Supplemental Observations on Structural Changes in Stigmal Tissue of Hen's Ovarian Follicle in the Process of Ovulation

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It is generally known that ovulation is caused in hens by the rupture of the stigma at the apical pole of the ovarian follicle, and that it is induced under control of gonadotropic hormones. Although the hormones play an essential role in the follicular rupture, the occurrence of the rupture is ultimately attributed to the destruction of the stigmal tissue. In the mammalian ovulation, many workers[1-5] have examined structural changes in the follicular wall occurring at the time of the ovulation in order to elucidate the mechanism of ovulation. No such examination has been made fully for the avian ovulation as yet. Only a few workers, including Phillips and Warren (1937)[6] and Kraus (1947)[7], have observed briefly structural changes in the stigmal wall in spontaneously or artificially induced rupture, either macroscopically or microscopically.

In their previous study, the authors[8] observed that the stigmal wall, where rupture occurs, had a specialized structure favorable for rupture. They[9] also found remarkable structural changes in the tissue of the stigma, particularly in its stratum granulosum, a few minutes before ovulation. The present study was performed to obtain more detailed information on morphological changes in the stigmal tissue in the process of ovulation. Observation was made mostly by scanning electron microscopy and partly by conventional light microscopy.

MATERIALS AND METHODS

Follicles in different stages of the process of ovulation, or preliminary, proper, and post-ovulatory stages were examined in the present study. They were collected, by laparotomy from White Leghorn hens laying daily, under anesthesia with sodium pentobarbital. Since ovulation is known to occur in hens about 30 minutes after oviposition[6], follicles were collected at a time counted from the time of oviposition.

Follicles in the preliminary stage were harvested 10 hours before expected ovulation to clarify the normal structure of the follicle. It was not easy to obtain successfully follicles immediately before ovulation or just ovulated. Therefore, follicles were removed from hens in the following manner within 15 minutes before the time of expected ovulation.

Hens were laparotomized under anesthesia to observe carefully the signs of ovulating

appearing in the largest follicle. Follicles showing the enlargement and clarification of the stigmal wall were selected as specimens of the ovulating stage.   Immediately after the appearance of ovulatory signs, the follicles were fixed at once *in situ* by dripping of a 2.5% glutaraldehyde solution (pH 7.4).   This fixing procedure was to arrest the progress of rupture, since rupturing of the stigmal wall proceeds rapidly.   Follicles at the post-ovulatory stage were collected from hens laying daily, which usually contained successive-ly regressed post-ovulatory follicles in the ovary.

The excised follicles were used for different examinations.   For light microscopy, they were fixed in a 10% formalin solution as a whole for 48 hours.   Then the follicular wall, including the stigma, were cut into small pieces, from which paraffin sections were prepared routinely.   The sections were stained with hematoxylin and eosin or by azan staining.   For scanning electron microscopy, follicles were prefixed as a whole in a 2.5% glutaraldehyde solution for 5 minutes.   The prefixation was to prevent the follicular wall from wrinkling and to make the separation of its layers easy.   Then the follicular wall, including the stigma, was cut into small pieces.   The perivitelline layer adhering to its inner surface was stripped off gently.   The remaining part of the wall was separated further into membranous specimens to expose the surface for examination.   This procedure was done carefully in a jar containing a 0.9% NaCl solution by the aid of needles under the dissection microscope.   The separated membranous specimens were refixed with the same fixative as before.   After fixation, they were treated by the conventional method and examined by the scanning electron microscope at an accelerat-ing voltage of 15 KV.

OBSERVATIONS

Pre-ovulatory follicle

The normal structure of the stigma of pre-ovulatory follicles has been described in a previous paper[8].   This paper deals with only ovulatory changes in the stigmal tissue.

Light microscopy of mature follicles revealed that the wall generally was composed of five layers, which were, from outside to inside, the superficial epithelium, loose con-nective tissue coat, theca externa, theca interna, and stratum granulosum. The superficial epithelium enclosed the whole surface of the follicle and consisted of a single layer of epithelial cells.   These cells appeared to be flattened cuboidal ones.   Their nuclei were compressed at the base of the cells..   The loose connective tissue coat underlying the epithelium was a layer of loose tissue containing abundant large blood vessels, lymphatic vessels, smooth muscle fibers, and nerve fibers.   It was thick in the proximal half of the follicle and became thinner as it approached the stigma.   Finally, it fused with the theca externa of the stigma.   The smooth muscle fibers were bound together in a thin membranous bundle and ran up to the equatorial region of the follicle, accompanying large blood vessels.   The theca externa constituted the main portion of the follicular wall. It was a dense layer of fibrous connective tissue.   A few flattened fibroblasts were scatter-ed in the network of fibers.   The theca interna was a relatively narrow zone of loose

connective tissue containing various types of cells and abundant networks of blood capillaries. The stratum granulosum, or the innermost layer, was composed of a row of granulosa cells. These cells were cuboidal and contained distinct nuclei. Just above the stratum granulosum, there was a basement membrane well stainable by azan staining (Fig. 1).

