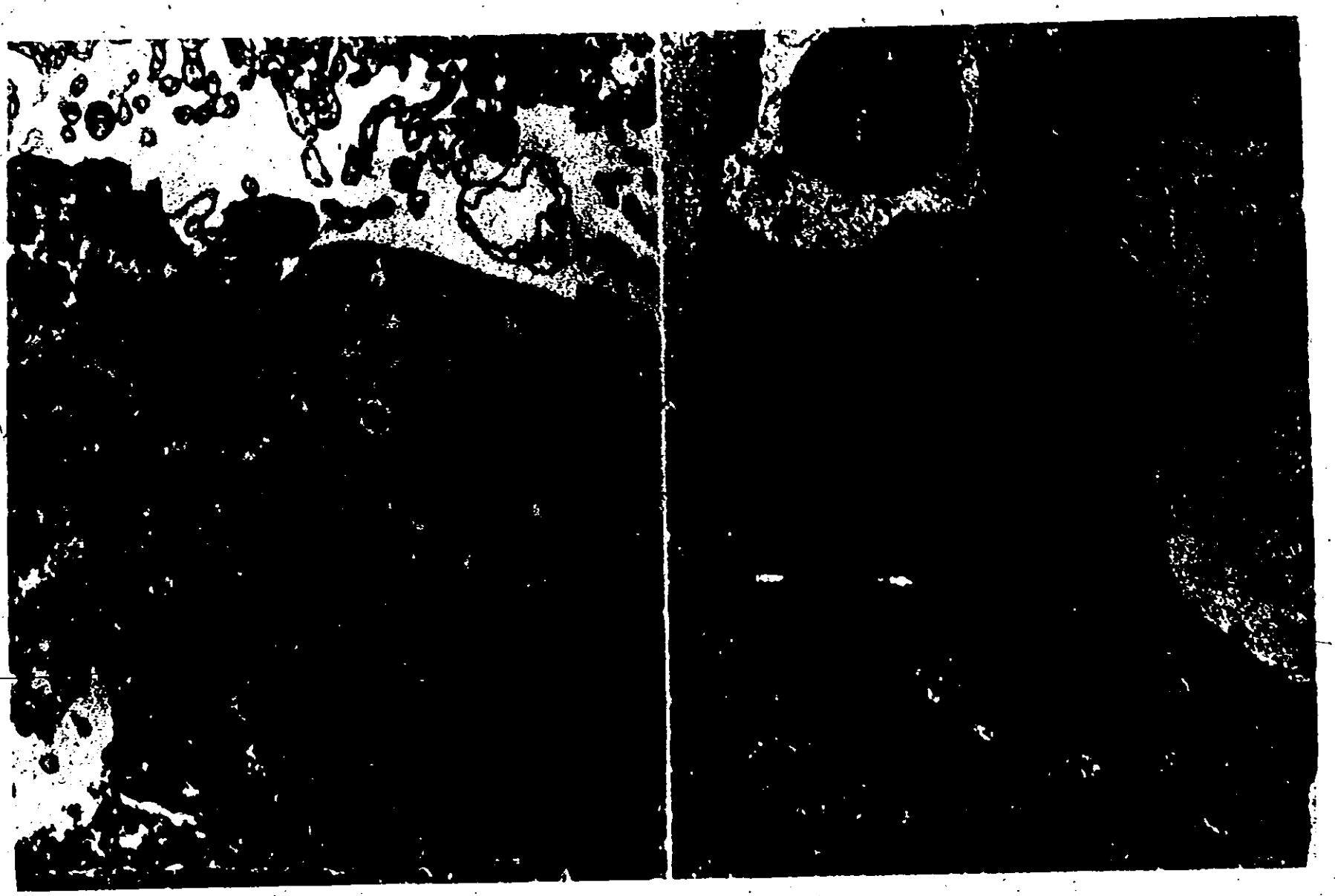
Fig. 18. Same follicle. Microvilli have decreased considerably as compared to smaller follicles as shown in Fig. 13. BM basement membrane, F follicular cell, O oocyte, T theca. X 8200.

Fig. 19. Portion of a oocyte from a 5 mm follicle. Large numbers of coated vesicles are shown. X 25000.

Fig. 20. Follicle 10 mm in diameter. A large space can be seen between adjacent follicular cells (F) with no tight junctions. The perivitelline space has decreased considerably. Very few microvilli are seen arising from the oocyte (O). BM basement membrane. X 20000.

Fig. 21. Follicle in stage 3. After 2 hours of treatment with amylase all the glycogen-like granules have disappeared (see empty spaces at arrowheads). O oocyte. X 25000.

Fig. 22. Follicle in stage 3. Control section incubated in buffer alone. Glycogen-like granules are well preserved. X 25000.



B- ULTRASTRUCTURE OF THE GROWING FOLLICLES OF THE ECHIDNA, TACHYGLOSSUS ACULEATUS.

Both the left and the right ovaries from the echidna had follicles of various sizes ranging from less than 50μ to about 2.5 in diameter. Whereas the smaller follicles were embedded in the ovarian cortex, the larger ones were prominent at the surface of the ovary and were attached to it by short stalks.

The description of various follicles has been divided into four groups on the basis of characteristic changes occuring at each stage.

1- Follicles 50-100μ in diameter:

The follicles of this category were observed in groups in the ovarian cortex; some of them were located in the connective tissue layer surrounding the larger follicles. In the smaller follicles the oocyte nucleus was rounded and centrally located (Fig. 23); it became ovoid and eccentrically placed and came to lie near the follicular envelope in the larger follicles of this group (Figs. 24 and 25).

The oocytes at this stage were surrounded by a single layer of flat follicular cells which were very elongated (Figs. 24 and 25). The follicular cells rested on a thick basement membrane which often appeared wavy (Fig. 26) and separated them from the cells of the theca.

The follicular cells were thicker in the middle portion where the nucleus was located and tapered towards the ends adjoining the adjacent cells (Fig. 25). Evaginations of the oocyte cytoplasm towards the follicular cells were frequently observed and were usually located at the junctions of follicular cells (Fig. 25).The plasma membranes of the oocytes and the follicular cells were in direct apposition with each other and tight junctions were present (Arrow in Fig. 26); no true desmosomes were observed connecting both cell types. Desmosomes were, however, observed joining follicular cells berween themselves. Both the perivitelline space and the zona pellucida were absent at this stage.

The elongated nuclei of the follicular cells had clumps of chromatin attached to the nuclear membrane. Their cytoplasm contained small Golgi complexes, profiles of endoplasmic reticulum and few mitochondria. Distributed throughout the cytoplasm were fine microfilaments and numerous dark granules morphologically similar to glycogen (Fig. 26).

In the larger follicles of this group the follicular cells became low cuboidal and the plasma membranes of these cells interdigitated with that of the oocytes (Fig. 27). Some of the processes of the follicular cells in the form of microvilli penetrated deep into the

superficial cytoplasm of the oocyte.

Specialized structures were observed in the superficial layers of cytoplasm of the oocytes from larger follicles of this group. These were rounded or ovoid structures bounded by a unit membrane and ranged in size from 1-2 μ in diameter. One of these structures is shown in Figure 27; it can be seen that a unit membrane surrounds bundles of irregularly arranged fibrils 150-200 Å° in diameter. In the following description these structures will be referred to as 'fibrillar bodies'.

2- Follicles 100-500μ in diameter:

A characteristic change occurring in the follicles of this stage was the appearance of the material of zona pellucida between the follicular epithelium and the oocyte (Fig. 29). This material appeared for the first time in 100μ follicles and seemed to be laid down in small patches (Fig. 28). In larger follicles it formed a continuous layer separating the oocyte from the follicular epithelium (Figs. 29 and 30).

The above mentioned material appeared as filamentous or amorphous and was traversed by microvilli both from the follicular cells and the oocyte (Fig. 29). Large cytoplasmic processes of the follicular cells which penetrated the zona pellucida, branched and contacted with the oocyte plasma membrane. Figure 29 shows such a contact

in a 150μ follicle. These contacts were not observed in larger follicles although microvilli were even more extensive. The width of the zona pellucida increased from 1.5μ in 0.25 mm follicles to 18-20 in 0.5 mm follicles.

The follicular cells became at this stage cuboidal or low columnar (Figs. 31 and 32), their nuclei were ovoid and there was an apparent increase in the number of mitochondria and rough endoplasmic reticulum (Fig. 32).

The perivitelline space appeared only in the larger follicles of this group and was confined to the vicinity of the oocyte (Fig. 33), the material of the zona pellucida being in direct contact with the plasma membranes of the follicular cells (Fig. 32). The microvilli arising from the follicular cells were fewer and were much longer than those of the oocyte (Figs. 32 and 33). The oocyte microvilli were profusely branched and their free ends were embedded in the material of the zona pellucida(Fig. 33). The striate layer described by Flynn and Hill (1939) at the optical microscope level and represented by s.l. in figure 31.

The fibrillar bodies described in the preceeding stage were now more numerous and formed a layer in the cortical portion of the oocyte (Fig. 31). These structure seemed to contribute to the formation of yolk granules. Figure 34 shows several of these structures in the

process of being incorporated into yolk granules.

3- Follicles 0.5-1.5 mm in diameter:

In the smaller follicles of this stage the zona pellucida attained its maximum width of about 22-25 μ (Fig. 35); this width started to diminish in follicles larger than 1 mm in diameter. The follicular cells retained their cuboidal or columnar aspect throughout this stage. Due to an increase in the number of follicular cells, the follicular epithelium became two layered in certain areas (Fig. 36) and reached its maximum height of 12-15 μ.

The submicroscopical characteristics of the follicular cells were similar to those found in the preceding stage (Fig. 37). In addition, two types of microvilli of follicular cell origin were observed; the first type was represented by long microvilli with contents similar to the rest of the cytoplasm (Fig. 38) and the second type by shorter processes which were thick microfilaments (Fig. 39). The abundance of microvilli of follicular cell origin is well demonstrated in figure 36. The microvilli arising from the oocytes were always thinner and more numerous than those of follicular cells and were confined to the striate layer only (Fig. 40). The number of oocyte microvilli started to decrease in the larger follicles where the perivitelline space had

disappeared and the zona pellucida occupied the entire space between the follicular cells and the oocyte (Fig. 41).

Another interesting characteristic was the appearance of tubular elements in the superficial cytoplasm of the oocytes from follicles larger than 1 mm in diameter (Fig. 41). These tubular structures were about 0.38 μ in diameter and their bulbous ends were about 0.58 μ in diameter. They were found associated either with pinocytotic vesicles or yolk granules.

An active process of pinocytosis was always observed at the plasma membrane of the oocytes. The fibrillar bodies were not observed at this or later stages.

4- Follicles 1.5-2.5 mm in diameter:

After reaching a maximum height the follicular epithelium started to become flatter at this stage (Fig. 42). In the largest follicles of this group the follicular cells were reduced to a width of 3 μ and the zona pellucida to 2 μ. The follicular cells, however, still showed signs of activity and were rich in Golgi complexes, rough endoplasmic reticulum and mitochondria (Fig. 43).

The number of microvilli arising both from the oocyte and the follicular cells was greatly reduced (Fig. 43) and they were never observed traversing the whole width of the zona pellucida as in earlier stages.

deep invaginations of the oocyte plasma membrane with pinocytotic vesicles were still observed (Fig. 43).

Fig. 23. Follicle 50 microns in diameter. Follicular cells (F) are flat with elongated nuclei. Oocyte nucleus (N) is spherical and centrally located. n nucleolus. X 600.

Fig. 24. Follicle 100 microns in diameter. The oocyte nucleus (N) has lost its spherical form and is eccentrically located. F follicular cells. X 1500.

Fig. 25. Same follicle as in Fig. 24. An evagination (arrow) of the oocyte (O) cytoplasm is shown. F follicular cell, N nucleus, n nucleolus, T theca. X 12000.

25

Fig. 26. Follicle in stage 1. Elongated follicular cell (F) with a wavy basement membrane (BM) is shown. A tight junction can also be observed at the arrow. Go Golgi complex, O oocyte, T theca. X 21000.

Fig. 27. Follicle 80 microns in diameter. Interdigitations of the plasma membranes of the follicular cell (F) and the oocyte (O) are shown. A fibrillar body can be seen in the oocyte. FB fibrillar body. X 32000.

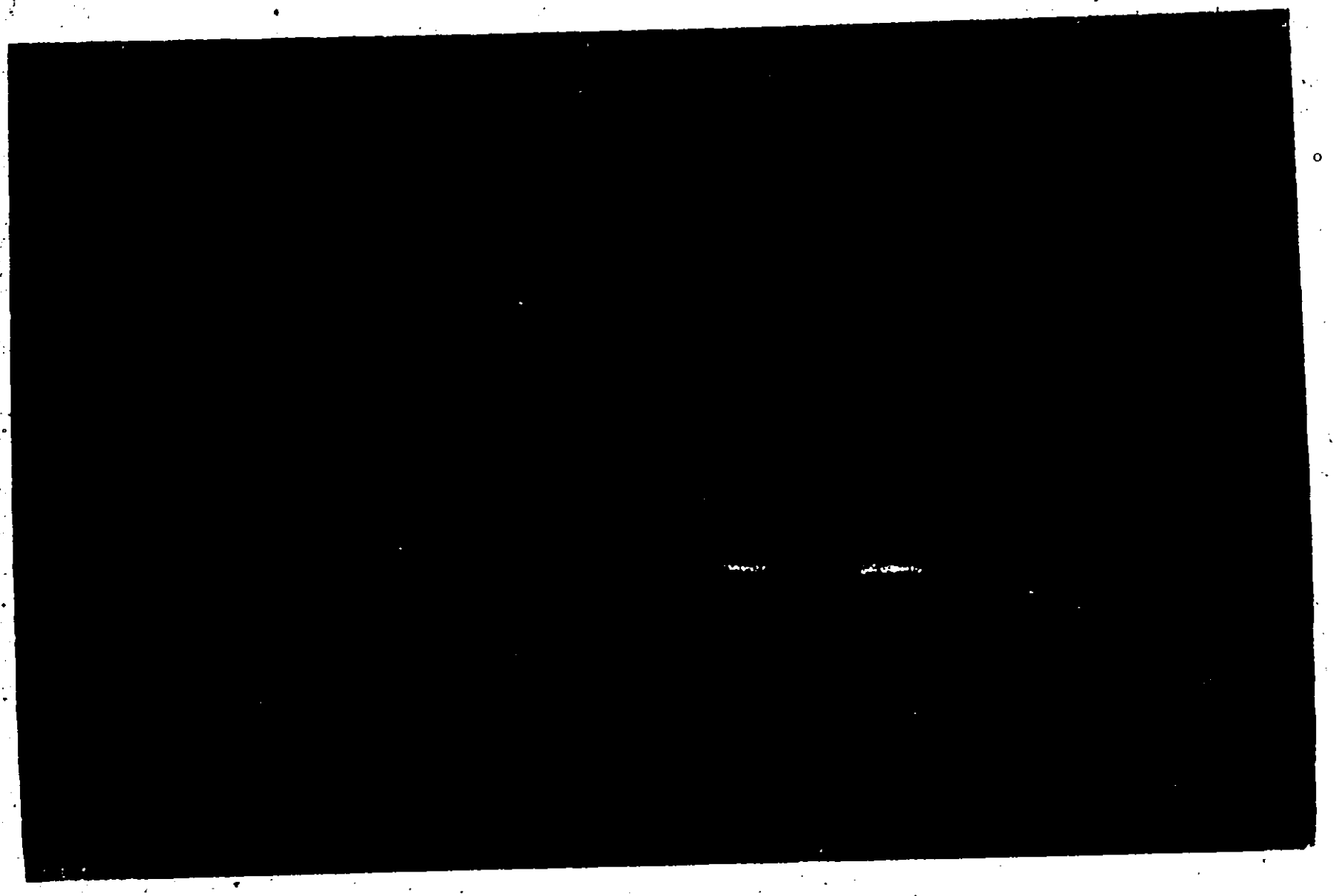


Fig. 28. Follicle 100 microns in diameter. The material of zona pellucida (arrowheads) has started to appear in patches between the follicular cells (F) and the oocyte (O). BM basement membrane, T theca. X 8300.

