

# STUDIES ON THE INFLUENCE OF THE ZONA PELLUCIDA IN ATRESIA <sup>1</sup>

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THREE PLATES (SIXTEEN FIGURES)

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## INTRODUCTION

In a previous paper the author has reported on the follicular cycle of the striped ground squirrel (*Citellus tridecemlineatus* Mitch.) Pliske ('38). An attempt was made to follow the course of differentiation of egg cells from their initial appearance in the germinal epithelium through their subsequent developmental history leading to maturity.

The present report embodies a study on the vast number of follicles of all developmental stages which undergo atresia

<sup>1</sup> The ovarian material used in this study was obtained from the Department of Zoology, University of Minnesota, Minneapolis Minn.

in the ground squirrel ovary at different periods of the oestrous cycle. The main purpose of this investigation has been to ascertain the type or types of atresia which unilaminar, multilaminar and mature follicles undergo and to correlate the presence of possible atretic types with the various periods of the oestrous cycle.

Although the contributions of Asami ('20), Kingery ('14), Stockard and Papanicolaou ('17), Evans and Swezy ('29), and more recently Garde ('30), Guthrie and Jeffers ('38a), Pincus and Enzmann ('37), and Kingsbury ('39) have shed much light on many of the perplexing problems relative to atresia, there still remain problems which require elucidation. The behavior of the zona pellucida from the onset of atresia to its final disappearance among invading connective tissue cells is a matter which is not clearly understood. In this connection special consideration has been given to the zona pellucida in an attempt to determine what, if any, influence it may have on the course of atresia.

#### MATERIALS AND METHODS

The materials and methods employed in this study are the same as those presented in a previous publication. (Pliske, Edward, C. 1938, *Journal of Morphology*; Vol. 63, no. 2, pp. 263-287). Measurements were accomplished by use of a 7.5  $\times$  eye micrometer. In all cases measurements were computed from a point on the basement membrane of the follicle to the nearest surface of the germinal epithelium.

#### OBSERVATIONS

Since considerable variation exists in the literature in defining the various developmental stages of ovarian follicles, it is desirable to define the developmental stages referred to throughout the text of this presentation. Primary follicles and unilaminar follicles are used synonymously and refer to ones which possess but one layer of investing follicle cells. Multilaminar follicles are defined as immature and mature types. The former contain two or more investing layers but

no follicular cavity. The latter refers to a highly cellular follicle in which the follicular cavity and discus proligerus are well-defined.

### *1. Types of atresia in primary follicles*

*Type I.* Primary follicles which undergo type I atresia manifest initial changes in the ovum. Nuclear changes usually precede those of the cytoplasm; however, the reverse is occasionally encountered. Degenerative changes in the nucleus are indicated by wrinkling of the nuclear membrane, dissolution of the chromatin material and disappearance of the nucleolus. These changes are rapidly followed by changes in the cytoplasm. The deutoplasmic material, which in the developing ovum has a flaky appearance and uniform distribution, exhibits a clumped granular appearance in the early degenerating condition. Coincident with these changes in the nucleus and cytoplasm, obvious ones occur in the zona pellucida.

The delicate protoplasmic processes which span the hiatus between the inner end of the follicle cells and the zona pellucida expand far beyond the condition of those in a normal follicle. Examination of the processes reveals that their increase in diameter is caused by accretive growth from materials of the zona pellucida which in an apparently liquefied condition, flows down the processes as an hyaline investment. The identity of the protoplasmic processes is quickly lost once they become hyalinized. Expansion of the processes continues until adjacent ones fuse to form wide bands which are frequently much wider than the zona pellucida itself (figs. 1 and 2). Such processes, varying greatly in width, may develop at any point on the zona pellucida.

The broader processes are observed to surround from one to three follicle cells and then attach to the basement membrane of the follicle (fig. 1). Follicle cells thus enclosed become intensely pycnotic and release their nuclear contents into the substance of the process which encloses them. Fol-

liele cells which have not been surrounded remain normal for a longer time.

While expansion and fusion of the processes are occurring, changes on the inner surface of the zona pellucida (immediately adjacent to the ovum) become apparent. Centripetal extensions from the zona pellucida in the form of long, slender filaments enter the substance of the ovum. From the free end of the filaments secondary and tertiary subdivisions develop which divide the ovum into pockets or compartments. Figure 3 shows several of these filaments spanning the space formerly occupied by the ovum. Once the zona pellucida has produced broad processes from its outer surface and slender ones from the inner surface, it becomes vacuolated and increases slightly in width (fig. 3).

Coincident with the vacuolation of the zona pellucida proper, vacuoles appear in the peripheral processes immediately adjacent to the enclosed follicle cells. Coalescence of the vacuoles forms irregular but continuous channels from the follicle to the interior of the ovum. Chromatin debris from the degenerating follicular epithelium enters these channels and migrates into the ovum. In some follicles this phenomenon is striking, while in others it is greatly reduced or entirely absent.

The next stage of atresia occurs with the collapse of the zona pellucida (figs. 2 and 3). Collapse may occur at one or more points on its surface. The invaginating surfaces are usually restricted, however, to those sectors from which broad processes fail to develop. As collapse of the zona pellucida progresses the broad bands adjacent to the collapsing surface withdraw from the follicular epithelium and by so doing, rupture the basement membrane. Continued collapse of the zona pellucida results in a more or less complete fusion of its opposing walls (fig. 2). The compressed, fragmentary remains of the ovum stain intensely with basic dyes. It appears likely that this staining quality of the ovum is augmented by nucleic acids derived from debris of the follicular nuclei. Subsequently the egg substance progressively loses its staining

quality and disappears by autolysis. No cells which could be regarded as phagocytes assist in the disorganization of the ovum.