By scanning electron microscopy, the superficial epithelium presented a pavement-like arrangement of epithelial cells. These cells were squamous and possesed a few short microvilli (Fig. 7). They were connected with neighboring cells by cytoplasmic projections. This structure may enable the epithelium to stretch itself without breaking cells. The theca externa consisted of striated layers of compact collagenous fibers running parallel to the surface of the follicle.

The collagenous fibers were bound compactly together with one another into a sheet-like bundle with interfibrillar matrix. As a result, these collagen bundles were so vague in appearance that few individual fibers were distinguishable (Fig. 11). Flattened fibroblasts were embedded among the bundles. The theca interna was composed of loose connective tissue. It contained various types of cells, such as elongated smooth-muscle-like cells, fibroblast-like cells, and interstitial cells (thecal gland cells). It was also richly supplied by a network of capillaries, which contained a mass of closely packed erythrocytes.

The granulosa cells of the stratum granulosum appeared cuboidal in shape and were separated from one another by a clear gap. Most of them possessed a large cylindrical cytoplasmic process and numerous fine and tall microvilli on their free surface (Figs. 13 and 14). Some cells were devoid of the process. Probably, they must have been broken by the time when the perivitelline layer was separated from the wall.

Ovulating follicle

A few minutes before ovulation, the stigmal wall increased in width and became transparent. In the cross section, it became thinner as it approached the center of the stigma (Figs. 2 and 3). Changes in the wall were restricted to the area of the stigma. No changes were observed in the non-stigmal region. The inside wall of the stigma became conspicuously thin (Fig. 3). The granulosa cells became flattened as they approached the center of the stigma (Fig. 2). They were completely absent in the place where the rupture may have initiated (Fig. 3).

Scanning electron microscopy revealed more distinct structural changes. The outer epithelial cells became elongated and fusiform, extending transversely along the longer axis of the stigma (Fig. 8). In an intensely injured area, they were detached entirely from the wall by breaking of the junction among them and the underlying thecal layer was exposed (Figs. 8 and 9). This distortion of epithelial cells must have been induced only mechanically by the distension of the wall, because no obvious lytic or necrotic changes were observed in the cells. In the exposed theca externa, collagen bundles, which were originally dense and compact, turned to a sparse network of fibers by dissociation into fibers or fibrils (Fig. 12).

The internal granulosa cells also underwent characteristic structural changes. In the bordering region of the stigma, they were swollen and lost the cytoplasmic processes and microvilli. Those near the center of the stigma protruded a single or a few large vesicular blebs. These decomposed cells often retained empty holes suggestive of the liberation of vesicles (Figs. 15 and 16). Those close to the splitting site were remarkably flattened and amorphous, elongating transversely to the longer axis of the stigma (Fig. 17). At the center of the stigma, granulosa cells disappeared completely, and the thecal layer was exposed.

Post-ovulatory follicle

Immediately after manifestation of the ovulating signs, a small protrusion bulged up on the surface of the stigmal wall. It became large rapidly with the extension of splitting along the stigma and abruptly the ovum squeezed out. After expulsion of the ovum, the follicle shrunk in a bud-like sack reducing its size. In releasing the ovum from the follicle, the stigmal wall did not rupture through its entire length, because the length of the stigma permitted the ovum fully to pass through. In consequence, ruptured follicles often retained a part of the unruptured stigmal wall at one end (Fig. 4).

When observation was made microscopically on post-ovulatory follicles just after ovulation in a cross section, the stigmal wall tapered to a point, losing its original structure (Fig. 5). The wide base of the tapered wall retained the almost complete structure of the follicle. The arrangement of connective tissue fibers, however, became rough and wavy, and fibroblasts round in shape. These changes in ruptured follicles were brought about by a decrease in tensile strength of the follicles. Just after rupture, the granulosa layer was partially detached from the thecal layer together with the basement membrane and thrown into folds. After that, the granulosa cells regressed in vacuolar changes, as observed already by many workers[10-13].