Fig. 29. Another follicle in stage 2. The material of the zona pellucida now forms a thick layer between the follicular cells (F) and the oocyte (O) and appears filamentous. A microvillar process of the follicular cells can be seen traversing the zona pellucida (ZP). X 20000.

Fig. 30. Follicle 300 microns in diameter. Follicular cells (F) are separated from the theca (T) by the basement membrane (BM). The zona pellucida (ZP) is occupied by microvilli both from the oocyte (O) and the follicular cells. Fibrillar bodies (FB) can be seen in the oocyte cytoplasm. X 18000.

Fig. 31. Follicle 500 microns in diameter. The follicular cells have become cuboidal and are separated from the oocyte (O) by a thick zona pellucida (ZP). The striate layer (sl) corresponds to the layer SL in Fig. 33. Fibrillar bodies (FBs) form a layer in the peripheral cytoplasm of the oocyte. X 600.

Fig. 32. Same follicle as in Fig. 31. Submicroscopical characteristics of follicular cells (F) are shown. The zona pellucida (ZP) is traversed by microvilli from the follicular cells. X 12000.

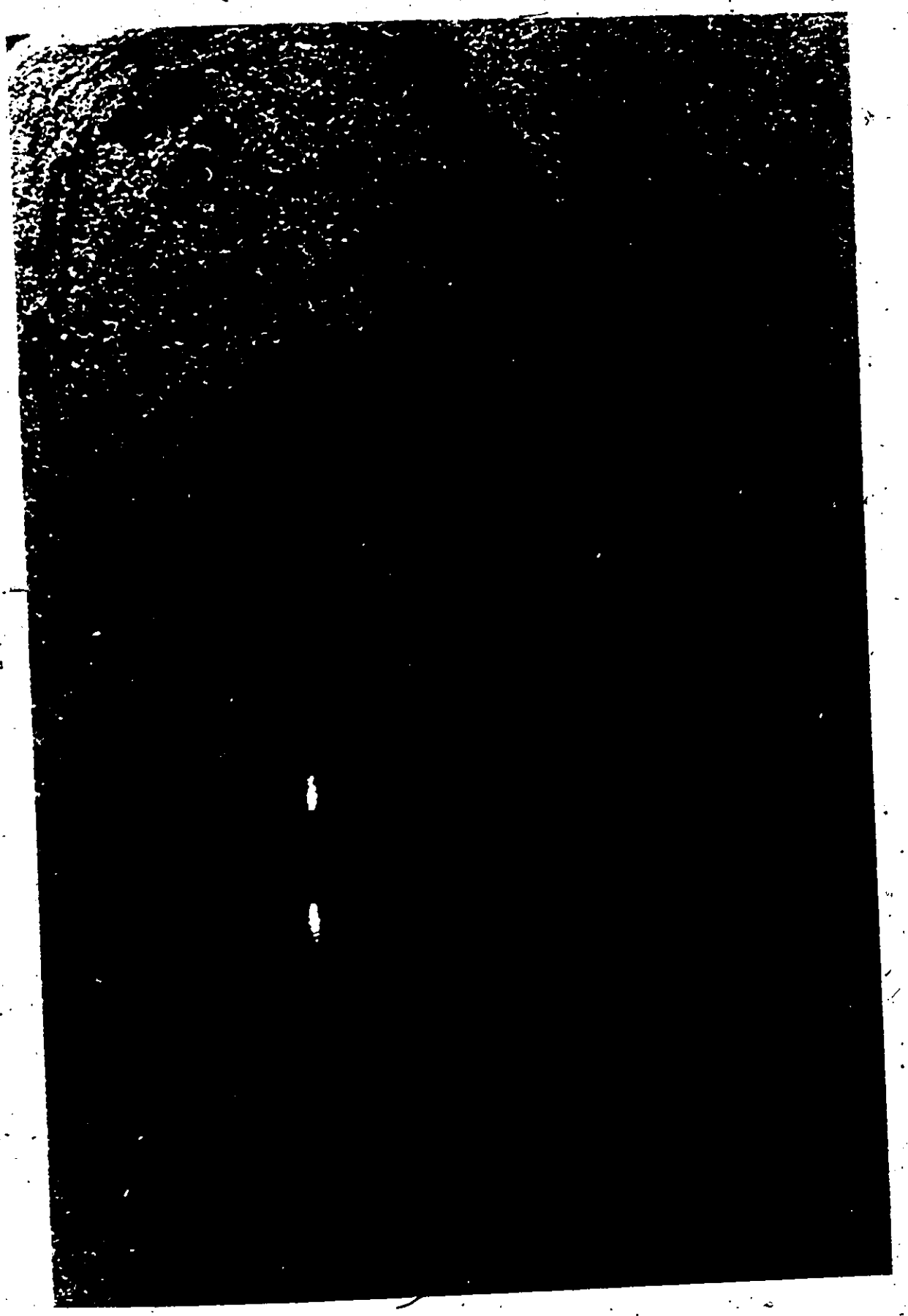
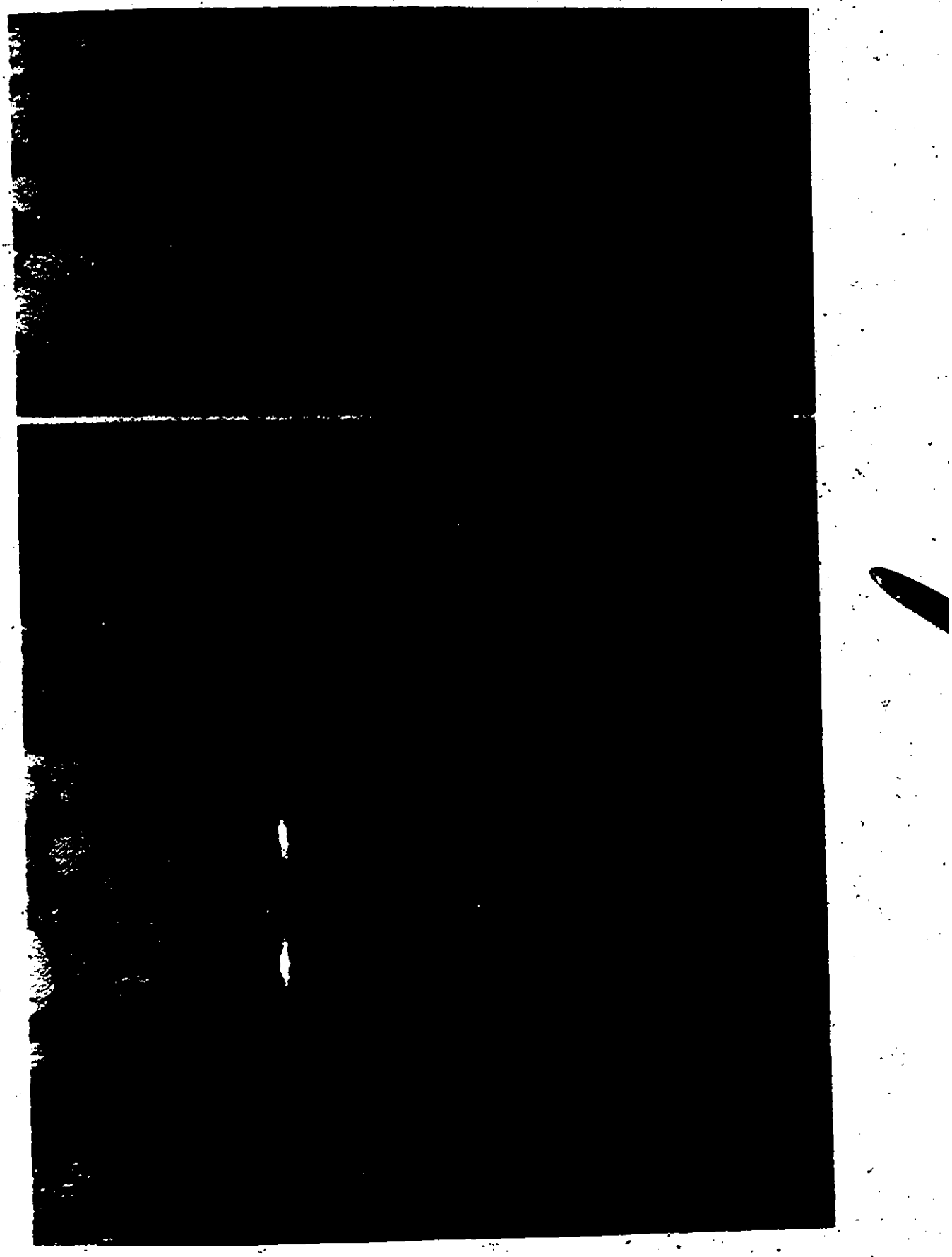


Fig. 33. Same follicle as in Fig. 31. The layer represented by SL in this figure corresponds to the layer marked by sl in Fig. 31. A perivitelline space has appeared in the vicinity of oocyte (O) plasma membrane. X 21000.

Fig. 34. Same follicle as in Fig. 31. Shows the portion of oocyte cytoplasm marked by FBs in Fig. 31. Several of the fibrillar bodies appear to be in the process of being incorporated into yolk granules. The progressive stages have been marked from 1-5; number 1 being the most primitive. X 21000.



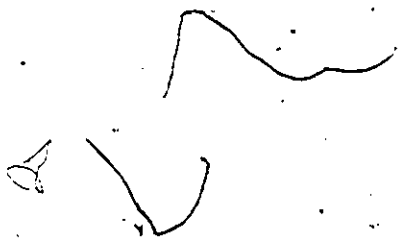


Fig. 35. Follicle 0.5 mm in diameter. The zona pellucida separating the follicular cells (F) and the oocyte (O) has attained its maximum width. The oocyte cytoplasm is devoid of fibrillar bodies but contains large numbers of yolk granules. T theca. X 600.

Fig. 36. Another follicle in stage 3. The follicular cells (F) have become columnar and at certain places their nuclei are arranged in two rows. Thick microvilli (arrows) arising from follicular cells are shown. T theca, O oocyte. X 1500.

Fig. 37. Follicle 1 mm in diameter. Submicroscopical charateristics of follicular cells (F) are shown. T theca, ZP zona pellucida. X 10000.

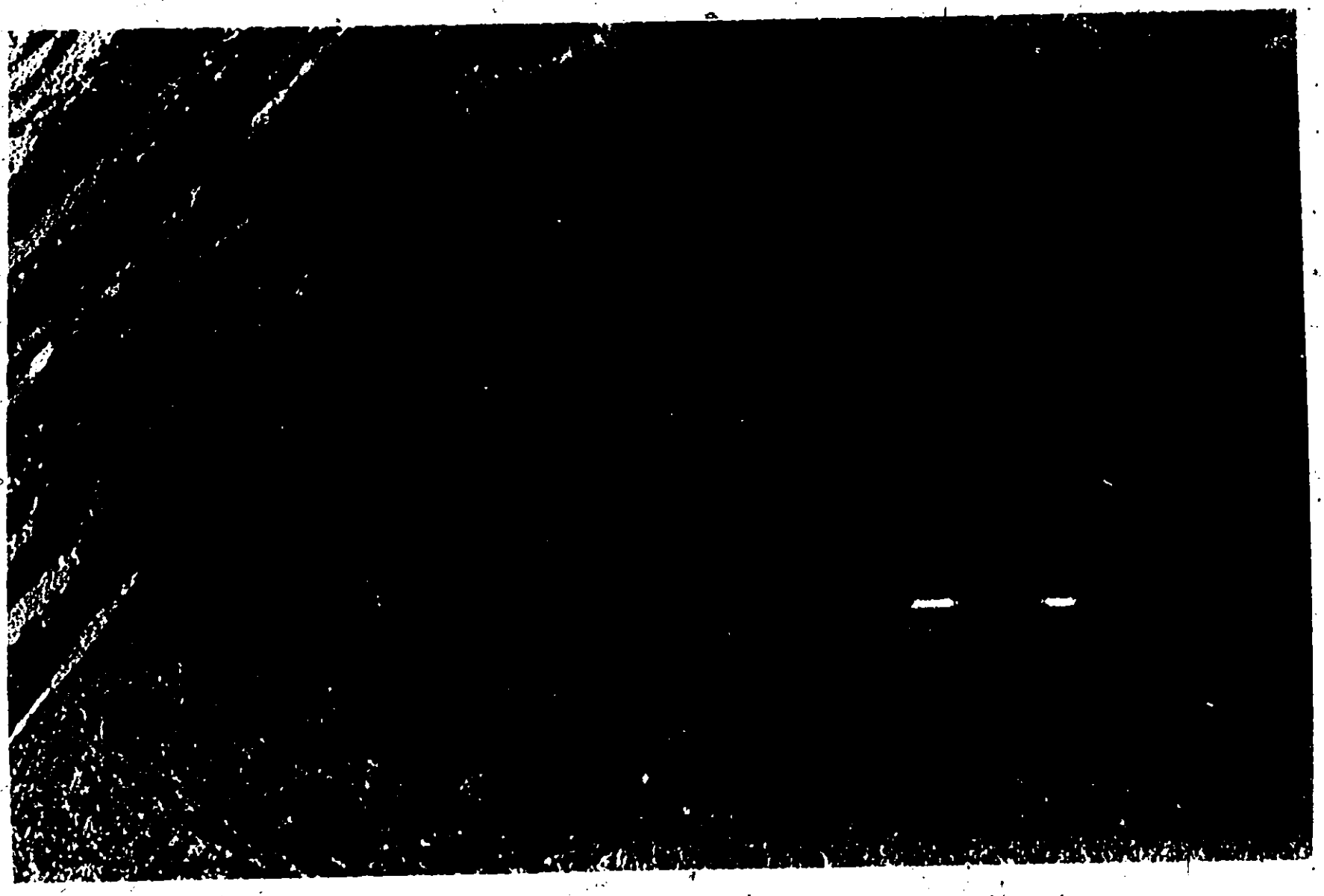


Fig. 38. Follicle 0.7 mm in diameter. A long microvillus with contents similar to those of follicular cell cytoplasm is shown. X 25500.

Fig. 39. Follicle 0.7 mm in diameter. A second type of microvillus arising from the follicular cell is shown. Thick microfilaments can be seen in the microvillus. X 25500.

Fig. 40: Follicle 0.7 mm in diameter. The oocyte microvilli are shown. Although more numerous, these microvilli are much smaller than those of follicular cells as shown in Figs. 37, 38 and 39. Note that the oocyte cytoplasm has very few tubular elements. X 25500.