When the zona pellucida is in a collapsed condition, the most striking events which occur in the final involution of the follicle are observed in the remaining follicular epithelium and connective tissue of the simple theca. Directly opposite a collapsed surface of the zona pellucida a cone of cells develops (figs. 2 and 3). The cone is composed at first of follicular cells only (fig. 2); later by a mixture of follicular cells and fibroblasts (fig. 3); and ultimately of fibroblasts entirely. The fibroblasts appear to be derived from the simple theca. They migrate through the ruptured basement membrane to mingle with and eventually replace the follicular elements of the cone. When the large majority of cells in the cone are fibroblasts, the follicular elements degenerate. The pycnotic nuclei in the cone of figure 3 are those of degenerating follicular cells.

The low incidence of mitoses in the invading fibroblasts suggests that their growth into the follicle is caused more by pressure from expanding structures in the stroma rather than by cell multiplication. As the cone becomes highly cellular, the zona pellucida through continued vacuolation becomes the structure of a delicate, hyaline reticulum (fig. 13). This structure is, however, rapidly obscured as the connective tissue cells increase in number. Subsequent orientation of these cells so that their axes conform with those of the surrounding stroma marks the end of atresia.

Garde ('30) studying the degenerative sequence in follicles of all sizes in the ovary of *Ornithorynchus*, observed that the ova of small follicles show initial changes in the nucleus and cytoplasm. Epithelial ingrowths and progressive complicated foldings of the zona pellucida indicate a later stage in the degenerative sequence. Garde further observed that the cells of the epithelial ingrowths pass through the zona pellucida and enter the egg cytoplasm where they act as typical phagocytes. Guthrie and Jeffers ('38a) reported early pycnosis and retrogression of the oocyte in atretic unilaminar and

early multilaminar follicles of the bat. They noted that destruction of the ovum is accomplished by phagocytosis and fragmentation at the center of a mass of granulosa cells. These cells were not observed to divide or degenerate. They were unable to determine the final fate of the zona pellucida from their material.

Although prominent cones develop in type I follicles of the ground squirrel, none were observed to contain phagocytic cells in excess of those normally found in the surrounding connective tissue. In no instance were phagocytes found to penetrate the zona pellucida directly opposite the cone or at any other place.

*Type IA.* Primary follicles which undergo type IA atresia closely resemble those described as type I. They follow a similar course of atresia with regard to nuclear, cytoplasmic and follicular changes. The zona pellucida of type IA follicles, however, show such marked changes from type I that they require separate consideration as a group.

The zona pellucida of this type usually shows a high degree of vacuolation in one sector of its wall before any signs of collapse are apparent. The vacuolated area extends peripherally until it fuses with the basement membrane of the follicular epithelium. The strength of the wall appears to be weakened in the vacuolated sector to such an extent that the remaining parts of the zona pellucida become compressed into an elongate horseshoe-shaped structure by the surrounding stroma. The ovum thus compressed undergoes progressive cytolysis until it entirely disappears. A cone of cells may or may not develop at the vacuolated end. When it does, the invasion of follicular cells and fibroblasts occurs in precisely the same manner as in type I. When a cone does not appear, fibroblasts enter the vacuolated end of the zona pellucida through widely separated apertures in the basement membrane. Progressive vacuolation of the remainder of the zona pellucida and continued invasion by connective tissue cells ends the process of atresia.

*Type II.* Primary follicles which follow type II atresia reveal early changes in the nucleus. The nuclear membrane fragments into a number of minute filaments and permits the nuclear contents to be released into the cytoplasm. Nuclear remains are perceptible for only a short time once atresia begins. They rapidly lose their staining qualities and disappear.

Cytoplasmic changes progress very slowly. The earliest atretic changes occur in the deutoplasm. These consist in the clumping of yolk granules to form anastomosing cords (fig. 4). As involution progresses the cords become increasingly heavier until they finally fuse to form darkly staining patches of variable shape and size. An irregular clear space develops between the periphery of the ovum and the inner surface of the zona pellucida. Figure 4 shows an ovum in which this space has developed to a slight degree. In later stages of atresia the space becomes very prominent (figs. 5 and 6). Shrinkage of the ovum is the factor which appears to be responsible for the formation of this halo-like space.

Mossman ('37) studying the ovary of *Geomys*, observed that a space always forms around an atretic ovum regardless of whether it was contained in a primary or mature follicle. This observation is in agreement with only type II atretic primary follicles of the ground squirrel ovary. In types I: IA and III no space was observed.

As the foregoing changes occur in the ovum, the zona pellucida increases slightly in diameter. It is of interest to note that a portion of the zona pellucida reveals well-defined stomata even though atresia has progressed to the state depicted in figures 5 and 6. The development of broad processes from the zona pellucida to the follicular epithelium which are so characteristic in types I and IA, do not occur in type II. A few delicate fibrils barely within the limit of vision span the space between the follicle and the zona pellucida. The ovum as a matter of fact appears to float freely in the follicular cavity (figs. 5 and 6).

While the ovum and zona pellucida undergo the changes just described, the cells of the follicle rapidly involute and disappear. Degeneration of the follicle cells is manifested by progressive karyolysis. Free chromophilic granules which appear between the zona pellucida and the follicular epithelium suggest that karyorrhexis may also occur in some sectors of the follicle.

Although the follicular elements undergo rapid involution, the basement membrane of the follicle persists for a longer period as a laminated, hyaline structure. Its identity remains as long as a few of the follicular cells remain attached to it. When the last of the follicular cells degenerate, the basement membrane appears to be incorporated into the collagenous and elastic fibers of the surrounding connective tissue. Whether the basement membrane exerts any influence on the manner in which connective tissue enters the follicle, is difficult to determine.