Scanning electron microscopy revealed that the tapering tip of the wall had a blunt end with an extensively decomposed structure. In the marginal region of the fissure, the outer epithelial cells, as well as the inner granulosa cells, sloughed off completely (Fig. 20). Cells far from the fissure were intact, although their form became round and reduced in size. The thecal layer exposed to the tapered tip looked like a complex network of collagen fibers.

Noticeable changes were observed in the unruptured stigmal wall mentioned above. The inner surface of the unruptured site was destroyed into an ulcerative groove along the stigma to keep only the thin thecal layer (Figs. 6 and 18). The remaining thecal layer was decomposed into a sparse network of collagen fibers (Fig. 19). On the contrary, the marginal region of the groove was rather ridged by a cluster of various tissue elements. Probably, these changes may have been brought about by the drawing of destroyed tissue toward the bilateral sides.

Regressive changes were clearly noticed in the granulosa cells of the ovulated follicle by scanning electron microscopy. Immediately after ovulation, the stratum granulosum wrinkled partially (Fig. 21). Occasionally, a small area of hemorrhage was observed in

the theca interna near the splitting site. The regressive change of the granulosa cells was initiated by the bulging of the small cytoplasmic vesicles accompanied with the loss of cytoplasmic processes and microvilli (Fig. 22). By 48 hours after ovulation, most cells had been detached from the basement membrane and a mass of vesicated cells appeared (Fig. 23). By 72 hours after ovulation, vesicular changes progressed further in the cells. On the other hand, the vesicles broke into small fragements varying in size. The granulosa cells shrunk largely due to the release of vesicles. As a result, the follicular cavity was filled with clusters of cellular fragements (Fig. 24).

DISCUSSION

A large number of workers[13-17] have examined in detail the structure of the ovarian follicle of hens. The findings of the present investigation may lend support to the opinions of these previous workers. Few of them, however, paid attention to the structure of the stigma which ruptures at the time of ovulation. GUZSAL (1966)[13] studied the structure of the follicle, particularly its stigma, with special reference to the mecahnism of rupture. He found that the stigmal wall had neither elastic fibers nor muscular elements which might be responsible for follicular rupture. His observation led to the presumption that the muscular tissue present in the loose connective tissue coat might play an important part in splitting of the stigma. He stressed that the smooth muscle tissue was well developed in the proximal half of the follicle and absent in the distal half.

Whether smooth muscle fibers are present in the theca externa or not has been a subject of much controversy. Phillips and Warren (1937)[6] asserted that these fibers were contained in the follicular wall, including the stigma. ROMANOFF and ROMANOFF (1949)[18] and KRAUS (1947)[7] proposed the presence of a thick layer of smooth muscle fibers, but GUZSAL (1966)[13] denied the presence of this layer. We agreed to his negation, since muscle fibers were observed not in the theca externa of the stigma, but in the loose connective tissue coat of the non-stigma.

At any rate, there remains the question why the follicle ruptures limitedly in the area of the stigma. As described in the previous paper[8], the stigmal wall had a characteristic structuer which differed from that of the non-stigmal wall. First, the stigmal wall was composed mainly of the theca externa, which was a collagenous connective tissue. Nevertheless, the collagen fibers of the stigma were arranged in a relatively regular way and tended to run parallel to the longer axis of the stigma, while they were interlaced complicatedly with one another in the non-stigma. The regular running of these fibers in the stigma was considered to be ascribed to the pronounced avascularity at this site. This specific arrangement of fibers suggests that the stigmal wall may be weaker structurally to tensile strength than the non-stigmal wall, and that it may tend to split parallel to the longer axis of the stigma.

Secondly, the stigmal wall was very poor in blood supply, as pointed out by many previous workers[19-21]. The avascularity of the stigma was mainly due to a lack of

veins penetrating vertically the thecal layer of the stigma. These penetrating veins derived from capillary beds in the theca interna were usually abundant in the thecal layer of the non-stigma. In the stigma, they traversed obliquely through the thecal layer along the border of the stigmal part and drained into large veins circumscribing the stigma. This specific vascular architecture suggests that the stigmal wall may be weak mecahnically. At the same time, it may serve for the prevention of bleeding when the stigma splits.