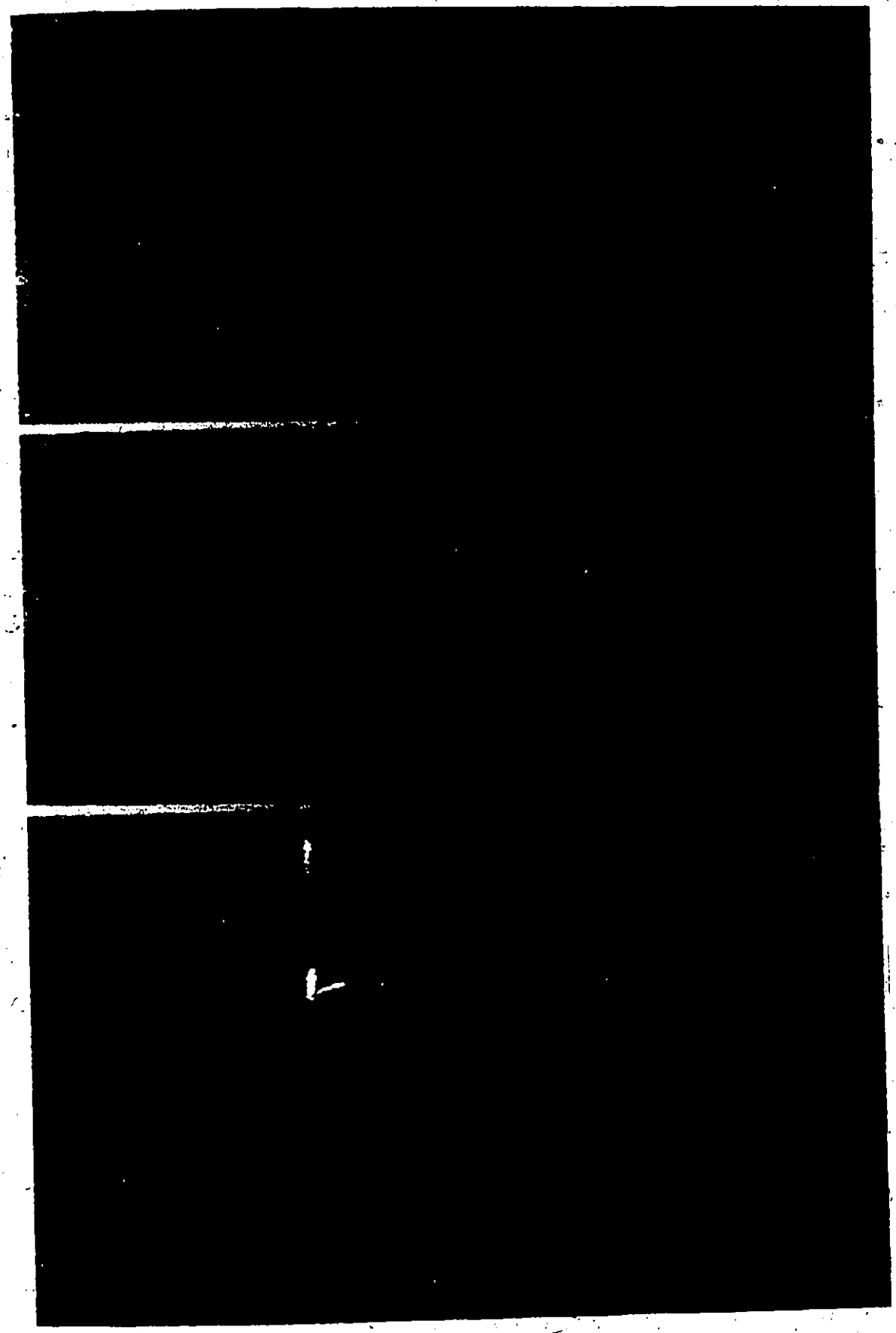


Fig. 41. Follicle 1 mm in diameter. A portion of the peripheral cytoplasm of the oocyte is shown. Note the abundance of tubular elements. Compare this figure with Fig. 40. X 27000.

Fig. 42. Follicle 2 mm in diameter. The follicular cells (F) have become flat and the zona pellucida is also reduced in its width. O oocyte, T theca. X 600.

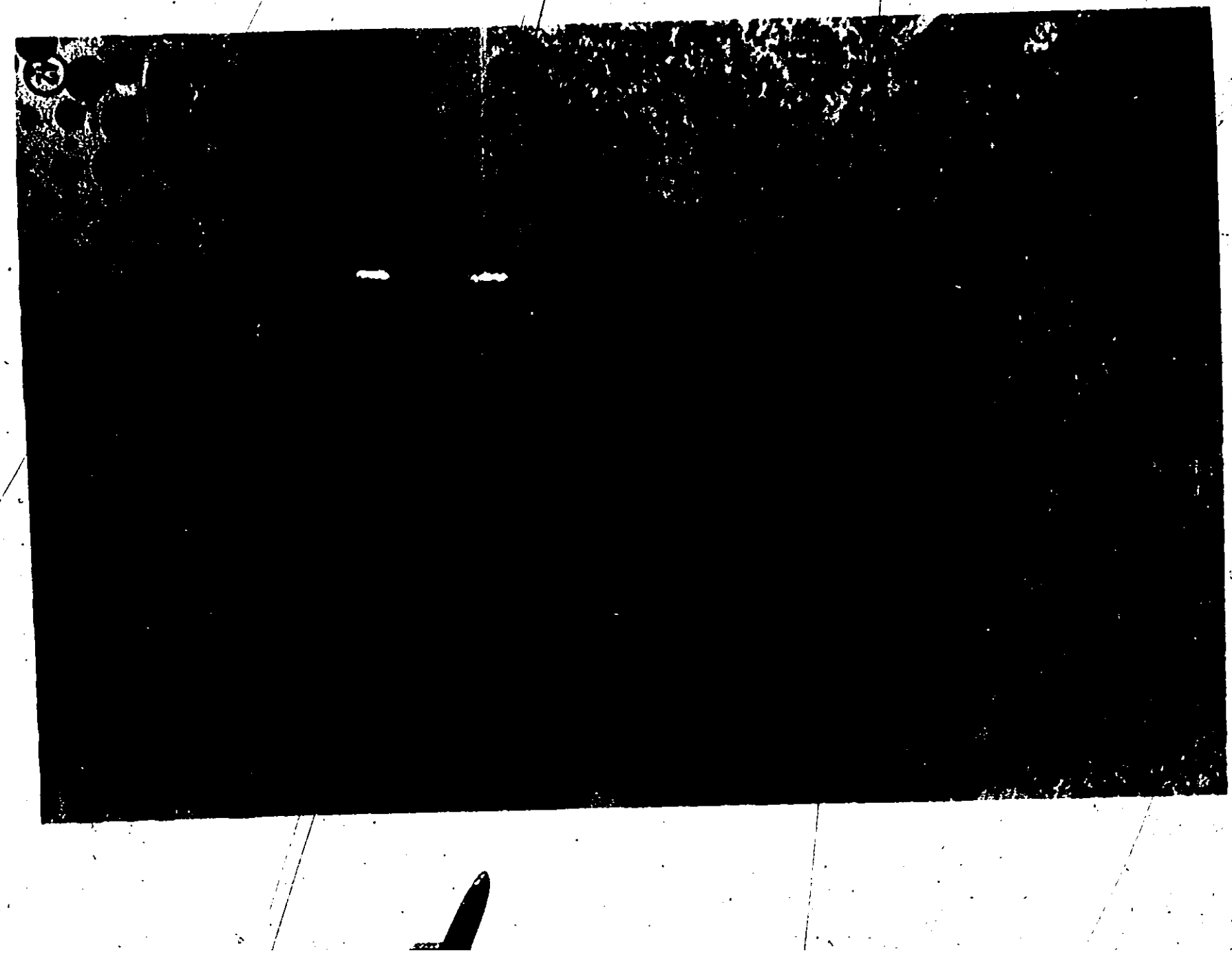
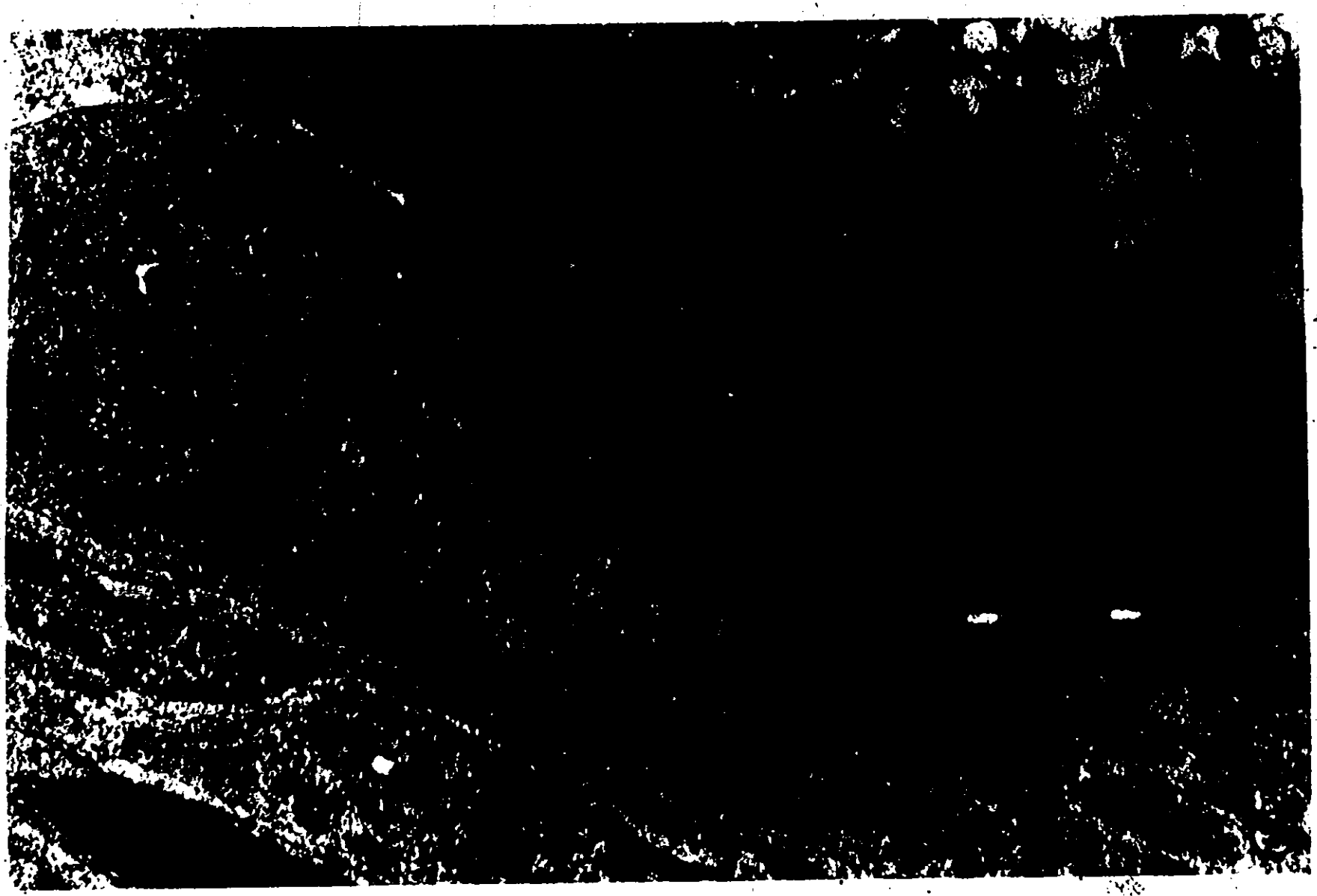


Fig. 43. Another follicle in stage 4. The number of microvilli has greatly reduced. The follicular cells are rich in rough endoplasmic reticulum (ER), mitochondria and have an extensive Golgi complex (Go). BM basement membrane, O oocyte, T theca. X 18000.



C- ULTRASTRUCTURE OF THE OVARIAN FOLLICLES OF THE HEN.

The relationship between the follicular cells and the growing oocytes in the hen ovary has been described in detail (see introduction). The present observations agree in general with those previously described by several authors. The presence of specialized organelles known as the lining bodies or transosomes was confirmed and additional observations were made on their origin and cytochemical nature.

Figure 44 shows two such organelles. It can be seen that the lining bodies consisted of an outer unit membrane which was continuous with the plasma membrane of the follicular cell (represented by A in Fig. 44) and subjacent to it there was a thicker membrane which under high magnification could be resolved into three separate membranes (represented by B in Fig. 44). A row of large granules 150-250 Å in diameter was attached to the innermost membrane.

The granules of the lining bodies seemed morphologically similar to ribosomes. The inner membrane of the lining bodies with its granules was occasionally observed in association with the membranes of the rough endoplasmic reticulum (Fig. 45).

Arrows in figure 46 indicate the sites of formation of lining bodies in the follicular cells. It is

shown that these organelles may be formed in any zone of the plasma membrane of these cells. In a second step lining bodies were pinched off from the follicular cells and came to lie either in the intercellular or the perivitelline space and were finally engulfed by the oocyte (thick arrows in Fig. 46). They were occasionally seen incorporated into the follicular cells.

When incorporated into the oocyte lining bodies were always surrounded by a unit membrane (Figs. 46 and 47) and several lining bodies were generally clustered to form macrobodies (Fig. 47). Macrobodies were not only confined to the peripheral cytoplasm of the oocyte but seemed to move inwards in the oocyte cytoplasm as new ones were being formed at the periphery. The lining bodies appeared to loose their characteristic structure and became homogeneous with the dense content of the macrobodies (Fig. 47).

In the past, speculations have been made as to the nature of the granules present in the lining bodies and it has been suggested that these may be constituted by RNA. To test this hypothesis tissues treated with ribonuclease were observed.

Although all the structures previously described could also be observed in the sections of ovaries embedded in glycol mathacrylate the quality of the images was lower;

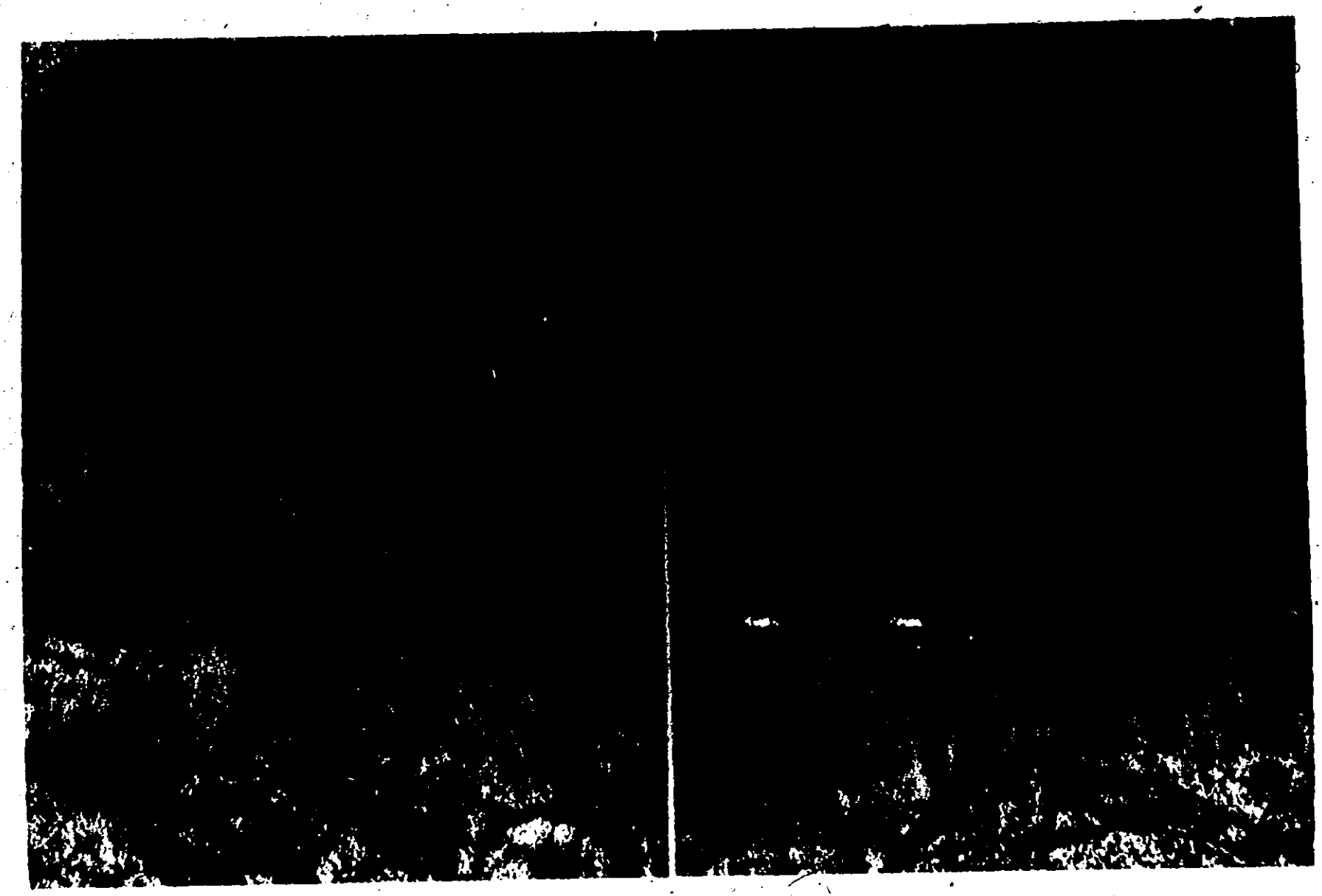
due to lack of post-fixation in osmium tetroxide, the cellular membranes gave negative images (Figs. 48 and 49).

Few profiles of rough endoplasmic reticulum are shown at arrows in figure 48, the membranes appear white and the ribosomes attached to them are darkly stained. In the lining bodies the outer membranes appeared white but the inner thick membrane and the granules were darkly stained (Fig. 49).