Invasion by connective tissue in type II follicles proceeds very slowly. It will be noted subsequently that type II follicles which begin atresia in prooestrus frequently do not complete their final stages until oestrus or later. After the follicular epithelium disappears, the follicular cavity is lined with extremely flattened fibroblasts. The actual ingrowth of connective tissue occurs at a considerably later period. When it does occur, however, it follows one of the two following courses. (1) Contraction of the delicate processes of the zona pellucida appears to loosen the fibroblastic lining as shown in figure 6. Loosening of the lining was observed repeatedly in those regions where processes are abundant. As the cells approach the zona pellucida, others follow. Amoeboid cells and fibroblasts eventually form a loose syncytium throughout the follicular cavity. Although a variable number of amoeboid cells enter the ovum, they quickly undergo pycnosis and degenerate. Later the zona pellucida ruptures and releases its contents into the cavities of the syncytium. The dark, granular remains of the ovum persist for a considerable time among the invading cells and then disappear. (2) Follicles in



which the delicate processes are few or entirely absent show a different course of connective tissue invasion from that described above. Amoeboid cells span the narrowest region of the follicular cavity and enter the ovum through stomata of the zona pellucida before a syncytium develops. The ovum in figure 6 contains a number of amoeboid cells within its substance. Several others are in the act of entering the stomata. A clear space forms around each amoeboid cell contained within the ovum. These spaces increase in size until adjacent ones fuse. The activity of the amoeboid cells appears to be one of extracellular digestion rather than of actual phagocytosis. Although amoeboid cells appear to be a primary factor in removal of the egg, the zona pellucida also plays an important role. It will be noted in figure 6 that the zona pellucida is indistinguishable from the darker staining masses of the ovum. When figures 5 and 6 are compared it becomes evident that much of the egg substance in figure 6 has diffused into the zona pellucida, whereas in figure 5 this has not occurred. Figure 7 shows a later stage in which no trace of the ovum remains except that which is contained within the zona pellucida.

There appears to be a wide variation in the consistency of the zona pellucida of type II follicles. In instances where amoeboid cells effect an early entrance into the deeply staining mass of the ovum, the zona pellucida absorbs much of the debris. If, however, no amoeboid cells are present, the substance of the ovum and zona pellucida remain entirely separate. In the latter instance, the zona pellucida may be so brittle that it is impossible to section such follicles without completely destroying their structure.

Regardless of whether invasion follows course (1) or (2), the zona pellucida fragments and disappears among the cells. The latter orient themselves so slowly that evidences of the former follicle site remain for some time after the zona pellucida has disappeared.

*Type III.* The earliest signs of atresia in follicles designated as type III begin in the nucleus and cytoplasm in precisely the same manner as described for type II. The be-

havior of the zona pellucida, however, suggests a type of atresia different from the types previously described.

Coincident with early signs of nuclear and cytoplasmic changes, the zona pellucida liquifies and diffuses into the marginal region of the ovum. The influx of the zona pellucida proceeds from all sides until it converts the ovum into a hyaline, amorphous mass (fig. 8). As the ovum is destroyed in the manner just described; broad, pseudopod-like processes develop from the periphery of the zona pellucida. These extend peripherally until they contact and fuse with the free surface of the follicular epithelium (fig. 8). Ultimately adjacent processes fuse and thereby obliterate the remaining cavities within the follicle. That the zona pellucida is the source of the hyalinizing substance is shown by the fact that its walls rarefy and disappear as the above described events occur.

When the follicular cavity is completely filled with the abortive zona pellucida, the ovum is entirely masked. Rarefaction continues until all evidences of its original structure vanish (fig. 8). Figure 9 shows a follicle at higher magnification in which rarefaction of the zona pellucida is in progress. The mottled appearance of the zona pellucida rapidly passes over into a more uniform texture as shown in figure 8. The follicular cavity at this stage of atresia contains nothing but a highly refractive, uniform sheet. Sections through a lesser diameter of the follicle show a striking resemblance to an anovular follicle. When nothing but the hyaline sheet remains within the follicular confines, small groups of follicular cells become chromophobic and disappear without leaving any trace of their degeneration products. As the follicular epithelium disappears, the hyaline mass expands until it is firmly adherent to the basement membrane of the follicle. It will be noted in figures 9 and 10 that many of the follicular elements have disappeared and that others exhibit a chromophobic condition. In most instances the entire follicular epithelium degenerates before connective tissue invasion takes place.

Closure of the follicular cavity by connective tissue occurs simultaneously with the formation of vacuoles in the hyaline sheet. The first vacuoles develop at the junction of the connective tissue with the hyaline sheet; later throughout the entire sheet. Tufts of cells bulge into the vacuoles from all sides of the follicle. Actual ingrowth by the cells, however, is usually restricted to one point on the follicle as shown in figure 10. It is difficult to determine if the basement membrane acts as a barrier to the ingrowing cells. The bulging nature of the cellular tufts prior to actual ingrowth suggests that they are being restrained by some force; either by their own fibers, the basement membrane or both.

Fibroblasts and amoeboid cells eventually leave some of the tufts and migrate inward. Continued vacuolation of the sheet produces at first a coarse reticulum into which fibroblasts migrate. Later the reticulum becomes delicate and cellular. In figure 10 the large oval nuclei are those of fibroblasts which are entering the reticulum at one side of the follicle. There is no evidence that phagocytes assist in the atresia of type III follicles. The final stage of atresia sharply resembles the condition shown in figure 13.