Although the stigmal wall is the weakest portion of the follicular wall, it is a prerequisite for the rupture of the stigma that destruction first occurs to the theca externa constituting the main portion of the stigmal wall. The present scanning electron microscopy showed that the theca externa was made of a compact sheet-like bundle of collagen fibers arranged in a striated manner. Immediately before the time of ovulation, this bundle was distorted into a sparse network of fibers accompanied by dissociated elemental fibers or fibrils.

In the ovulation of mammals, ESPEY (1967)[2] and OKAMURA and TAKENAKA et al. (1980)[5] found ultrastructurally that the collagenous connective tissue at the apex of the ovulating follicle was decomposed markedly into loose networks. Taking these changes of the follicle into consideration, ESPEY (1971)[3] postulated that the decomposition might have been induced by the action of certain proteolytic enzymes. He presumed that these enzymes might be endogenous ones of fibroblasts in the thecal layer, since the cells of the follicle became ultrastructurally multivesicular prior to the occurrence of ovulation.

Upon the distortion of collagen bundles of the stigma, it is possible that these bundles may be broken by a mechanical force originated from the expansion of the stigmal wall. It seems, however, that the distortion may be induced hydrolytically by certain enzymes in the same manner as suggested by ESPEY[3], since changes in the collagen bundles were so conspicuous and characteristic.

Other marked structural changes occurred in the stratum granulosum of the stigma. A few minutes before ovulation, the granulosa cells were involved in a curious vesicular change characterized by the bulging of large vesicles and by their release. Particularly, such morphological changes as flattening and elongation of the cell body, which were distinct at the site of splitting, may have been induced mechanically by the expansion of the stigmal wall. It is considered, however, that this "blebby phenomenon" of the granulosa cells may have been followed autolytically by the subsequent decomposition of the inner layer of the stigma, since the release of vesicles seemed to suggest the extrusion of some cytoplasmic inclusions. This suggestion may be supported by the fact that these morphological changes of the stigmal tissue occur extensively in the area of the ruptured, as well as unruptured stigmal wall, as mentioned above. The reason for this is unknown. Probably, it may be concerned with the avascularity of the stigma.

At present, the exact mechanism of ovulation in hens is not yet clear, although various hypotheses have been proposed; that is, the muscular contraction theory of PHILLIPS and WARREN (1937)[6]; the vascular degeneration theory of NALBANDOV (1961)[22]; the intra-follicular pressure theory of some earlier workers[23-24]. Certainly, ovulation is a complex

mechanism. It is not induced by a single physiological action. The following process is suggested for ovulation from the present morphological studies on the stigma during the process of ovulation : (1) the stigmal region where rupture occurs may be originally composed of a structure fragile to tensile strength, (2) the tissue of the stigma may be decomposed previously by some enzymic action to reduce tensile strength prior to ovulation, and (3) the reduction of the strength may be intensified by the contractile force of the follicular wall and result in rupture.

SUMMARY

Structural changes occurring in the process of ovulation were examined morphologically in the stigmal tissue of the hen's ovarian follicle to clarify the mechanism of ovulation. The examinations were carried out mostly by scanning electron microscopy and partly by conventional light microscopy. The findings obtained are as follows.

1. The stigma of the follicle, where rupture occurred, had a characteristic structure favorable for rupture. Its wall was occupied by the theca externa for the most part. The theca externa was composed of collagenous connective tissue. In it, collagen fibers tended to run parallel to the longer axis of the stigma. The blood supply was very poor in the stigma. Particularly, the theca externa lacked blood vessels running vertically.

2. In the theca externa, collagen fibers were bound together into sheet-like bundles, arranged densely in layers. Granulosa cells lining the inner surface of the stigmal wall were cuboidal in shape and possessed a prominent cytoplasmic process and numerous fine microvilli.

3. Immediately before the time of ovulation, the compact bundles of collagen fibers were dissociated into a sparse network of fibers or fibrils. The change was presumed to have been induced by the action of some proteolytic enzymes. The granulosa cells of the stigma were involved in characteristic vesicular changes suggesting the release of cytoplasmic inclusions.

4. The regressive changes of the granulosa cells in the post-ovulatory follicle were characterized by marked vesicular changes accompanied with the decomposition of the cell body.

5. From the findings mentioned above, ovulation was assumed to be induced in hens by a combination of the preceding decomposition of the stigmal tissue by certain proteolytic enzymes and the tensile strength of the follicular wall.