In the sections treated with ribonuclease, the ribosomes from the endoplasmic reticulum had completely disappeared, leaving the naked membranes (see arrows in Fig. 50). The same figure shows that the lining bodies have persisted but there was a partial dissolution of their granules.

Fig. 44. Shows two lining bodies attached to the plasma membrane of the follicular cell. The outer membrane of the lining bodies is a unit membrane and is continuous with the plasma membrane of the follicular cells (represented by A in this figure). The inner thick membrane (B) can be resolved into three different membranes (arrows at B). Large granules (arrowheads) are attached to the innermost membrane. X 133000.

Fig. 45. Shows the association of lining bodies (LB) with the membranes of the rough endoplasmic reticulum (arrows). X 83000.



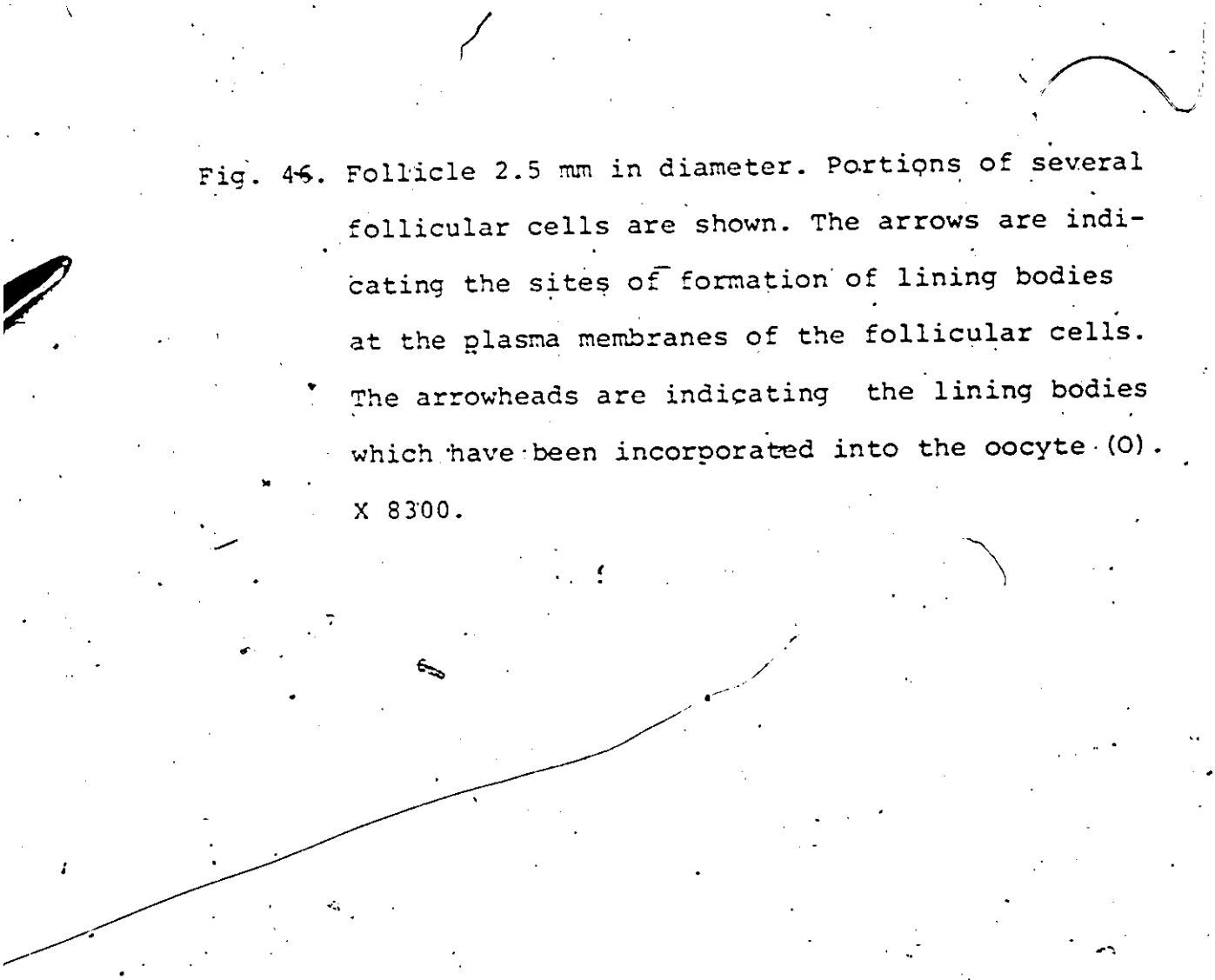


Fig. 46. Follicle 2.5 mm in diameter. Portions of several follicular cells are shown. The arrows are indicating the sites of formation of lining bodies at the plasma membranes of the follicular cells. The arrowheads are indicating the lining bodies which have been incorporated into the oocyte (O). X 8300.

Fig. 47. Same follicle as in Fig. 46. A deeper part of the oocyte cytoplasm is shown. Lining bodies have clustered to form macrobodies (MBs). Note that the lining bodies have started to loose their characteristic structure. X 32000.

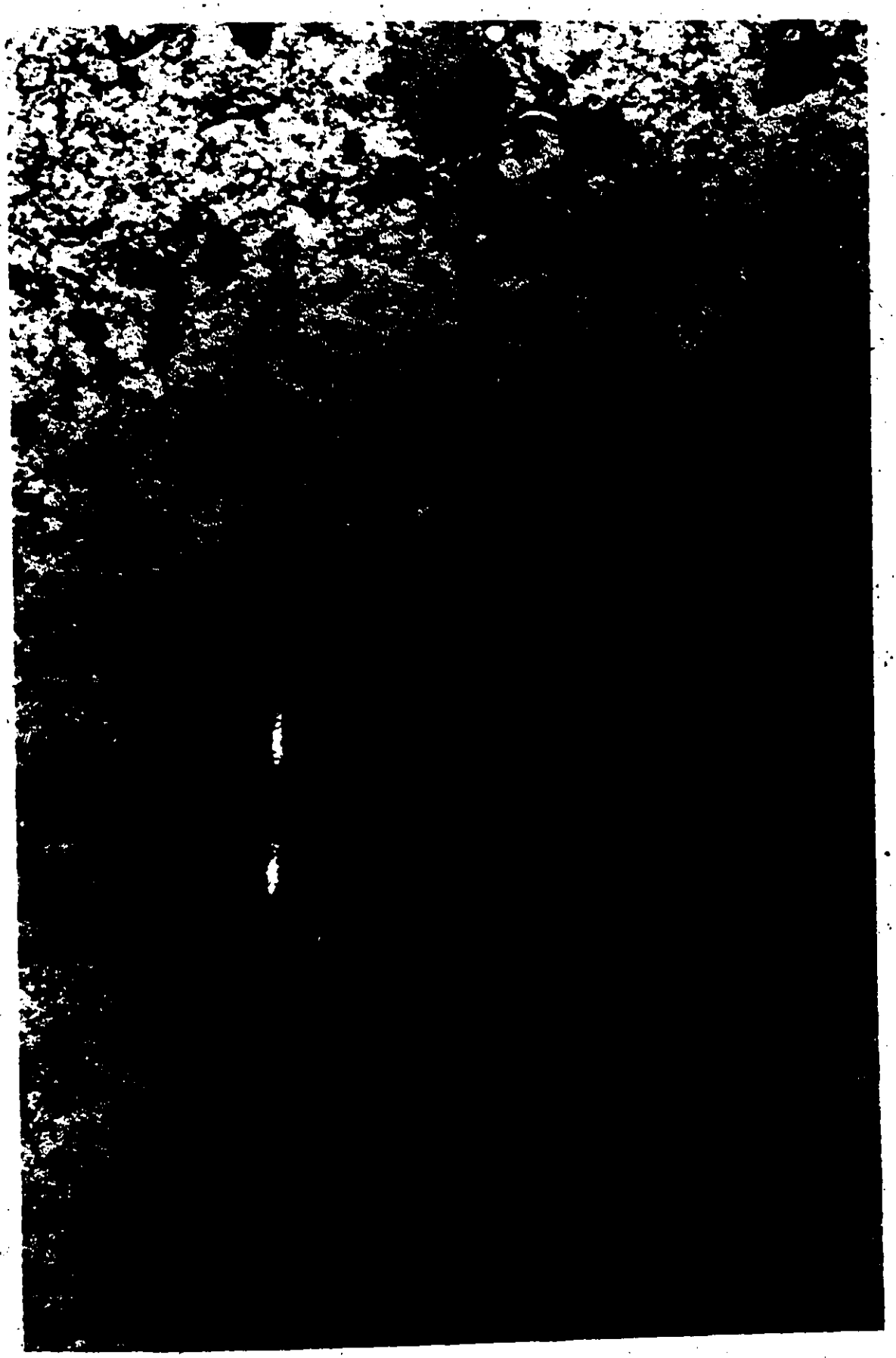
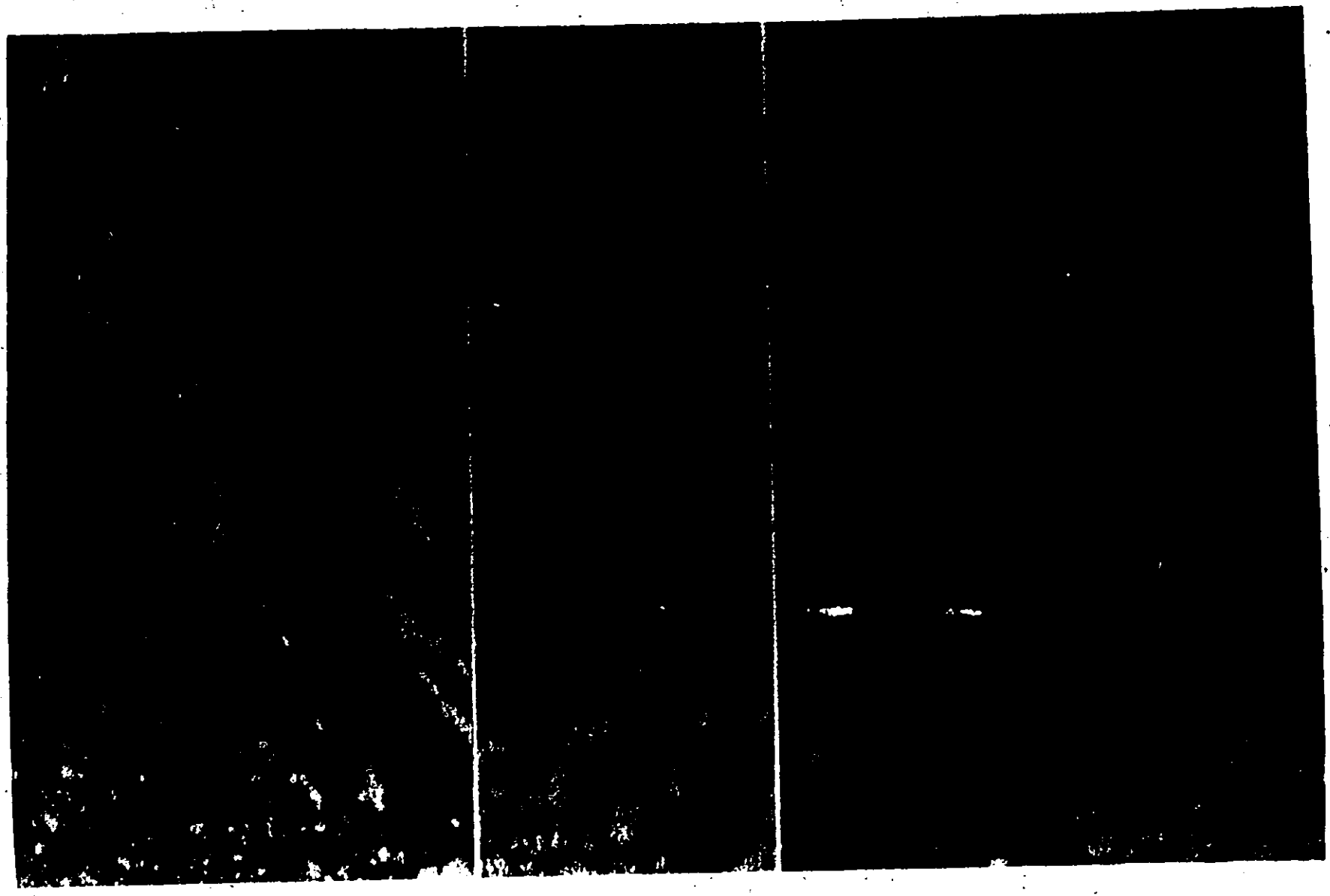


Fig. 48. Shows a section from glycol methacrylate embedded tissues. Since no post-fixation was done in osmium tetroxide, the membranes have negative images. The arrows indicate the profiles of rough endoplasmic reticulum. The ribosomes can be seen as dark granules (arrows). X 32000.

Fig. 49. Shows another section from glycol methacrylate embedded tissue. The granules of the lining bodies (LB) are clearly shown. X 53000.

Fig. 50. Shows a section from glycol methacrylate embedded tissue which had been treated with ribonuclease. Note that the granules of the lining bodies (arrows) and the ribosomes (arrowheads) have been extracted. X 27000.



PART - II EMBRYOLOGICAL STUDIES

A - ULTRASTRUCTURE OF THE GONADAL GERMINAL EPITHELIUM IN THE CHICK EMBRYO.

It is known that the follicular cells of the adult ovary are derived from the epithelial cells of the cortex of the embryonic gonad (see introduction). In the following study special attention was thus focussed on these cells.

4-day gonads:

The gonads at this age were elongated, transparent structures and appeared on the medial surface of the mesonephros. They were constituted by a two or three-layered germinal epithelium surrounding a compact mesenchymal medulla (Fig. 51). The present ultrastructural studies were confined only to the germinal epithelium.

The germinal epithelium was separated from the mesenchymal tissue by a continuous basement membrane and was constituted by few large germ cells and more numerous smaller somatic epithelial cells. The germ cells could be easily recognized by their large round vesicular nucleus and a large body of cytoplasm (Fig. 51). Figure 52 shows the submicroscopical structure of these cells and it is shown that their cytoplasm contained numerous polyribosomes, few profiles of granular endoplasmic reticulum and mitochondria. The Golgi complex was well

developed and small secretory vesicles were observed. Lipid inclusions and pinocytotic vesicles were not uncommon.

The epithelial cells facing the coelom were connected at their apical ends by typical junctional complexes (Fig. 53). The cells in the deeper layers had typical desmosomes. In contrast to what was found in the germ cells, there was less cytoplasm in relation to the nucleus. The cytoplasm was rich in profiles of granular endoplasmic reticulum; free polyribosomes and few mitochondria with a dense matrix were also observed (Fig. 53).

A network of fine microfilaments was found distributed throughout the cytoplasm of the epithelial cells with special abundance at the apical poles. In addition, microfilaments were also found forming large bundles. While some of these bundles seemed to be emanating from the junctional complexes, the others were seen isolated in the cytoplasm (Fig. 53). Microtubules were also a common occurence.

Short and thick cellular processes emerged from the apical ends of the superficial epithelial cells facing the coelom. These were irregular projections of the cytoplasm, some of which branched and contained polyribosomes; they were devoid of any organelles (Fig. 53). Intercellular

spaces were frequently observed among the epithelial cells and were occupied by short microvilli from them.

5-day gonads :

The gonads became more prominent at this age. The germinal epithelium was multilayered and there was an apparent increase in the number of germ cells (Fig. 54). A thick basement membrane separated the germinal epithelium from the underlying mesenchymal tissue. Primary sex cords could be seen arising from the germinal epithelium and penetrating the underlying mesenchymal tissue (Fig. 54).

Figure 55 shows a part of the germinal epithelium at this age. The ultrastructural characteristics of both the germ cells and the epithelial cells were essentially similar to those described for four-day gonads. However, the number of microfilaments distributed throughout the cytoplasm of epithelial cells seemed to increase (Fig. 56). Small organelles with a structure similar to the 'lining bodies' described in ovaries of later ages appeared for the first time in some of the gonads examined at this age.

6-day gonads :

Left and right gonads were fixed and examined separately at this age. Signs of asymmetry berween the left and the right gonads appeared for the first time

at this age. While the germinal epithelium in the left gonads continued to grow and form the sex cords (Fig. 57), the right germinal epithelium was extremely reduced (Fig. 58).