## 2. *Relation of atretic types of primary follicles to the oestrous cycle*

*a. Prooestrus.* Animals in nature enter prooestrus early in April. The ovary at this time contains large numbers of primary follicles. A few multilaminar follicles are also present, but few of these have well-defined antra. During the first few days of prooestrus atresia overcomes a large number of primary follicles. By the end of the period approximately 75% of these young follicles are in some stage of atresia. The rampant atresia of follicles during prooestrus offers excellent opportunities for a study of all the early stages described above under the various types.

Primary follicles deep in the cortical region undergo type I atresia. The follicles are usually from 140 to 200 micra

below the surface of the germinal epithelium. All stages of type I atresia are observed in a single ovary; however, there is a predominance of early stages. Atretic follicles described as type IA are also common during early prooestrus. They occupy the same position within the ovary as type I. During the last week of prooestrus types I and IA are greatly reduced in number, although a few of them are always found. In the ten ovaries studied for this period of the oestrous cycle a surprising uniformity in the ovarian position of types I and IA was noted. Their distance from the germinal epithelium remains so constant that they are easily distinguished from other atretic types.

Follicles which undergo type II atresia in prooestrus are characteristically located just below the germinal epithelium. Although their depth from the surface of the ovary may range from 30 to 140 micra, the great majority lie from 90 to 140 micra below the ovarian surface. Only the early atretic stages of type II follicles occur during prooestrus; later stages leading to their final disappearance are found during oestrus and early pregnancy.

Primary follicles in the medulla of the ovary follow type III atresia. These follicles are always in close relation to one or more of the many blood vessels in the medulla. This relationship is clearly shown in figure 8. Occasionally type III follicles lie deep in the cortical region. In such cases they are always in close proximity to larger cortical blood vessels. Both early and final stages of type III atresia occur in prooestrus, although as in the case of types I and IA, the earlier stages predominate.

*b. Oestrus.* There appears to be a wide variation in the condition of the ovary of different female ground squirrels at this period. In 50% of the ovaries examined large numbers of primary and multilaminar follicles were completing their final stages of atresia. The remaining 50% showed but few of these. Foster ('34) studying the reproductive cycle of the ground squirrel ovary, found only a few atretic follicles during oestrus. Although most animals in nature enter oestrus

at approximately the same time, such variations in the condition of the ovary are plausible when such factors as age of the animal; location of the burrow, and weather conditions are considered.

Few follicles of type I atresia are present in the ovary during this period. Their position within the ovary is influenced to a large extent by growing multilaminar and mature follicles. In sectors of the ovary where mature follicles fail to develop, follicles of type I and IA retain the same position as in prooestrus. In other regions, however, the rapid growth of maturing follicles forces them toward the periphery of the ovary so that they occupy the same position as that of type II follicles. Many of the type II follicles which begin atresia in prooestrus disappear before ovulation takes place. Others which remain for a longer time assume a more peripheral position in the ovary as a result of pressure caused by growing follicles. Many of them are actually forced into the fibers of the tunica albuginea. Although most of the type II follicles at the periphery of the ovary begin degeneration during prooestrus, a few always resist atresia until the succeeding period. These follicles complete their terminal stages of atresia during late pregnancy or early anoestrus.

A number of type III follicles are always present in the medullary region of the ovary during oestrus. As in prooestrus the large majority lie close to the medullary blood vessels. Others, however, depart from this position. The reorganization of the ovarian stroma which results from the rapid growth of maturing follicles, displaces many of the type III follicles so that they surround the thecal layers of the former. Their distribution around a mature follicle is not uniform, but appears to be restricted to those sectors where the circumfollicular blood supply is most abundant.

*c. Pregnancy.* Atresia of primary follicles during pregnancy is at its lowest ebb. The picture presented by the ovary at this period is notably one of monotony. Enormous, expanding corpora lutea almost completely fill the ovary. The ovarian stroma is reduced to thin, compressed strands which

contain but few degenerating follicles of any type. Types I and IA, as a rule, are entirely absent. A few examples of type II are usually found in the tunica albuginea. These are so tightly compressed that they become greatly elongated structures. In those regions of the ovary where the stroma is more abundant (usually at the periphery between adjacent corpora lutea), intergrades between types I and II may occur. Follicles which obviously began atresia as type II exhibit a partially collapsed zona pellucida and the ingrowth of a more or less distinct cone of cells. Neither the condition of the zona pellucida or cone of cells suggests typical type I atresia. The shifting in ovarian position of atretic follicles due to pressure and changes in the vascular supply appears to be responsible for the production of intergrades.

Terminal stages of type III atresia are contained in the greatly reduced medullary region and connective tissue which surrounds the corpora lutea. The relationship of type III follicles to the ovarian blood vessels is not as sharp during pregnancy as in the two preceding periods of the oestrous cycle. Although fewer in number, their position is essentially similar to that found in oestrus.

*d. Anoestrus.* The ground squirrel usually parturates about the last of May or in early June. There is little change in the condition of the ovary from the time of parturition to the time when the animals seek their burrows for hibernation in late September or early October. The long period of anoestrus begins in July and extends into March. Ovarian material used for this study from October to March was obtained from captive females.

Animals sacrificed during early December showed prominent though regressing corpora lutea. Few atretic follicles of any type were observed at the periphery of the ovary. The medulla of the ovary, however, usually contains several terminal stages of type III. Ovaries taken in late February and early March showed regressing corpora lutea and enormous numbers of oogonia in the cortical region. The ovary at this time also contains large numbers of primary follicles in the

inner regions of the cortex. Many of these are in early stages of atresia. Early stages of types I and III predominate. In the interval between early March and the first few days of April the large majority of oogonia develop into primary and multilaminar follicles. The number of atretic follicles correspondingly increases so that by the end of prooestrus all three types of atresia (I, II and III) are apparent.