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Explanation of Figures

Plate I

Fig. 1. Transverse section of the stigma in a pre-ovulatory follicle. The wall of the stigma is composed of the thick and compact theca externa. The loose connective tissue coat is hardly visible. Hematoxylin and eosin staining. x400.

Fig. 2. Transverse section of the stigma at the time of ovulation. Granulosa cells become gradually flat and disappear as they approach the center of the stigma. The thecal layer becomes thin and rough. HE staining. x400.

Fig. 3. Transverse section of the stigma at a site where rupture is initiated. The stigmal wall is very thin, lacking the granulosa layer, theca interna, and most part of the theca externa. The dark streak-like structure is the perivitelline layer. HE staining. x400.

Fig. 4. Post-ovulatory follicle immediately after ovulation. It shrinks into a bud-like sack with a wide fissure. Arrow shows the unruptured stigmal wall.

Fig. 5. Transverse section of the tapered stigmal wall of the newly ovulated follicle shown in Fig. 4. The follicular wall tapers to a point with shaving of the tissue. The underside surface shows the outside of the follicle. HE staining. x100.

Fig. 6. Transverse sectione of the unruptured stigmal wall shown in Fig. 4. The inside layers of the wall are shaved off largely. HE staining. x100.
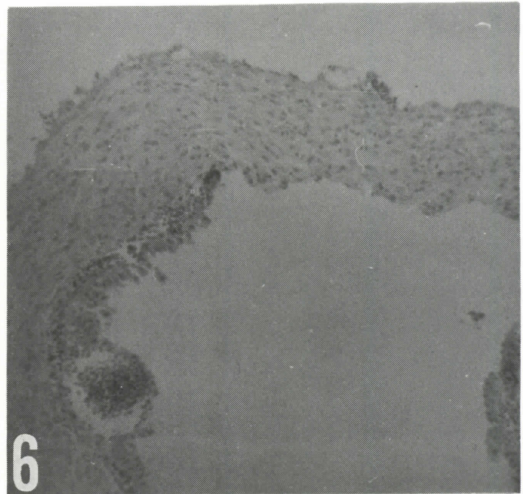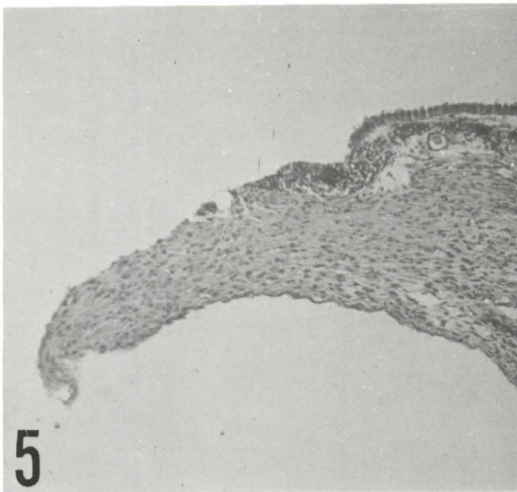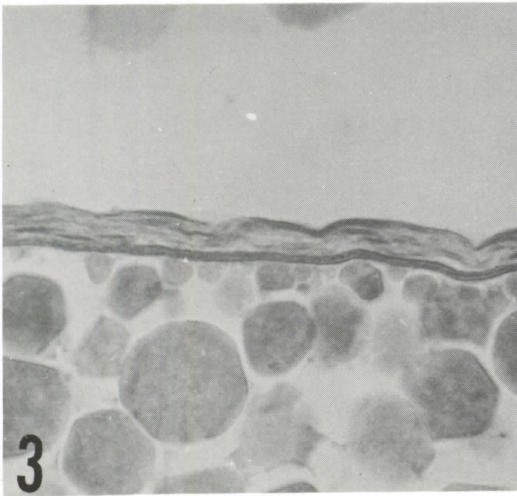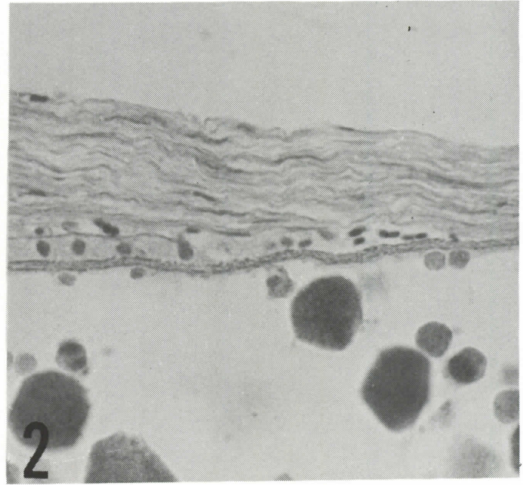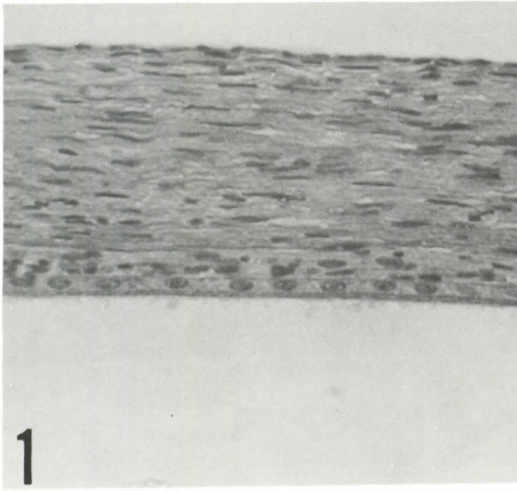
Plate I