When examined with the electron microscope all fifteen left gonads of this age had lining bodies. These were observed in different locations and stages of evolution: the smaller lining bodies were usually crescent shaped: they were always embodied in the plasma membrane of the cell of origin and measured between 0.1-0.15μ in length (Fig. 59). In a second step the lining bodies seemed to be in the process of being budded off from the epithelial cell. They were horse-shoe shaped, sligtly larger and while still attached to the epithelial cells protruded either into the intercellular space (Fig. 60) or the coelom. Finally the large lining bodies were rounded, ovoid or elongated in shape and were found either lying freely in the intercellular space or were contained in the cytoplasm of other epithelial cells (Fig. 61). When incorporated into other cells the lining bodies were always surrounded by a third membrane (Fig. 61); and probably represent organelles formed by one epithelial cell and engulfed by another. Lining bodies were never observed to be incorporated in the cytoplasm of the germ cells at this age.

In contrast with the regular presence of lining bodies in all the left gonads observed, not a single one of these organelles was found in the ten right gonads of this age which were studied. In addition to this, apical cytoplasmic processes described in the epithelial cells of 4- and 5-day gonads, were still present at this age but were clearly shorter and less numerous in the right gonads. Microfilaments were also present at this age but no difference in their distribution could be established between the left and the right gonads.

The epithelial cells from left gonads were rich in rough endoplasmic reticulum and an active Golgi complex with secretory vesicles was also observed (Fig. 62). Occasionally lining bodies were seen associated with such organelles as lysosomes (Fig. 62) or inclusions like lipid droplets.

8-day gonads :

At this age ovaries and testis can be distinguished macroscopically; left and right ovaries are also clearly different both in size and aspect.

When left ovaries were examined histologically, two regions could be distinguished (Fig. 63); an outer thick cortex which contained most of the germ cells and an inner, larger region, medulla. Ultrastructural aspects of the epithelial cells from left ovarian cortex were similar

to those described for previous ages. However the lining bodies increased both in size and number. The medullary portion had a typical lacunar structure (Fig. 63).

The cortex in the right ovaries was considerably thinner than in the left ones and was formed only by epithelial cells (Fig. 64). Figure 65 illustrates the ultrastructure of the right ovarian cortex at this age. A single layer of epithelial cells rested upon a irregular basement membrane. The cells were rich in polyribosomes, mitochondria and rough endoplasmic reticulum. Few 'boursouflures' could still be observed. Lining bodies were not found in these gonads.

The testes at this age contained numerous seminiferous cords and a cortex, thickness of which varied from one area to another (Fig. 66). This cortex was formed only by the epithelial cells; these contained numerous ribosomes lying free in their cytoplasm and had very little rough endoplasmic reticulum (Fig. 67). Microtubules and microfilaments were also present in their cytoplasm. Lining bodies in different stages of development were seen only in the cortex of left testes.

A careful search for lining bodies in other regions of the gonads was conducted and not a single one of these organelles was found in the medullary zone of either the testes or the ovaries.

10-day gonads :

While the cortex of the left ovaries had continued to grow, the germinal epithelium of the right ovaries was reduced to a squamous layer.

The cortex of the testes had also diminished in thickness. However, in those places in which it kept its original thickness, lining bodies were still observed.

Fig. 51. Shows a left gonad from a 4-day old embryo. The germinal epithelium (GE) is two or three layered and contains few germ cells (GC). m medulla. X 600.

Fig. 52. Same gonad. A germ cell in the germinal epithelium. Go Golgi complex, L lipid, N nucleus. X 18000.

Fig. 53. Apical portions of epithelial cells from a 4-day left gonad. Lipid droplets (L), rough endoplasmic reticulum, mitochondria, microfilaments, apical cellular processes or 'boursouflures' (B) and junctional complexes (arrows) are shown. N nucleus. X 27000.

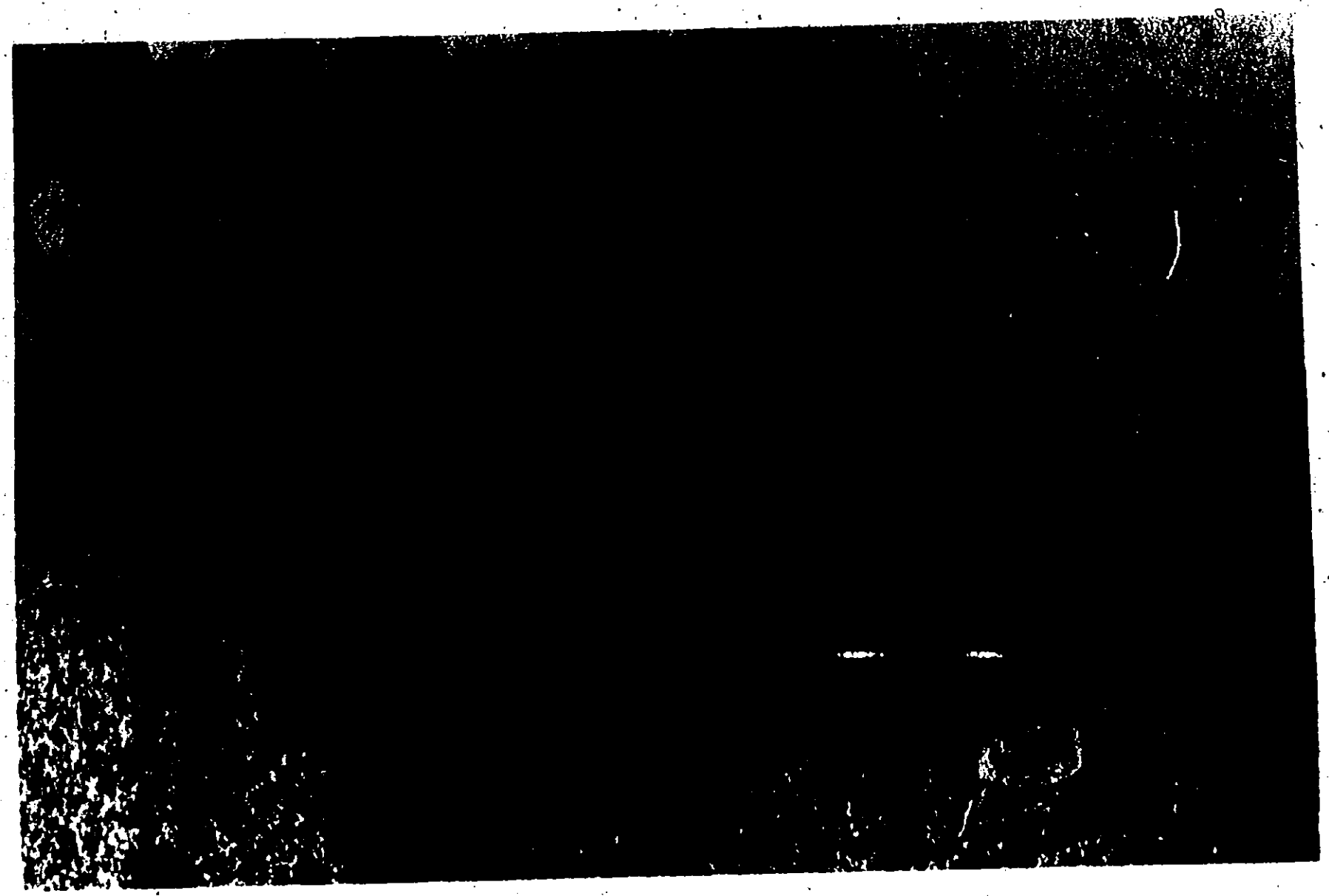


Fig. 54. Five-day left gonad. The height of the germinal epithelium (GE) and the number of germ cells (G) has clearly increased than in the 4-day gonads. Primary sex-cords (SC) have started to appear. X 600.

Fig. 55. Same gonad as in Fig. 54. Epithelial cells (E) and germ cells (G) are shown. X 8300.

Fig. 56. Portions of two epithelial cells from a 5-day gonad. Network of fine microfilaments can be seen. X 40000.

Fig. 57. Six-day left gonad. The thick germinal epithelium (GE) continues to form primary sex-cords (SC). X 600.

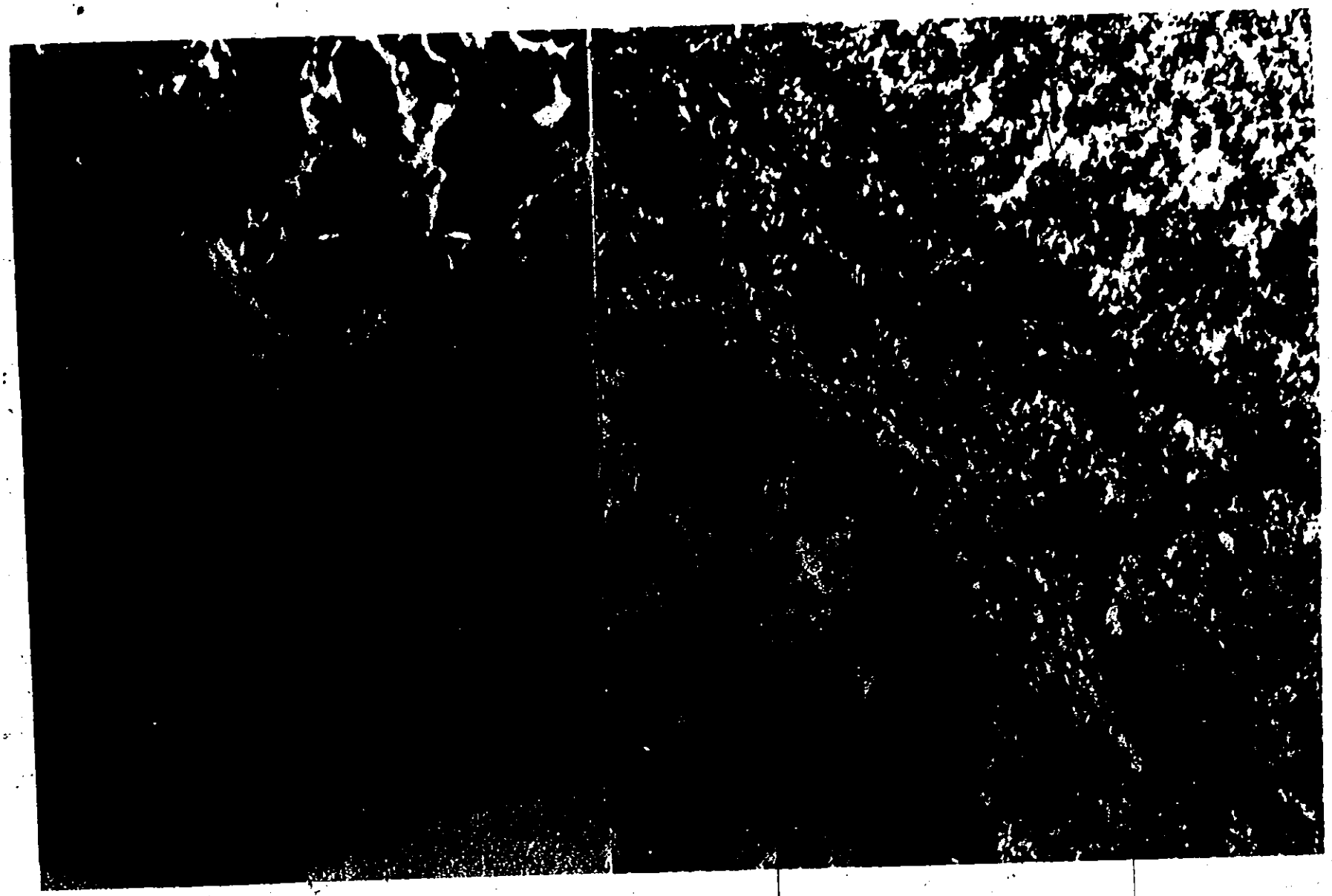


Fig. 58. Six-day right gonad. The germinal epithelium (GE) is extremely reduced. X 600.

Figs. 59-61. Three stages in the evolution of lining bodies in the 6-day left gonads. In Figs. 59 and 60, lining bodies are seen attached to the cell of origin (arrowheads), while Fig. 61 shows a lining body incorporated into another cell and has a third membrane (arrow). Figs. 59 and 60, X 53000. Fig. 61, X 80000.

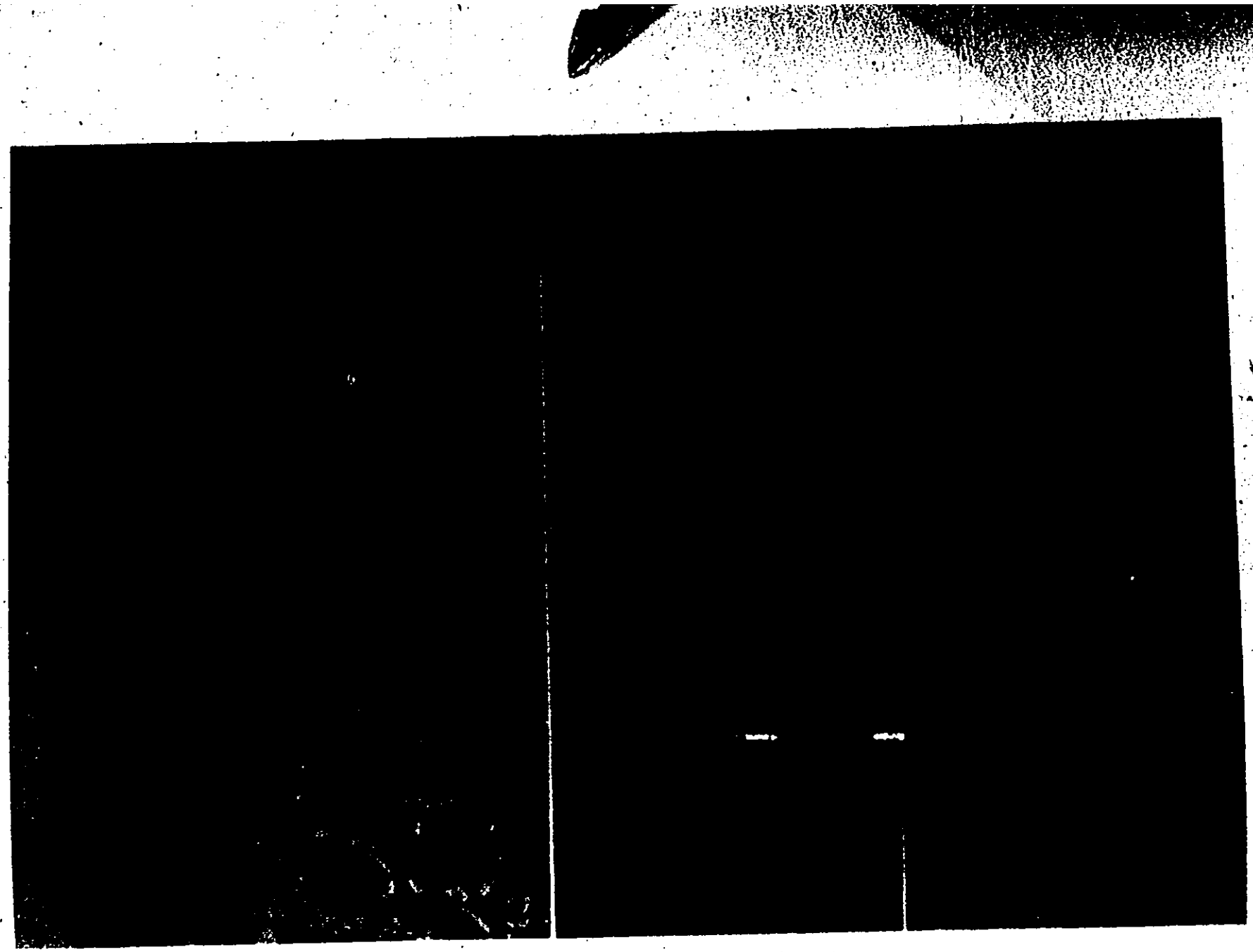


Fig. 62. Epithelial cells from a 6-day gonad. Lining bodies engulfed by epithelial cells are shown (LB). Those at the right are incorporated into a lysosome-like body. An active Golgi complex is also shown (Go). X 32000.