3. *Influence of the zona pellucida on the atresia of immature and mature multilaminar follicles*

a. *Immature multilaminar follicles.* The factors which permit a small percent of primary follicles to resist atresia and grow toward maturity are not clear. Many of the developing multilaminar follicles in the ovary at prooestrus are eliminated by atresia at various stages in their development; only a few develop to maturity.

The expression which atresia will take in any developing multilaminar follicle appears to vary with the developmental stage and the intraovarian position of the follicle. Young multilaminar follicles in the early stages of growth are overcome by atresia in a manner similar to that described for primary follicles. Their only advance over the primary type is the presence of an incomplete, intermediate layer of cells in the follicular epithelium.

Multilaminar follicles which possess from two to five intermediate layers of cells (fig. 11) reveal a different course of events in the atretic phenomenon from that described for primary follicles, although similarities exist. The earliest evidences of atresia occur in the ovum. The follicle is affected much later. Fragmentation of the nuclear membrane and chromatolysis of the nuclear material is rapidly followed by changes in the cytoplasm. The deutoplasmic material becomes arranged into coarse, granular clumps; first, in the center of and later at the periphery of the ovum (fig. 11). When the cytoplasm has become distinctly granular, the inner surface of the zona pellucida rarifies and diffuses throughout the ovum as shown in figure 12. Although the ovum becomes hyalinized,

its original structure is not immediately masked. Subsequently, however, the hyaline mass becomes vacuolated (fig. 12) and all signs of the egg disappear. When the ovum has reached the stage of degeneration just described, the innermost layer of follicular cells undergo pycnosis and disappear. A centrifugal wave of degeneration progressively overcomes the remaining intermediate layers. Meanwhile the outer surface of the zona pellucida rarifies and extends into the debris of the degenerating cells. Internal disorganization of the follicle so weakens its structure that it is unable to withstand the pressure of the surrounding stroma. Irregularities in the contour of the follicle result as shown in figure 12. As the peripheral or basal layer of the follicle degenerates, slender tufts of connective tissue pass through the basement membrane. The remains of the zona pellucida and follicular debris are eventually obscured among the invading cells (fig. 13).

Multilaminar follicles of the type just described exhibit some variation in the rate with which atresia is accomplished. Follicles which are located in the medulla or medullary-cortical region of the ovary appear to pass through atretic stages more rapidly than ones in the cortex. In this connection it is interesting to note that type II primary follicles in the cortex of the ovary likewise show a retardation of the atretic process.

*b. Mature multilaminar follicles.* Follicles which approach maturity, but in the strict sense are not mature appear to follow the same course of atresia characteristic of mature follicles. The following description is therefore applicable to both of these developmentally similar types.

Ovaries taken just prior to the ovulatory period contain about a dozen mature follicles and a lesser number of ones which approach maturity. Most of the latter show early signs of atresia, while only a few of the former are thus affected. Shortly following ovulation all of the mature follicles which remain in the ovary show signs of atresia. The course of atresia in all of these mature forms is essentially similar.

The earliest signs of atresia are manifested by nuclear pycnosis and fragmentation of the follicular cells which surround



the ovum and follicular cavity. Changes in the ovum which are indicative of atresia do not occur until its follicle is in a late stage of disorganization. Destruction of the innermost three or four layers of the follicle usually liberates the ovum from the discus proligerus. As pycnosis occurs in each successive layer of the follicle, the cells of the layer separate from one another individually or in groups. The number of free pycnotic cells increases as successive layers of the follicle degenerate.

The nuclei and cytoplasm of the free cells exhibit many interesting variations. In some instances the pycnotic nuclei attenuate and become lobed; in others they appear as rings or irregular blobs. The cytoplasm is usually finely granular and shows a weak affinity for the acid stain. The fate of these cells is complete degeneration. Freud and Vedder ('38) observed an infiltration of eosinophilic leucocytes in the follicular cavity of atretic follicles of the rat. They further observed that the leucocytes had ring shaped nuclei. Cells which match their description in the present study are plainly degenerating follicular cells and show no resemblance to leucocytes whatsoever.

When all but the three or four most peripheral layers of the follicle have become disorganized, irregularities appear in the contour of the follicle. Pressure from the surrounding stroma and thecal layers progressively collapses the remainder of the follicle wall and at the same time disrupts the continuity of the basement membrane. Slender connective tissue tufts enter the follicular cavity through apertures in the basement membrane, however, closure of the follicular area appears to depend more on a sort of "sphincter action" of the thecal layers. Progressive reduction of the follicular area compresses the remaining follicular epithelium into a compact mass about the ovum. Subsequent cytolysis and disappearance of the follicular debris completes the cytomorphosis of the follicular epithelium.

Changes appear in the ovum following the collapse of its follicle. Those in the cytoplasm usually precede those in the nucleus. The deutoplasmic material passes from a heavy flaky

condition into a coarse granular one. The most striking changes are to be observed in the nucleus. In some instances the nuclear membrane fragments and releases its chromatin material into the cytoplasm. A small vesicle develops around each chromatin particle thereby resembling a nuclear membrane. From six to eight pseudonuclei such as these may appear in a single ovum. Two such structures may be observed in figure 15 (marked by "X"). In other instances the nucleus may divide amitotically into two equal parts. Cleavage of the cytoplasm may or may not follow. When it does the ovum resembles a typical two cell stage (fig. 14). Kingery ('14) reported in his studies on the mouse ovary that a disturbance in the nutritive supply of the ovum due to degeneration of its follicular elements, in all probability initiates an attempt on the part of the ovum to divide and establish a new nucleocytoplasmic relationship. Several clear cut cases of intraovarian cleavage were observed in the ground squirrel ovary, but the large majority of multinucleated ova maintained an undivided cytoplasm.