Plate II

Fig. 7. Scanning electron micrograph of the superficial epithelium of a mature follicle. Epithelial cells are flat and polygonal in shape. They are connected with one another by the aid of cytoplasmic process (arrow). x1,000.

Fig. 8. The superficial epithelium of the stigma immediately before ovulation. Epithelial cells are deformed into a flat and elongated shape and detached from the wall in a wide area. x200.

Fig. 9. Higher magnification of the portion similar to Fig. 8. x550.

Fig. 10. The superficial epithelium of the follicle immediately after ovulation. Epithelial cells become round due to a decrease in tensile strength of the follicular wall. x1,150.

Fig. 11. Bundles of collagenous fibers of the theca externa in a mature follicle. They look like compact and thin sheets. Individual fibers are hardly recognized, since they are bound together by inter-fibrillar matrix. x2,350.

Fig. 12. Bundles of collagenous fibers of the theca externa in the stigma immediately before ovulation. They are dissociated into a sparse network of fibers. x5,200.

Plate II
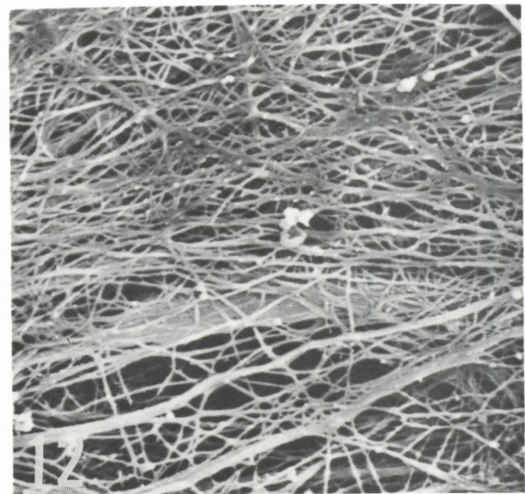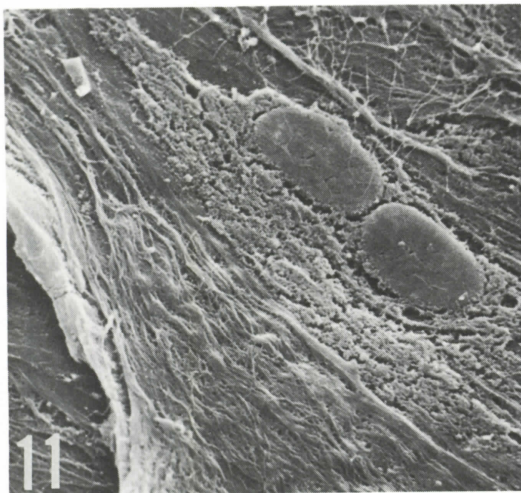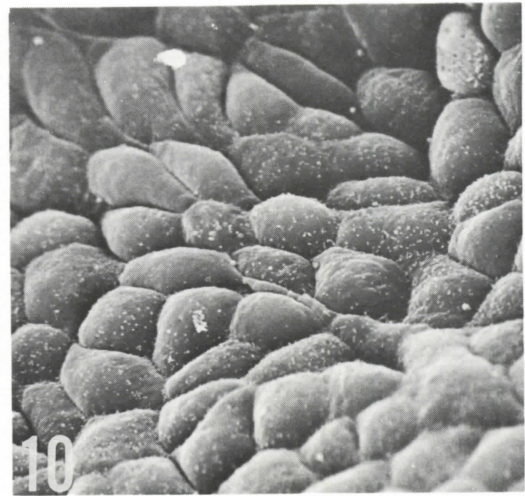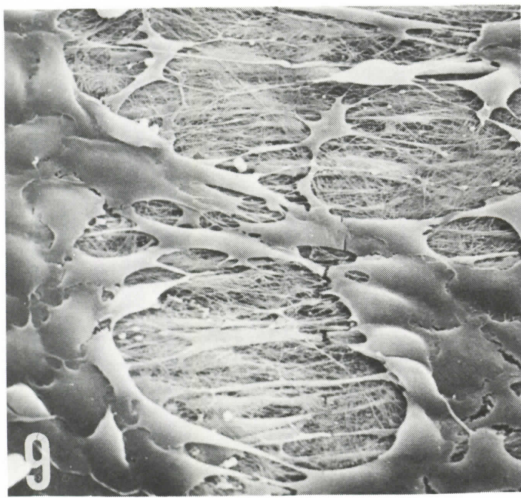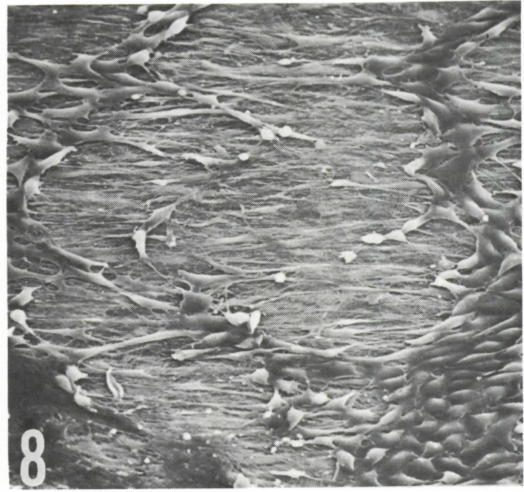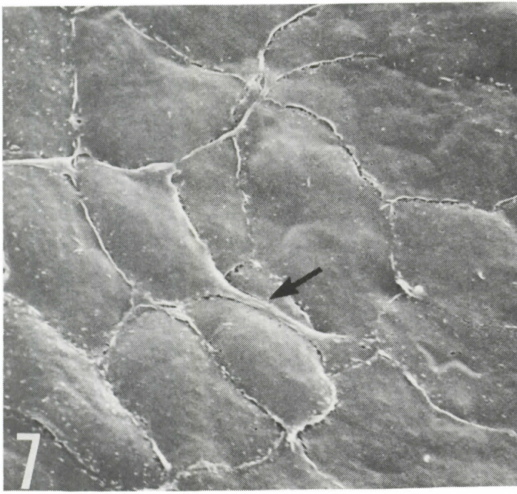
Plate III

Fig. 13. Stratum granulosum adjacent to the stigma in a pre-ovulatory follicle. Granulosa cells are cuboidal. Each cells has a long cytoplasmic projection and numerous fine and tall microvilli. x1,000.

Fig. 14. Higher magnification of the portion similar to Fig. 13. x3,250.

Fig. 15. Stratum granulosum of the stigma near the center of the stigma immediately before rupture. Most granulosa cells have largely bulged vesicles and retain empty holes after the release of vesicles. x1,000.

Fig. 16. Higher magnification of Fig. 15. x3,100.

Fig. 17. Stratum granulosum of the stigma at the site of rupture immediately before ovulation. Granulosa cells become flattened and elongated, and disappear finally. x1,200.