Fig. 63. Eight-day left ovary. A thick cortex surrounding the lacunar medulla is shown. X 360.

Fig. 64. Eight-day right ovary. The cortex is represented by a squamous layer of epithelial cells. The ovary seems to be entirely constituted by the medullary tissue. X 360.

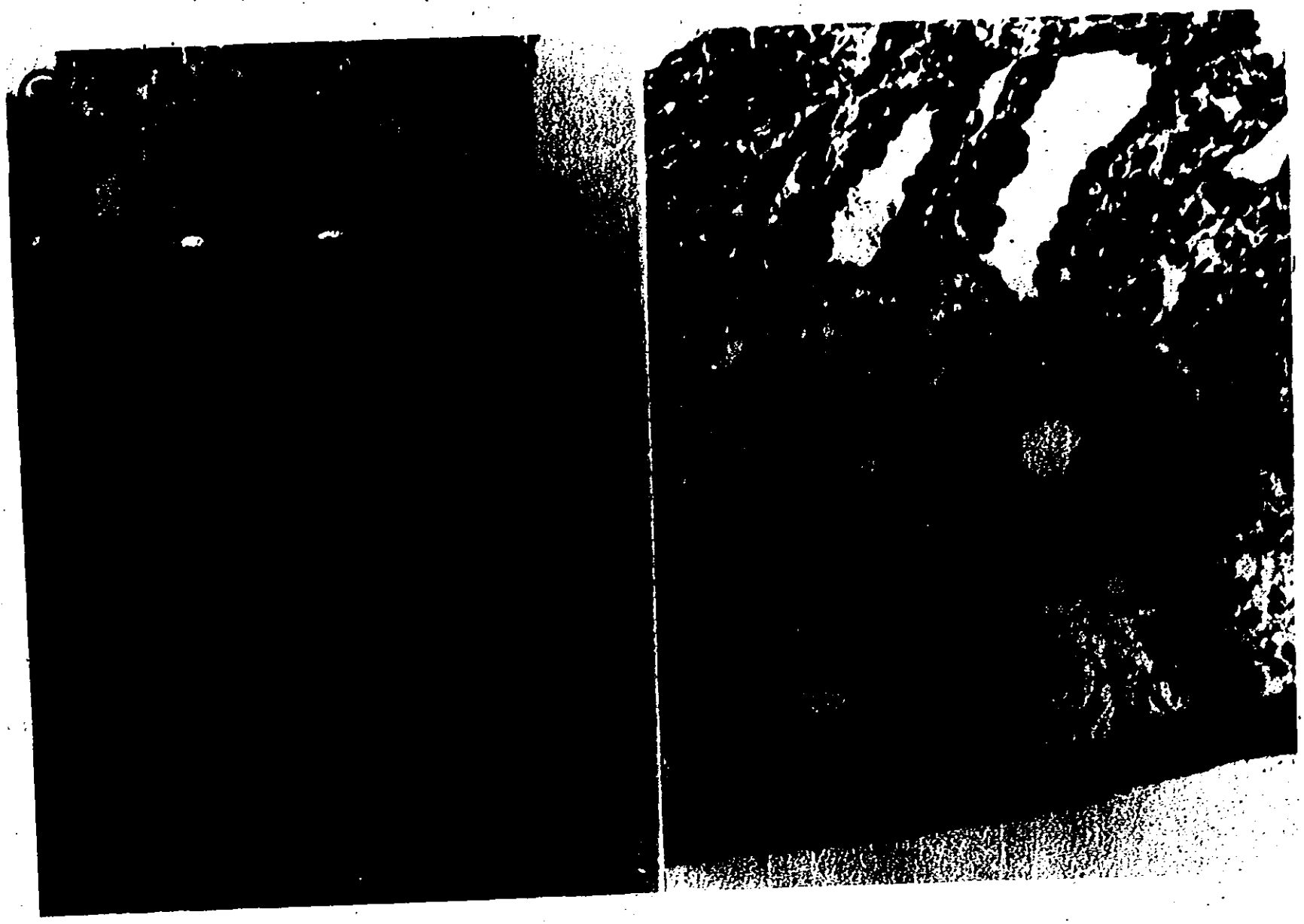
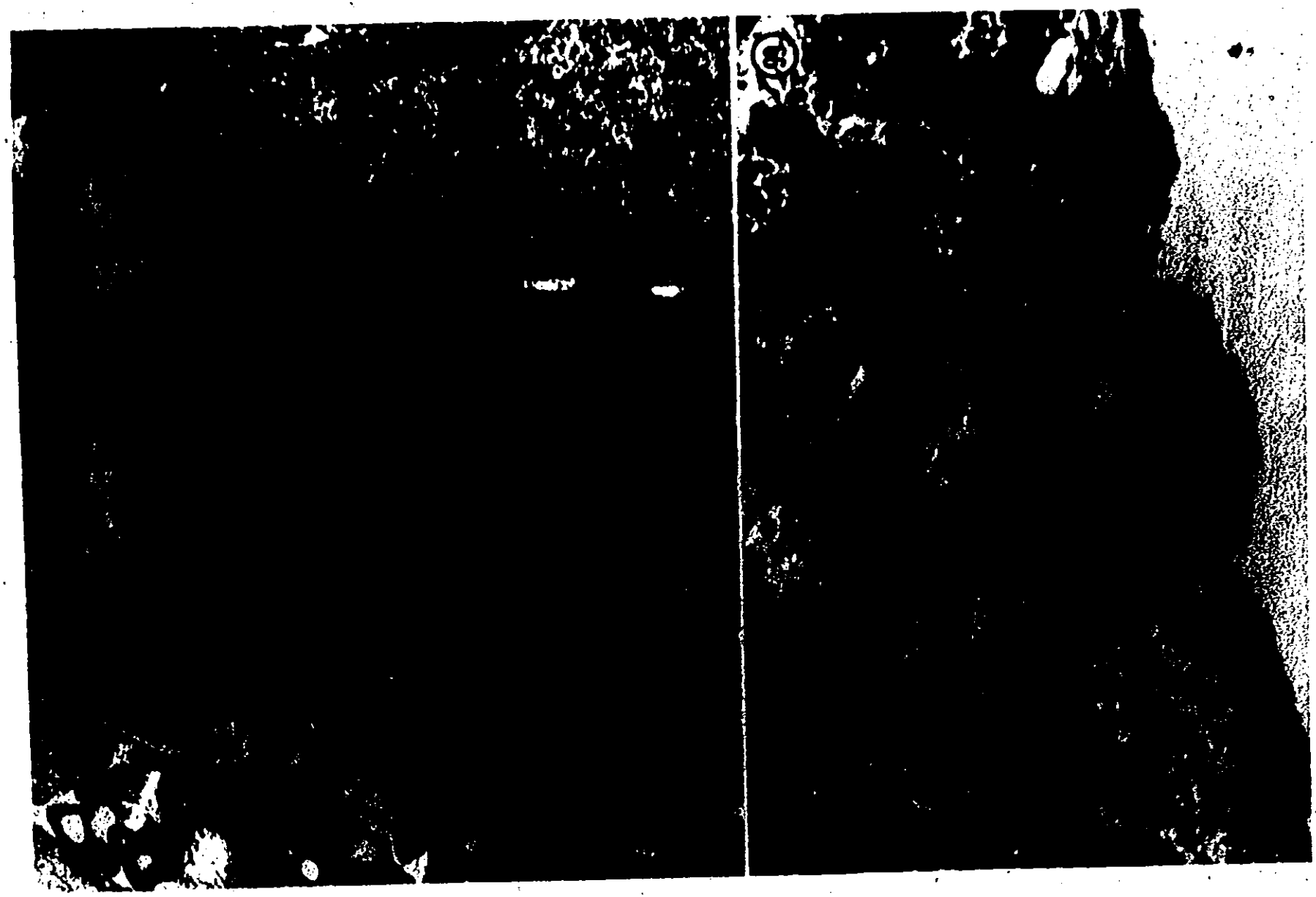


Fig. 65. Eight-day right ovary. The germinal epithelium is represented by a single layer of epithelial cells. BM basement membrane. X 14500.

Fig. 66. Eight-day left testis. The germinal epithelium is constituted only by the epithelial cells and varies in thickness from one area to another. ST seminiferous tubule. X 360.

Fig. 67. Germinal epithelium of the same gonad. Lining bodies (arrowheads) can be seen at the plasma membranes of the epithelial cells. X 21000.



B - ROLE OF VARIOUS HORMONES IN THE DIFFERENTIATION OF FOLLICULAR CELLS IN THE CHICK EMBRYO.

1. Effects of estrogen :

In a first series of experiments chick embryos which had received a single injection of estradiol propionate on the second day of incubation were sacrificed on the fourth and fifth days. Cytological characteristics of somatic cells of the germinal epithelium appeared to be similar in the estradiol treated and in the control embryos of this age (see description on page 79). These experiments were designed in order to find out if estrogens could stimulate the cortical cells to differentiate and form lining bodies before the normal age. The results were negative and no lining bodies were observed.

In a second series, chick embryos that had been injected on the fourth day of incubation were sacrificed on the 6th, 7th, 8th, 10th, 12th or 14th day. The examination of the left gonads of this series failed to disclose any difference between injected and control embryos.

Differences were however, evident in the germinal epithelia of the right gonads from the experimental and the control groups. Thus in the right gonads from 6-, 7- and 8-day embryos treated with estradiol, the germinal epithelium consisted of two or three layers of cuboidal epithelial cells with very few germ cells among

them. Figure 68 shows the germinal epithelium from a 6-day estradiol stimulated gonad. The epithelium is considerably thicker than that of a normal 6-day right gonad as shown in figure 58. Lining bodies were never observed in right gonads of treated embryos at this age.

In 10- and 12-day right gonads from injected embryos, small proliferations of the germinal epithelium started to appear. Lining bodies which were never found in the right gonads of control embryos, were present in about half of the gonads from the injected embryos.

The right gonads from 14-day old estradiol treated embryos were considerably larger than those of control group. The histological examination showed that whereas right gonads from control embryos had very flat covering epithelium (Fig. 69) a thick cortical layer could be observed in the right gonads from the estradiol treated embryos (Fig. 71). The ultrastructural study of these gonads showed that this epithelium, although less developed than the one of the normal left ovaries, was similarly organized. Thus it contained germ cells, some of which were in mitosis and epithelial cells very rich in polyribosomes, mitochondria, small Golgi complexes and little rough endoplasmic reticulum (Fig. 73). Lining bodies were present in all of these gonads (Figs. 72 and 73).

2. Role of pituitary gland in the differentiation of follicular cells:

In order to analyze the role of the pituitary gland in the differentiation of follicular cells, left ovaries from newly hatched chicks were cultured on a purely synthetic medium for periods of 5 and 10 days. The histological examination showed that the explants had cultured well on the synthetic medium and both the somatic and the germ cells survived adequately (Fig. 74).

Although follicles were not formed in the cultures, the ultrastructural aspects of the cells did not show alterations. The submicroscopical aspects of the oocytes showed typical chromosomal filaments of pachytene in their nuclei. Typical Balbiani bodies, constituted by a Golgi complex surrounding the centrioles and a peripheral layer of mitochondria, were present in a juxtanuclear position (Fig. 75).

The epithelial cells of the cortex (pre-follicular cells) from the explanted ovaries showed similar characteristics to those of non-cultured controls. These cells produced lining bodies, were rich in polyribosomes and had very few mitochondria and few profiles of endoplasmic reticulum (Fig. 76).

Fig. 68. Estradiol stimulated 6-day right gonad. The germinal epithelium (GE) is several layers thick in certain areas. m medulla. X 400.

Fig. 69. Right ovary from a 14-day old normal embryo. The cortex is represented by a squamous layer of cells. m medulla. X 400.

(63)

Fig. 70. Left ovary from a 14-day old normal embryo. Thick cortex is constituted by cortical cords (CC). X 360.

Fig. 71. Right gonad from a 14-day old estradiol treated embryo. The cortex is constituted by cortical cords (CC) and is thinner than in the normal left ovary (Fig. 70). m medulla. X 360.

70
71

Fig. 72. Shows at low magnification, a portion of cortex of right gonad from a 14-day old estradiol treated embryo. The basement membrane (BM) of the germinal epithelium is continuous with the cortical cord (CC). The arrowheads are indicating the sites where the lining bodies are being formed at the plasma membranes of the epithelial cells. X 8300.

Fig. 73. Higher magnification of the germinal epithelium of the same gonad as in Fig. 72. Lining bodies can be seen in the process of their formation as well as lying free in the intercellular space (arrows). X 17000.

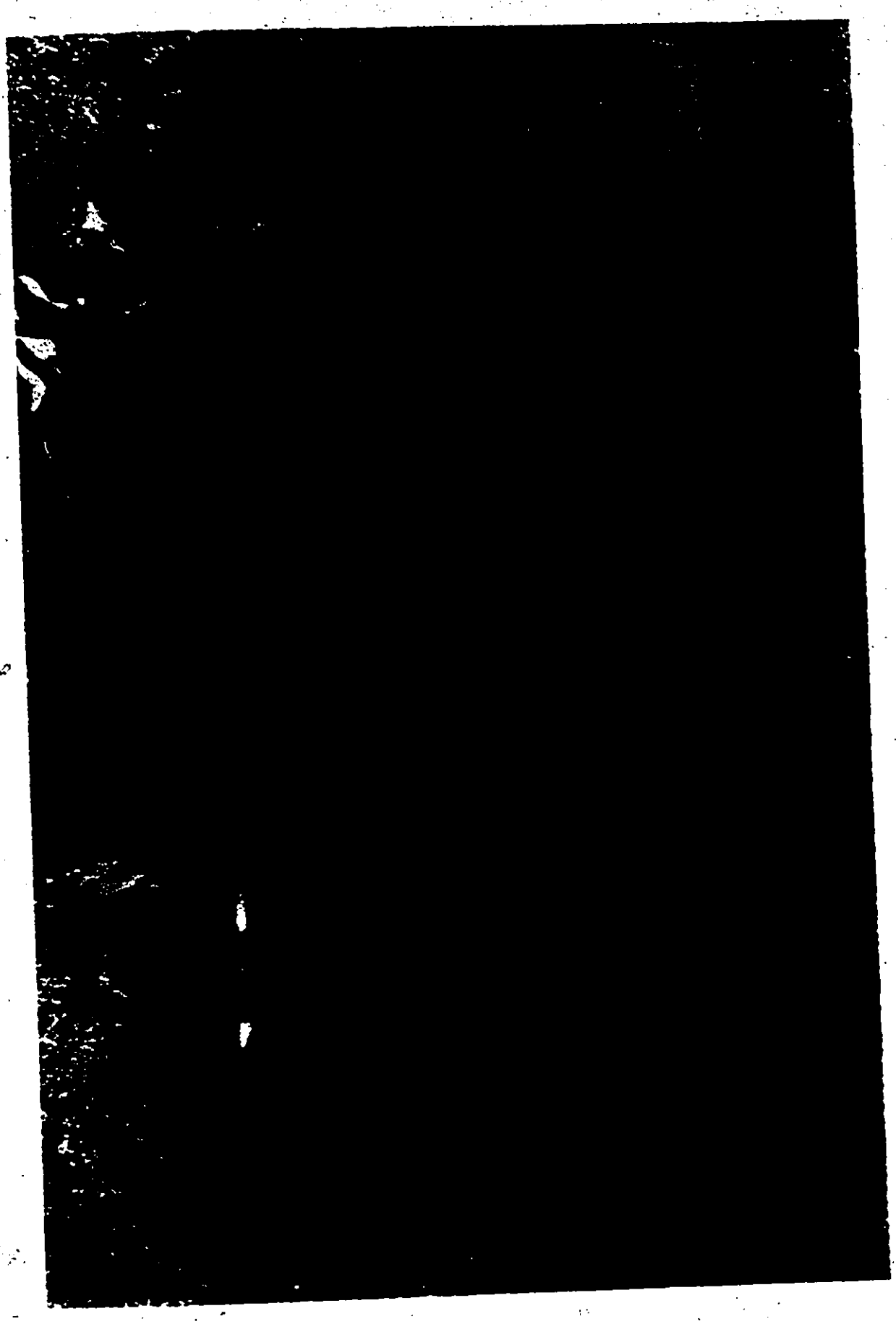


Fig. 74. An explant of left ovary from a newly hatched chick. After 10 days in culture both the pre-follicular cells (F) and the oocytes (O) have survived adequately. X 600.