Probably the most important factor which permits the ovum of mature follicles to resist atresia is the stability of the zona pellucida. This structure undergoes very little change from the onset of degeneration in the follicle until it is compactly surrounded by cells of the theca. When the innermost layer of the follicular epithelium degenerated, the protoplasmic processes of the cells were also destroyed. The irregular projections on the surface of the zona pellucida remain as rudiments of these former connections (figs. 14 and 15). The zona pellucida which surrounds a mature ovum is usually thick, pigmented and hyaline. Although numerous cells of the theca compactly surround it, none are observed to penetrate its walls. Eventually unequal pressure on the surface of the zona pellucida causes its wall to break and permits amoeboid cells from the thecal tissue to enter the ovum. The behavior of the cells within the ovum suggests that they digest its substance extracellularly rather than by phagocytosis. A final stage in the atresia of a mature follicle is shown in figure 16. Several

amoeboid cells are included in the egg substance, however, no signs of phagocytosis are apparent. Intracellular inclusions in the digesting cells are rarely seen. As the ovum becomes disorganized by the amoeboid cells, the zona pellucida fragments into minute pieces and becomes indistinguishable from the degenerating mass of the ovum. Fibroblasts from the theca slowly fill the area formerly occupied by the ovum and thereby obliterate the last trace of the follicle. Reorganization of the thecal layers into stroma progresses more slowly. Interstitial cell nests persist for a variable length of time.

Occasionally mature ova which are deeply situated in the ovary show a thin, poorly-defined zona pellucida. Every example of this type reveals a hyaline substance in the follicular cavity. The ovum stains intensely with the acid stain in its early degenerative stage, but it becomes vacuolated and disappears long before its follicle is disorganized. The complete atretic sequence of such ova is difficult to follow because of the lack of intermediate stages in the material studied. Follicles of this type usually show an extremely rich vascular supply in the thecal layers.

#### DISCUSSION

The foregoing observations strongly indicate that the expression of atresia in the ground squirrel ovary varies in follicles at different developmental stages. Less conspicuous, but none the less noteworthy, are the variations in the course of atresia which affect follicles of like or similar developmental level.

Asami ('20) reporting on follicular atresia in the rabbit ovary, noted that atresia is manifested differently in primary, medium-sized and mature follicles. He observed that in medium-sized and mature follicles degeneration of the granulosa is a primary factor, whereas in primary follicles the egg and granulosa are affected simultaneously. No mention was made whether variations of atresia occurred in follicles of each size group.

The observations which Kingsbury ('13), ('39) has made on atresia in the cat ovary, are indeed of fundamental significance. He concluded that the irregular wave of degeneration which follows a period of differentiation in the developing cat ovary is an expression of the inefficiency of the vascular system of the organ to nourish and supply the requisites to large numbers of follicles. His observations on the adult cat ovary relative to atresia have led to a similar conclusion. Kingsbury considers the ovary as a site of continuous vasculogenesis and marked vascular degeneration. He states, "In the complex factors which must be considered in interpreting atresia the vasculogenic factor must not be overlooked."

There appears to be a definite correlation between the vascular supply in the ground squirrel ovary and the manner in which atresia is expressed. In late anoestrus and early prooestrus vascular channels attain a high degree of development and distribution generally throughout the ovary. Late in prooestrus, however, vascular changes are pronounced. Capillaries, arterioles and venules are lacking throughout most of the cortical region of the ovary. Only in the medulla and roughly in the medullary-cortical region are vessels of a noticeable diameter to be found. Coincident with these vascular changes the large majority of primary follicles contained in the ovary at this time rapidly undergo atresia so that by the time of oestrus but few remain. The blood vessels which are first to disappear are those which supply the tunica albuginea and the overlying germinal epithelium. The large vessels of the deeper cortical layers disappear more slowly. At the approach of oestrus the vascular system appears to be restricted chiefly to the medullary region of the organ and to the thecal sinuses which surround large maturing follicles.

It is suggested that the types of atresia which primary follicles undergo are expressions of varying metabolic conditions in the ovary due to progressive vascular changes. Type II follicles which are brittle, hyaline structures are the first to be deprived of an adequate vascular supply. This type is always restricted to a narrow peripheral zone of the ovary. Types I

and IA, characterized by collapse of the zona pellucida and the ingrowth of a cellular cone, constitute a zone deeper in the cortex and are affected later than type I by vascular changes. Type III follicles lie deep in the medullary region, and as previously stated, are always found in close association with medullary blood vessels.

Changes in the vascular system which might affect the atresia of growing multilaminar follicles and mature follicles in oestrus and the postovulatory period progress more slowly and cannot be followed with any degree of certainty. However, by the time that atresia is pronounced in these more mature forms, degenerative vascular changes become increasingly obvious.

Whether the peculiar configurations which the zona pellucida exhibits in the follicular atresia of all developmental stages are caused by vascular changes, can only be speculated from the material and procedure employed in this study. It is important to note, however, that the condition of the zona pellucida appears to influence the course of atresia and the speed with which it is accomplished. Garde ('30) noted that the zona pellucida of atretic primary follicles in *Ornithorynchus* collapses opposite an ingrowing cone of cells. Guthrie and Jeffers ('38a) observed a similar phenomenon in the bat ovary. Garde considered these cells as the source of phagocytes which dispose of the egg. These observations are in striking agreement with primary follicles of type I atresia in the present study, although the activity of phagocytes was not observed.

Mossman ('37) believes that atresia of all developmental stages as well as mature follicles is similar in the ovary of *Geomys*. He reports that cessation of mitotic activity in the granulosa, hyalinization of the ovum and the final dissolution of the ovum by phagocytes constitutes the course of atresia. Once atresia has begun, he believes that atretic primary follicles cannot be distinguished from more mature forms except possibly by their intraovarian position. These findings are not in agreement with conditions observed in the ground squirrel.

There is little indication that phagocytes play an appreciable role in the atresia of primary follicles. Autolysis of the egg substance occurs so rapidly that little is left upon which phagocytes might act. Amoeboid cells were observed to enter the ovum through stomata in the zona pellucida of type II follicles in much the same manner as described by Branca ('25); however, it is doubtful if these cells function as true phagocytes. A more prominent role of phagocytosis in the atresia of young follicles has been reported by Garde ('30), and others.

The zona pellucida of atretic mature follicles appears to act as an efficient barrier against the penetration of amoeboid cells into the ovum during the degeneration of its follicular epithelium. At a later stage, however, when the zona pellucida ruptures, amoeboid cells migrate from the thecal connective tissue into the ovum and begin its disorganization. There is no evidence in the material studied that phagocytes are derived from cells of the follicular epithelium as has been suggested by Stevens ('04) for the human and Kingery ('14) for the mouse. The fate of all follicular cells appears to be one of complete degeneration. At no time were they observed to transform into fibroblastic scar tissue as reported by Mjassojedoff ('23) for the cat. The derivation of fibroblasts and phagocytes in late stages of atresia requires further study. In this connection it is hoped that *supra vital* studies recently begun in this laboratory will offer some clues to the problem.

Intraovarian cleavage which occurs in the ovum of some mature atretic follicles is interpreted as being an adjustment to nutritive disturbances. These observations conform with those of Kingery ('14) for the mouse. No difference was noted in the course of atresia from that described for mature follicles.

Factors in the ground squirrel ovary which influence the course of atresia in follicles at different developmental stages as well as those of a similar stage, are numerous. The vascular factor appears to be of primary importance and in all likelihood controls the expression of other factors which are more subtle, i.e. the zona pellucida.

## CONCLUSIONS

1. The course of atresia in follicles of all developmental stages is influenced to a large extent by the zona pellucida.
2. The manner in which the zona pellucida influences atresia appears to be correlated with the condition of the ovarian vascular system and period of the oestrous cycle.
3. Primary follicles undergo three distinct types of atresia depending on their intraovarian position.
4. The role played by phagocytes in the course of atresia in follicles of all sizes is influenced by the condition of the zona pellucida.
5. The follicular cells of atretic follicles possess no potentialities for transformation of cell type. The fate of the follicular epithelium regardless of its degree of development is complete degeneration.
6. Intraovarian cleavage in mature follicles, at the most, is an expression of atresia.

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## PLATE I

### EXPLANATION OF FIGURES

1 A primary follicle undergoing type I atresia. Note that the zona pellucida has collapsed and that no trace of the ovum remains. Broad processes of material from the zona pellucida have enclosed several pyknotic, degenerating follicle cells at the right of the figure.  $\times 540$ .

2 A characteristic cone of cells in type I follicles. The cells of the cone are obviously of follicular origin. All of the follicular epithelium has disappeared with the exception of a few chromophobic cells.  $\times 540$ .

3 A later stage of type I. The follicular epithelium has degenerated. The cone is composed of a mixture of degenerating follicle cells and normal fibroblasts. The broader processes of the zona pellucida show considerable vacuolation. A few slender strands extending from the inner surface of the zona pellucida are apparent.  $\times 252$ .

4 A type II primary follicle. The follicular epithelium has disappeared. The ovum has shrunk slightly from its zona pellucida.  $\times 252$ .

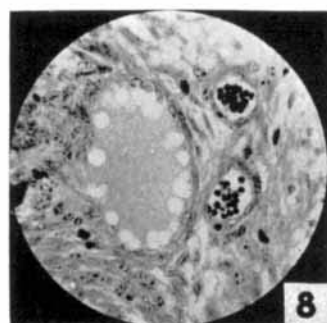
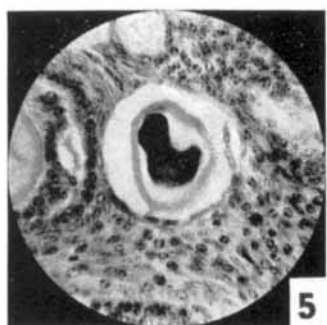
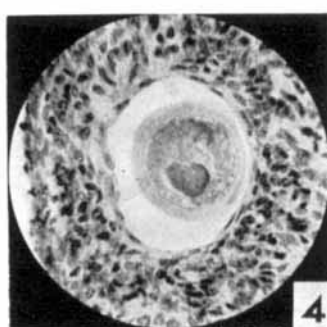
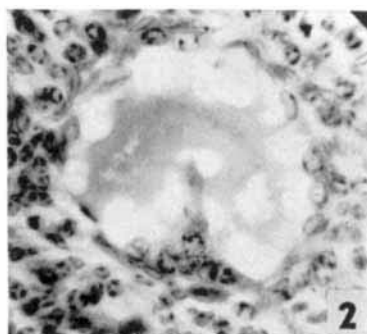
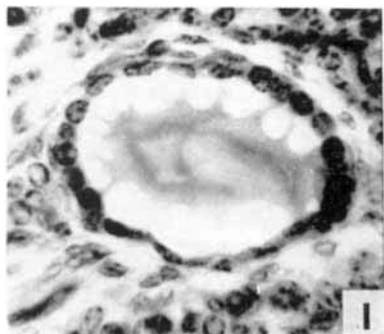
5 A later stage in atresia of a type II follicle. The dark material of the ovum shows considerable shrinkage. Stomata are apparent in the zona pellucida opposite the two ingrowing fibroblasts.  $\times 252$ .