Fig. 75. Shows an oocyte from the same explant. A typical Balbiani body (BB) and the pachytene chromosomes (Ch) can be seen. F follicular cell, N nucleus. X 14500.

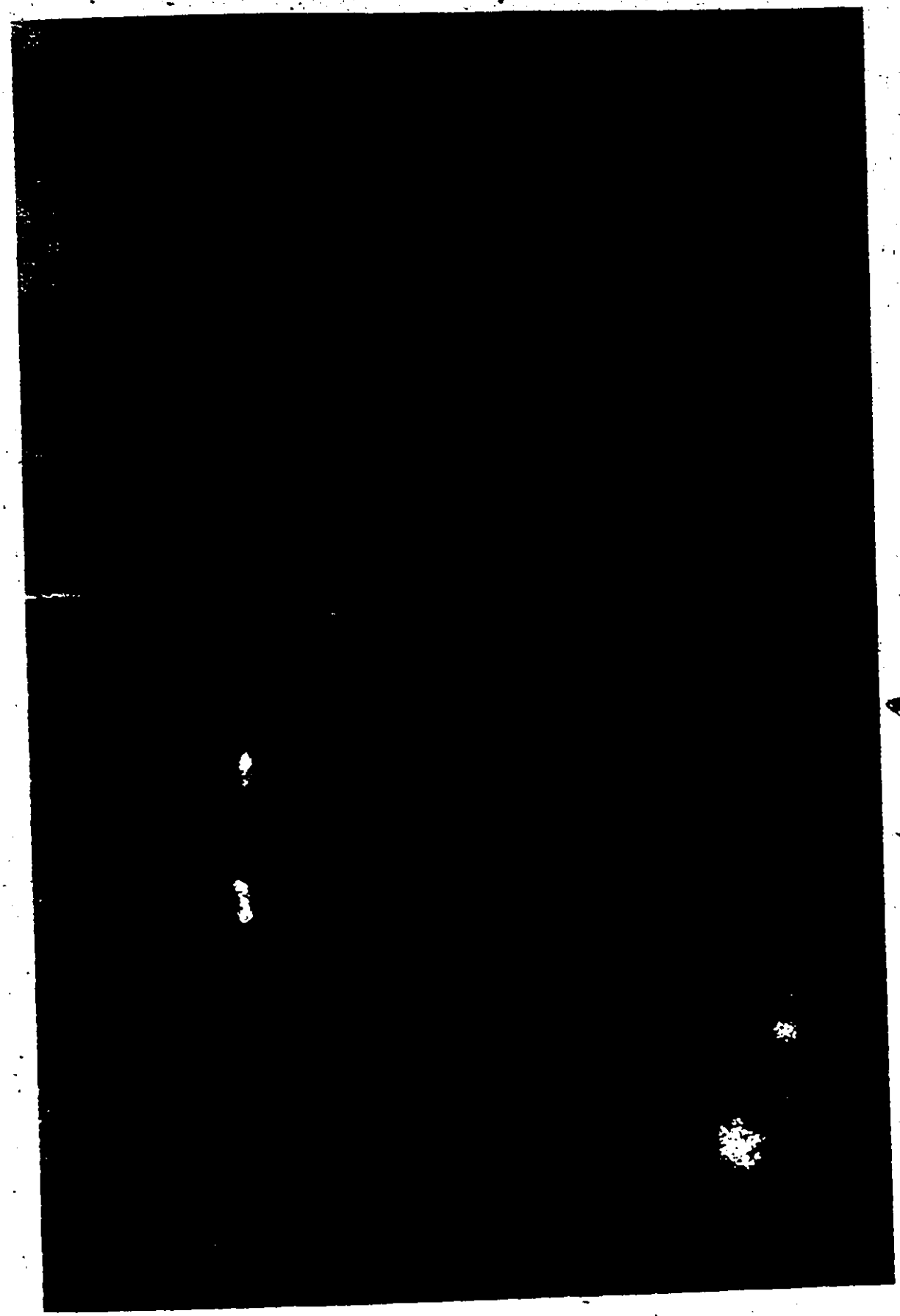


Fig. 76. Same explant as in Fig. 74. Portions of several pre-follicular cells are shown. Lining bodies in different stages of their evolution can be seen. X 18000.

PART-1- COMPARATIVE ASPECTS

The present results have provided a detailed ultrastructural analysis of the relationship between the follicular cells and the oocytes in the turtle and the echidna and have filled gaps in the information supplied by previous studies on the hen by other authors. The comparison of these findings with previously existing literature on other vertebrates has led to some conclusions of interest.

1. FOLLICULAR EPITHELIUM:

In agreement with the classical histological studies on chelonian ovaries (Munson, 1904; Thing, 1918; Witland, 1951), it was observed that in Pseudemys the follicles have a single layer of follicular cells throughout their development.

In the echidna it was observed that the follicular epithelium, which was constituted by a single layer of follicular cells in the smaller follicles, became two layered in follicles ranging from 0.5 mm to 1 mm in diameter and finally returned to a single layer of flat follicular cells in the larger follicles. These observations are contrary to those of Flynn and Hill (1939) in the echidna and Gatenby (1922) in the platypus who observed two layered follicular epithelium in the largest follicles. Garde (1930), however, has described a single

layer of follicular cells throughout the growth of the follicles in platypus. Multilayered follicular epithelium is typical of eutherian mammals. In lower vertebrates more than one layer of follicular cells has been described only in some reptiles such as lizards and snakes (see review by Brambell, 1956) but not in others such as the turtle.

More than one type of follicular cells appears to be present in some vertebrates. The present study has shown that in the turtle two types can be observed in the medium and large sized follicles. 'Clear' and 'dark' cells could be distinguished both with the optical and the electron microscope. Both types of cells showed similar ultrastructural characteristics, the only difference being the relative density of their cytoplasmic matrices. The possible significance of this difference has not been established. Similar observations have been made in the avian (Bellairs, 1965; Narbaitz, 1971) and mammalian ovaries (Weakely, 1966). Bellairs (1965) believes that the dark cells are degenerating; sometimes she observed whole follicles constituted by them and considered these follicles to be atretic. Narbaitz (1971) described dark and clear follicular cells in the ovaries from very young chicks and believes that they represent two stages in their differentiation. Weakley (1966)

found differences in the cellular densities of the follicular cells from hamster ovary and suggested that these differences may be related to the changes in the secretory activity of these cells.

Small, intermediate and large (pyriform) follicular cells have been described in the follicular epithelium of lizards (Porte and Zahnd, 1961; Hubert, 1971a, b; Neaves, 1971; Taddei, 1972) and snakes (Betz, 1968). The large pyriform cells are derived by mitotic divisions of smaller cells (Hubert, 1971a) and are confined only to the earlier stages in the follicle development. Pyriform cells have not been described in vertebrate groups other than lizards and snakes and were not presently observed either in the turtle or in the echidna.

Pyriform cells in the lizard follicles establish large intercellular bridges with the oocytes (Ghiara et al., 1968; Hubert, 1971b; Neaves, 1971; Taddei, 1972). Intercellular bridges between the follicular cells and the oocytes were not observed in the present study and have not been reported in vertebrates other than lizards.

2. SECRETORY ACTIVITY OF THE FOLLICULAR CELLS:

The cytoplasm of the follicular cells in the turtle and the echidna showed signs of synthetic activity as indicated by the presence of a well developed rough endoplasmic reticulum and an extensive Golgi apparatus

with secretory granules. This fact may mean that the follicular cells contribute to the development of the oocyte and/or the formation of the zona pellucida with substances elaborated in their own cytoplasm. Similar observations have been made in amphibians (Hope et al., 1963; Kessel and Panje, 1968), birds (Bellairs, 1965) and mammals (Anderson and Beams, 1960). Bellairs (1965) has suggested that in birds the secretory activity of these cells is restricted to the initial stages in the follicular growth. Contrarywise, both in the turtle and the echidna, although the height of the follicular cells decreases in the largest follicles, their cytoplasm continues to be rich in various organelles characteristic of secretory cells. In agreement with this, Kessel and Panje (1968) using autoradiographic techniques have shown in the ovary of necturus that the follicular cells synthesize proteins at later stages of follicle development.

3. EXTRACELLULAR GRANULES :

In 0.5 to 1 mm follicles of the turtle ovary numerous dense granules were observed in the perivitelline space. These granules were, the same as those present in the cytoplasm of the follicular cells, morphologically similar to glycogen β-particles. They appeared to be released into the perivitelline space by the follicular cells through an apocrine type of secretion. The fact

that these granules did not appear in tissues treated with amylase tends to confirm that they are constituted by glycogen. The significance of extracellular location of these granules has not been clarified but their presence in the intercellular fluid suggests uptake by the oocyte.

Morphologically similar granules have also been found in the echidna, although in a much smaller number and were never observed in an extracellular location as in the turtle. To my knowledge, lizards are the only group in which similar granules have been previously described in an extracellular location (Ghiara et al., 1970; Hubert, 1971b). These authors have suggested a mechanism of release of these granules which is different from that described for the turtle in the present study. Ghiara et al., (1970) believe that after the degeneration of the pyriform cells the dark granules contained in them are released into the perivitelline space. Hubert (1971b) is of the view that the degenerated pyriform cells leave spaces in the follicular epithelium, thus, allowing the granules present in the theca to pass freely into the perivitelline space. The latter author, after cytochemical staining, also concluded that in the case of the lizards these granules were not constituted by glycogen.

4. MICROVILLI:

Numerous microvilli have been presently observed

extending into the perivitelline space of both the turtle and the echidna follicles. In the initial stages of follicular growth microvilli arise both from the follicular cells and the oocyte but at later stages, while they are more branched and larger, they are almost entirely of oocyte origin. The number and size of the oocyte microvilli, however, was much greater in the turtle than in the echidna. Since the microvilli are responsible for the uptake of materials from the perivitelline space into the oocyte (see review by Norrevang, 1968), the more complex organization of this region in the turtle may be related to the fact that its eggs are larger than those of the echidna. This view is further substantiated by the fact that the oocyte microvilli are much less developed in eutherian mammals (Anderson and Beams, 1960; Baca and Zamboni,1967; Hope, 1965) which produce smaller eggs than the echidna and by the fact that in the hen, in which the rate of accumulation of yolk is very high, the microvilli are extraoridinarily well developed and constitute a specialized structure referred to as the zona radiata (Bellairs, 1965; Wyburn et al., 1965b).

The microvilli arising from the follicular cells in the echidna are especially long. Some of these large microvilli penetrate into deep infoldings of the oocyte plasma membrane. This arrangement is, however, restricted

to follicles less than 0.5 mm in diameter. Similar penetrations of elongated cytoplasmic projections of the follicular cells have been described in a variety of mammals including man (Anderson and Beams, 1960; Hope, 1965; Baca and Zamboni, 1967) and probably represent an arrangement to further increase the transporting surface.

Although the presence of interlocking microvilli tends to indicate that an important transport of materials between the follicular cells and the oocytes takes place, the possibility of substances reaching the oocyte directly without crossing the cytoplasm of the follicular cells has to be considered.

In the larger follicles of the turtle ovary, the follicular cells were separated by large intercellular spaces so that the perivitelline space had a continuous passage to the theca separated only by a basement membrane. Thus it would seem possible that the nutrient materials reach the oocyte directly instead of having to pass through the follicular cells. This process, however, does not seem possible in smaller follicles since tight junctions secure the adhesion between adjacent follicular cells. The passage of materials through the extracellular spaces in the follicular epithelium was experimentally demonstrated by Weaves (1972) in the lizards ovaries.This author showed that tracers such as lanthanum, saccharated iron oxide

and peroxidase pass freely through these spaces to reach the perivitelline space where they are finally taken up by the oocyte through pinocytosis. Large intercellular spaces have also been described in fishes (Droller and Roth, 1966) and amphibians (Hope et al., 1963; Kessel and Panje, 1968).

5. PINOCYTOSIS :

An active process of pinocytosis was observed at the plasma membranes of the oocytes both in the turtle and the echidna. In the medium and large sized follicles of the turtle, large oocyte microvilli were full of coated pinocytotic vesicles. This process was observed to a lesser degree in the echidna. Pinocytosis has been observed in oocytes from fishes (Droller and Roth, 1966), amphibians (Wartenberg, 1962), birds (Press, 1964) and mammals (Adams and Hertig, 1964; Lanzavecchia and Mangioni, 1964; Zamboni and Mastroianni, 1966). This phenomenon probably represents the uptake of materials from the perivitelline space by the oocyte and has been demonstrated experimentally by Wartenberg (1964) in the newt by injecting an iron containing dextran (Myofer) and registering its presence in the oocyte pinocytotic vesicles.

6. LINING BODIES, MACROBODIES AND TRANSOSOMES :

The present observations on the follicles of the hen agree in general with those previously described

(Press, 1964; Bellairs, 1965, 1967; Wyburn et al., 1965a, b; Greenfield, 1966; Paulson and Rosenberg, 1972) and have confirmed the presence of specialized organelles known as 'lining bodies'.

The origin and the nature of the granules attached to the inner membrane of the lining bodies is not known. During the present study it was found that, while in the process of their formation, lining bodies may be associated either with the membranes of the rough endoplasmic reticulum or with groups of polyribosomes. This association tends to suggest that the granules of the lining bodies may be derived from ribosomes. In agreement with this, no differences between the size and shape of these granules and those of ribosomes were observed. Bellairs (1965), however, found the granules of the lining bodies to be larger than the ribosomes.

In the past it has been conjectured that the granules of the lining bodies contain RNA, the same as the ribosomes (Schjeide et al., 1963b).

In the present study it was observed that the granules of the lining bodies from sections treated with ribonuclease had been partly dissolved while they were present in the control sections, indicating the presence of some RNA in these granules. This fact together with the previously described association of lining bodies

with the rough endoplasmic reticulum indicates that the granules of the lining bodies probably are ribosomes. (While this study was in progress similar results using RNAse were reported by Paulson and Rosenberg, 1972).

The lining bodies are known to be produced at the plasma membrane of the follicular cells and to be released either into the intercellular space separating the follicular cells or, most commonly, into the perivitelline space. The final fate of these bodies has been discussed and while some authors maintain that they are incorporated into the oocyte (Bellairs, 1965) where they become associated with yolk granules (Bellairs, 1967), the others believe that these organelles remain permanently attached to the follicular cells (Press, 1964; Wyburn et al.,1965a).

The present study has shown that the lining bodies are incorporated by the oocyte where they cluster to form large macrobodies. These macrobodies were not seen in association with other oocyte cytoplasmic organelles. The macrobodies have been previously described only in the ovaries from very young chicks (Greenfield, 1966). Since macrobodies appear to be predecessors of yolk granules the present observations support Bellairs (1967) findings in the sense that lining bodies are finally incorporated into yolk granules.

Since 'lining bodies' always contain, in addition

to the membranes and granules, a substantial amount of cytoplasmic matrix, their incorporation into the yolk granules in the oocyte probably represents a supply of raw materials for the formation of yolk. In addition, the presence of RNA in these granules as shown in this study suggests that they may also play an informational role in the organization of yolk granules.

The small, rounded or horse-shoe shaped organelles observed in the turtle ovaries have been termed 'transosomes' in the previous section. These organelles differed morphologically from those described in the hen as 'lining bodies' (Bellairs, 1965) or 'transosomes' (Press, 1964) in that they were much smaller in size and in that no granules were present on their inner membrane. Inspite of these morphological differences, since the organelles found in the turtle are similarly transported from the follicular cells to the oocyte, the term 'transosomes' seems appropriate. The fact that these organelles were seen incorporated into pinocytotic vesicles and into yolk granules suggests that they may play some role in yolk formation as happens with the lining bodies in the hen (Bellairs, 1967). Additional information is needed to prove this assumption.

Organelles comparable to bird and turtle 'lining bodies' or 'transosomes' were never observed in the echidna

ovaries. The fact that these organelles are complex and very numerous in the hen in which the rate of production of yolk is very high, that they are simpler and less numerous in the turtle which produces smaller eggs and at a slower rate and, finally, that they are not found in other vertebrates in which the rate of production of yolk by each oocyte is significantly lower, reinforces the belief that their main function is to contribute to vitellogenesis.

FORMATION OF THE ZONA PELLUCIDA:

In the turtle formation of the zona pellucida was expressed at the ultrastructural level by the accumulation of a filamentous material. In the echidna this material appeared as filamentous or amorphous in different follicles. These differences are probably related to the state of fixation of each follicle as has also been suggested in amphibians (Wartenberg, 1962).

The zona pellucida is a characteristic of developing follicles of all vertebrates and its origin has not been established (see review by Norrevang, 1968). Studies on mouse, rat, guinea-pig, hamster and cat follicles have shown that the material of the zona pellucida arises as discrete and separate parts which secondarily form a complete layer enveloping the oocyte (Chiquoine, 1960; Franchi, 1960; Odor, 1960). Chiquoine concluded that such an

origin suggests the activity of several cells rather than a single cell thus indicating the role of follicular cells rather than that of the oocyte in the formation of the zona pellucida.

In the echidna the material of the zona pellucida appears in a similar manner as described by Chiquoine for other mammals. In the turtle, however, since a perivitelline space appears before the formation of the zona pellucida such relationship could not be established. In this case the zona material was localized in the outer zone of the perivitelline space suggesting that it may be produced by the follicular cells. Similar material, however, could be found in the superficial layer of the oocyte cytoplasm indicating a possible participation of the oocyte as well. This would agree with the conclusions drawn by Wartenberg (1962), Hope et al. (1963) and Hope (1965) from studies in amphibians and mammals. These authors have described two morphologically (Wartenberg, 1962; Hope et al., 1963; Hope, 1965) and histochemically (Wartenberg, 1962) distinct zones in the zona pellucida and suggested a different origin for each of these two zones.

8. ADDITIONAL OBSERVATIONS:

Although special attention was paid to the ultrastructural aspects of the follicular cells and to the zone separating these cells from the oocyte, several morpholo-

sical details of the peripheral cytoplasm of the oocyte have been also analyzed and seem to be of some interest.

In the oocytes of the echidna, specialized structures referred to as 'Fibrillar bodies' and ranging in size from 1-2 μ in diameter were observed. These structures were bound by a unit membrane and contained irregularly arranged bundles of fibres 150-250 Å in diameter. Since many bodies with structures which appeared to be intermediate between these 'fibrillar bodies' and the fully formed yolk granules were frequently observed, it is concluded that those bodies represent initial stages in the formation of yolk granules.

Another interesting feature of the echidna oocytes was the presence of an unusual number of elongated tubular structures in their peripheral cytoplasm. These structures seemed to increase with the follicular size and were often found associated with large vesicles or primitive yolk granules, indicating their possible role in the formation of yolk. Bellairs (1967) has described somewhat similar but slightly larger structures in the hen oocytes (about 700 Å in diameter in hen as compared to 400-600 Å in the echidna) and called them yolk spindles. She found them associated with large vesicles leading to the formation of yolk granules.

PART - II EMBRYOLOGICAL ASPECTS

1. ULTRASTRUCTURE OF THE GERMINAL EPITHELIUM OF THE CHICK EMBRYONIC GONAD.

Several ultrastructural studies on the germinal epithelium of the chick embryonic gonad are available in the literature (Dubois and Cuminge, 1967, 1969; Cuminge and Dubois, 1969a, b, 1971; De Simone-Santoro, 1969). The present observations on the submicroscoical aspects of the germinal epithelium of gonads of earlier ages of chick embryo agree in general with these authors and in addition have revealed certain interesting aspects which had not been previously described.

Cuminge and Dubois (1969a, b, 1971) described characteristic short cytoplasmic processes of the somatic cells of the germinal epithelium, which they called 'boursouflures' (blebs), and which were eliminated from the epithelial cells by a merocrine type of secretion. These authors further state that a regular basement membrane is absent at the basal poles of epithelial cells of gonads from 2- and 3-day old embryos and that the 'boursouflures' are present at both the apical and the basal poles of these cells at this age. Thus, the content of the 'boursouflures' could be eliminated not only

towards the coelom but also towards the blood circulation. The idea of a secretion was further substantiated by autoradiographic studies of the same authors (Cuminge and Dubois, 1969b, 1971) indicating that the proteins are being synthesized by the epithelial cells and secreted through the 'boursouflures'. Dubois (1964, 1965, 1966) showed in his 'in vitro' studies that the germinal epithelium exerts a chemotactic attraction on the germ cells and it is possible that the secretion represented by the 'boursouflures' constitutes a chemotactic agent.

Cuminge and Dubois (1971) mentioned that the 'boursouflures' start to disappear after the fourth day. Contrarywise, it was presently observed that these cytoplasmic processes were still present in all 6-day gonads and in both the testes and the ovaries from 8-day embryos although in these cases restricted only to the apical portions of the epithelial cells. Another observation presently made was the asymmetrical distribution of 'boursouflures' between the left and the right gonads from six day old embryos; these cellular processes were always clearly larger in size and more numerous in the left gonads. The meaning of this asymmetrical distribution is discussed on page 122.

A network of fine microfilaments distributed throughout the cytoplasm of the epithelial cells of gonads

from 4-day old embryos was observed in the present study. The number of microfilaments seemed to increase in these cells from gonads of 5-day old embryos. Microfilaments have been described in other embryonic and undifferentiated cells as being contractile in nature and playing a role in cell shape changes that accompany several morphogenetic phenomena (see review by Wessels et al., 1971). Thus, in vitro studies have shown that by adding cytochalasin B (a fungal metabolite which disrupts microfilaments) to the culture medium, the formation of furrows and invaginations can be prevented (Wrenn and Wessels, 1970; Spooner and Wessels, 1972). Similarly in the case of embryonic chick gonads the presence of microfilaments may be related to the formation of sex cords since these start to be formed around the fifth day of incubation (Swift, 1915, 1916) correspondingly with the increase in the number of microfilaments.

Lining bodies appeared for the first time in gonads from 5- and 6-day old embryos. These organelles observed in the embryonic epithelial cells were much smaller in size and fewer in number than in the follicular cells of the adult hen and while they are 0.1-0.15μ in length in the embryonic cells, they may reach well over 1μ in length in the adult hen. It is evident from the present study on the growing follicles of the hen and

from the past literature on the same subject (Bellairs, 1963; Wyburn et al., 1965a; Greenfield, 1966) that the lining bodies are a characteristic product of the follicular cells. Since the follicular cells are derived from the somatic cells of the germinal epithelium of embryonic gonads, the presence of lining bodies in the epithelial cells of gonads from 5- and 6-day old embryos suggests that the initial steps in the differentiation of follicular cells begins at this early age.

The classical 'inductor' theory of sex differentiation first put forward by Witschi (see reviews by Burns, 1961; Witschi, 1963) maintains that an inductor substance (corticin) is secreted by the gonadal germinal epithelium which influences the differentiation of the medulla. In addition, it has also been shown that the differentiation of oocytes is dependent on the influence of neighbouring follicular cells (Haffen, 1963). Additional research is needed to find out if the production of lining bodies by the embryonic pre-follicular cells is related in some way to the corticin secretion or to follicular cell-germ cell interaction.

The present findings, showing that the lining bodies which are typically ovarian organelles are initially present in the cortex of both the male and the female embryos, tend to support the classical concept on the

bisexual nature of the vertebrate gonad (see reviews by Willier, 1939; Burns, 1961; Franchi, 1962).

It is known that gonads are asymmetrical in most adult birds. This asymmetry is especially noticeable in females in which only the left ovary is functional, the right one being rudimentary (see reviews by Domm, 1939; Van Tienhoven, 1961). It has also been shown that this asymmetry is already evident during the embryonic life and even before sex differentiation (Swift, 1914, 1915; Arndt, 1928; Witschi, 1935; Venzke, 1954). There is a clear difference in the height of the germinal epithelium and the number of germ cells between the right and the left gonads. It has also been shown that the germinal epithelium of the right gonads starts to regress after the fifth day (Wolff and Wolff, 1948). In agreement with this the present observations have shown that ultrastructural differences between both sides can be observed after the sixth day. Thus, at this age, 'boursouflures' appeared to be more numerous in the left gonads and the lining bodies were consistently absent in the right gonads.

The most common explanations for the atrophy of the right cortex in bird embryos are either that the right cortex is inherently deficient (Witschi, 1935; also see review by Domm, 1939) or that the right cortex

starts regressing before the left side thus not allowing time for the stimulatory action of the estrogens secreted by the medulla (Wolff and Wolff, 1948; Wolff and Pinot, 1961). The present findings, showing that the right cortex does not form lining bodies, would support the first view. Additional information on this point has been supplied by experiments with estrogen stimulation discussed in the following section.

2. ROLE OF ESTROGENS IN THE DIFFERENTIATION OF THE GERMINAL EPITHELIUM.

Classical works (Wolff, 1947; Mintz and Wolff, 1952, 1954) have shown that the growth and differentiation of the ovarian cortex is dependent on some factor secreted by the medulla. Since estrogens are capable of simulating this action of the medulla on the cortex (Wolff and Ginglinger, 1935; Willier et al., 1935, 1937; Dantchakoff, 1935, 1936) it was concluded that the estrogens are the natural inductor of cortical differentiation (Wolff and Haffen, 1951; Weniger, 1958a, b, 1961).

This assumption has received additional confirmation from the following facts: a) the synthesis of estrogens by the embryonic ovary has been demonstrated biochemically (Gallien and LeFoulgoc, 1957; Weniger and

Zeiss, 1971) and the site of synthesis has been localized by histochemical techniques in the medulla (Narbaitz and Sabatini, 1963a,b; Narbaitz and Kolodny, 1964; Chieffi et al., 1964; Boucek et al., 1966; Scheib and Haffen, 1967, 1968, 1969). Weniger and Zeiss (1971) have demonstrated that the estrogens are secreted by the undifferentiated gonad on the sixth day of incubation which is precisely the age at which the lining bodies start to appear in the cortical cells as shown in the present study.

The above conclusion suggested the need of establishing if the appearance of the lining bodies was a direct response to estrogen stimulation. This question appears to be negatively replied by the present experiments which show that the early injections of estrogen are neither able to cause the appearance of lining bodies in gonads from embryos younger than five days nor cause an increase in their numbers at later ages.

Since the rate of absorption of estrogens at early ages is not known these experiments should be considered as tentative. Especially so since they contradict earlier studies by Dahl (1971) in the adult hen which showed that the lining bodies do increase with estrogen stimulation.

Narbaitz (1971) has shown that after estrogenic stimulation the cortex of the genetical male left gonads persists and continues to produce lining bodies. The

present observations have shown, in addition, that estrogens are also capable of stimulating the appearance of lining bodies in the right cortex which normally does not form them. The response of the right side is, however, slower than the one of the left side since differentiation of lining bodies in stimulated right gonads does not occur untill the tenth day. This difference in response would also support the previously discussed idea of an inherent deficiency of the right cortex.

3. ROLE OF PITUITARY IN THE DIFFERENTIATION OF THE FOLLICULAR CELLS :

Experiments with partial decapitation (hypophysectomy) by Fugo (1940) showed that sex differentiation occurs in the absence of pituitary stimulation and similar conclusion has been reached by culturing undifferentiated gonads in synthetic media (Stenger-Haffen, 1957). Fugo (1940) also observed that some influence of pituitary secretion on gonads is present after the thirteenth day. However, this is true for certain aspects of gonadal differentiation such as steroid secretion (Woods and Weeks, 1969) but not for others such as oocyte maturation (Fugo, 1940; Narbaitz and Adler, 1966b).

Since the first stages in the differentiation of follicular cells occur at an age in which the pituitary

has not differentiated one must therefore assume that they do not require the stimulus of pituitary hormones. The present experiments showing that the lining bodies persist in ovaries of newly hatched chicks culture in the synthetic medium appear to indicate that the control of the pituitary over the differentiation of follicular cells is not required either at the end of the embryonic period.

In the adult hen, however, Dahl (1972) has shown that pituitary hormones are capable of increasing the production of lining bodies.

S U M M A R Y

The present thesis includes ultrastructural studies on comparative and developmental aspects of ovarian follicular cell differentiation.

The comparative studies were carried out in order to establish if special transporting organelles such as those described in bird ovaries as lining bodies or transosomes were also present in the ovaries of two other vertebrate groups producing heavy-yolked eggs. As a result of those studies it was established:

1. Small, rounded or horse-shoe shaped transosomes, constituted by two thick membranes separated by a material of lesser density, were observed in the follicles of the turtle Pseudemys scripta. These organelles probably are homologous to the transosomes or the lining bodies described in the bird ovaries. They differ morphologically from them in that they are much smaller in size and in that no granules are present on their inner membranes. No comparable organelles were observed in the ovaries of echidna.

The fact that lining bodies or transosomes are large and very complex in the hen in which the rate of yolk production is very high, that they are smaller,

simpler and less numerous in the turtle which produces fewer and smaller eggs and that they are absent in other vertebrate groups (including the presently studied echidna) in which the rate of production of yolk by the oocyte is lower, supports the idea suggested by several authors in the sense that their main function is related to vitellogenesis.

2. As a part of the comparative study, additional observations on adult hen ovaries were made. It was shown that the granules present in the lining bodies are partly digested by ribonuclease. This fact, together with the observed close relationship of some lining bodies with the rough endoplasmic reticulum, tends to support the view that the granules are ribosomal in nature. Their passage from follicular cells to oocytes may constitute a transfer of information. A definitive answer to the question of the function of lining bodies would require biochemical analysis of organelles isolated by differential centrifugation.

3. In addition to the information on the existence of transosomes in the turtle and their absence in the echidna, the ultrastructural details on the ovaries of these two species supplied interesting details of the morphology of follicles. Since no ultrastructural study had been made previously in other species of Chelonia

and Mesotremata, the obtained information is of particular interest to comparative morphologists. Among the numerous details analyzed, the demonstration of extracellular glycogen observed in Pseudemys is particularly intriguing and suggests uptake by the oocytes.

The developmental aspects analyzed in this thesis, were carried out in chick embryos and were designed to establish the age of appearance of lining bodies in the ovaries. It was shown that lining bodies are already present in the cortical epithelial cells in the first stages of ovarian differentiation. This fact indicates that differentiation of follicular cells starts at that age. Surprisingly enough, the lining bodies were also found in the cortical zone of undifferentiated gonads from both male and female embryos. In addition to satisfy the original purpose of the investigation, which was to establish the age of appearance of lining bodies, the study supplied interesting information bearing upon the current theories of gonadal differentiation:

1. The cortical origin of the follicular cells was confirmed.

2. The present observation on the presence of lining bodies in undifferentiated gonads of both genetical sexes, constituted the first demonstration, at the ultrastructural level, of the accepted principle of bisexual

nature of the undifferentiated vertebrate gonad.

3. The absence of lining bodies in the cortex of right gonads supplied an ultrastructural evidence of the defective differentiation of this cortex; this observation contributes to the understanding of the fact that in most birds ovarian cortex does not develop usually in the right side.

4. The possibility that the formation of lining bodies in the embryonic gonads may be due to a direct response to estrogen stimulation was investigated and ruled out since early injections of estrogen failed to cause the appearance of these organelles in gonads from embryos younger than five days and to cause an increase in their number at later ages. The estrogens were, however, capable of stimulating the appearance of lining bodies in the right gonadal cortex which usually does not form them.

5. The electron microscopical study of portions of left ovaries cultured on a purely synthetic medium showed that pre-follicular cells continue to form lining bodies in vitro. This fact appears to indicate that extra-gonadal hormonal stimulation (gonadotrophins) is not required for their differentiation.

6. The function of lining bodies during the embryonic period remains unclear. An analysis of the action

purified fractions isolated by differential centrifugation from adult ovaries and tested in vivo and/or in vitro on differentiating embryonic gonads appears as a logical approach to the solution of this question.

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UNIVERSITÉ D'OTTAWA / UNIVERSITY OF OTTAWA
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TITLE OF THESIS ULTRASTRUCTURAL AND CYTOCHEMICAL ANALYSIS OF THE DIFFERENTIATION OF OVARIAN FOLLICULAR CELLS: A COMPARATIVE AND EMBRYOLOGICAL STUDY

DEGREE Ph.D. YEAR GRANTED 1973

(Histology and Embryology)

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