6 Diffusion of the egg substance into the zona pellucida of a type II follicle. A number of amoeboid cells have entered the ovum; others are in the act of entering.  $\times 252$ .

7 A late stage of a type II follicle. Little remains of the ovum. Connective tissue ingrowth is more obvious in other sections of this follicle.  $\times 540$ .

8 A type III follicle. Little is left of the follicular epithelium and the ovum has been hyalinized by the zona pellucida. Note its close association with medullary blood vessels.  $\times 252$ .





## PLATE 2

### EXPLANATION OF FIGURES

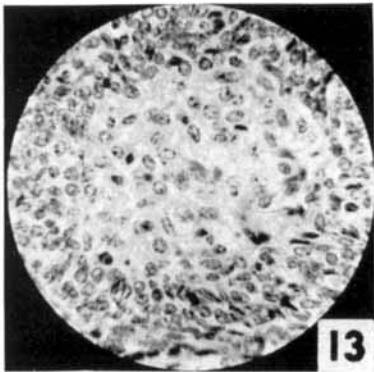
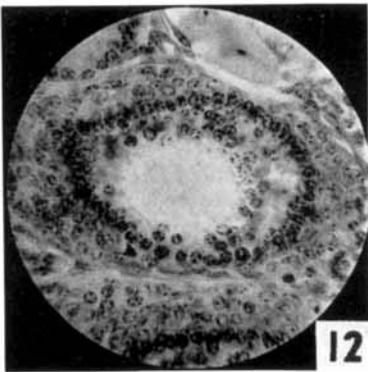
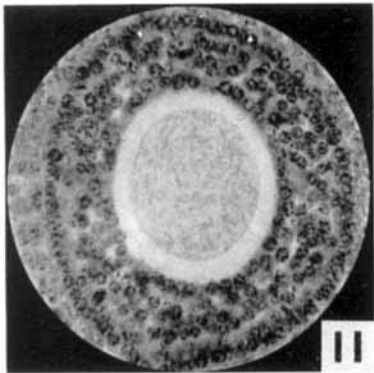
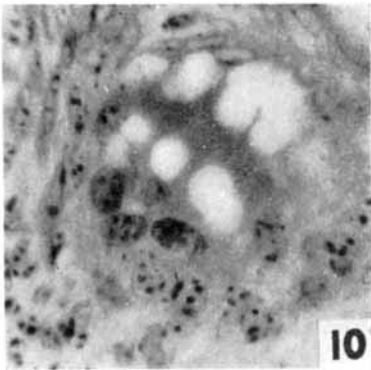
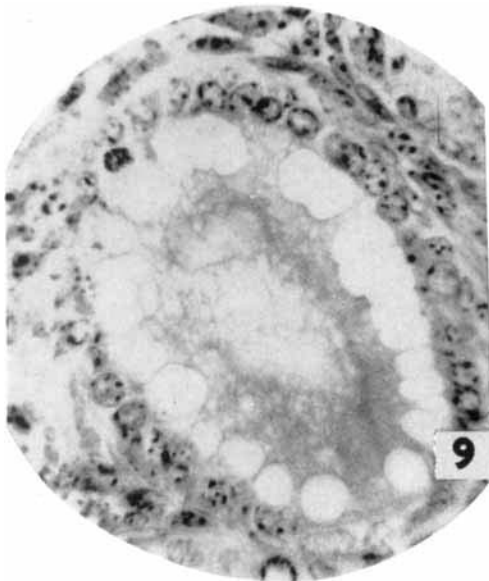
9 A high power view of a type III follicle. Note the mottled appearance of the zona pellucida and the processes which extend from it to the follicle cells. Many of the follicular elements are chromophobic; others have entirely disappeared.  $\times 540$ .

10 A late stage of a type III follicle. Fibroblasts are filling in the vacuolar cavities of the zona pellucida.  $\times 540$ .

11 An early stage in the atresia of a young multilaminar follicle. The follicular epithelium appears normal. The granular deutoplasm and diffuse zona pellucida indicate early atresia.  $\times 252$ .

12 A later stage in the atresia of a young multilaminar follicle. The zona pellucida has diffused into the ovum. The innermost follicle layers are degenerating; the outermost shows evidence of collapse.  $\times 252$ .

13 An end stage in the atresia of a young multilaminar follicle. Only minute strands of the zona pellucida remain among the invading fibroblasts. The end stages of types I, IA and III are strikingly similar.  $\times 252$ .



### PLATE 3

#### EXPLANATION OF FIGURES

14 An example of intraovarian cleavage in an atretic mature follicle. The cells adjacent to the zona pellucida are those of the theca. The follicular layers have disappeared.  $\times 700$ .

15 Late stage in the atresia of a mature follicle. Only the theca surrounds the zona pellucida. Two small pseudonuclei are barely discernible in the ovum (marked "X"). Note that the zona pellucida has ruptured.  $\times 650$ .

16 An end stage in the atresia of a mature follicle. The ovum and zona pellucida appear as a fragmented mass. Several amoeboid cells from the degenerating theca have wandered into the degenerating egg.  $\times 400$ .