Fig. 18. Inner surface of an unruptured area of the stigma. It is engraved distinctly in an unruptured area corresponding to the stigma. On the left side is a ruptured fissure. x50.
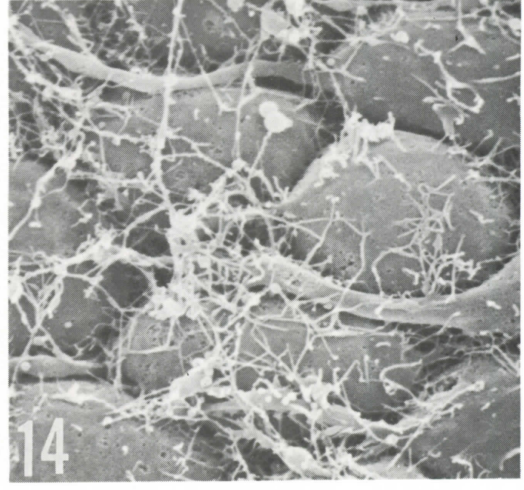
Plate III
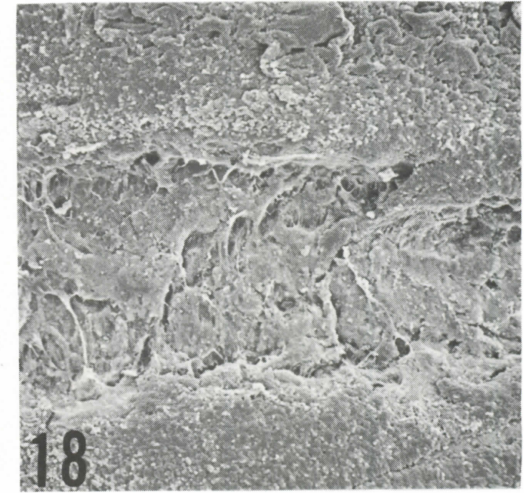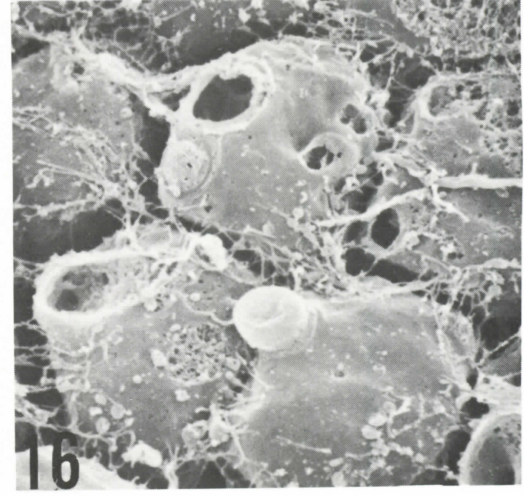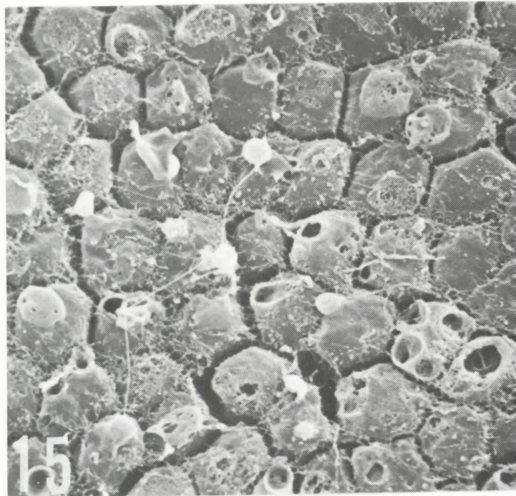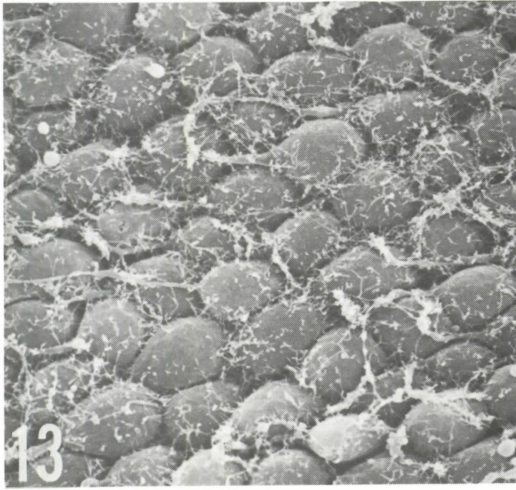
Plate IV

Fig. 19. Decomposed bundles of collagenous fibers of the theca externa in the unruptured stigmal wall shown in Fig. 15. x5,200.

Fig. 20. Tapered stigmal wall around the fissure of a ruptured follicle. The superficial epithelium sloughs off and the theca externa is exposed. x1,150.

Fig. 21. Stratum granulosum of a post-ovulatory follicle immediately after ovulation. Granulosa cells become round, and some of them slough off. x300.

Fig. 22. Stratum granulosum of a post-ovulatory follicle about 6 hours after ovulation. Granulosa cells are swollen and lose cytoplasmic processes. x2,350.

Fig. 23. Stratum granulosum of a post-ovulatory follicle 48 hours after ovulation. Granulosa cells cluster in a mass. They are involved in marked vesicular changes. x2,300.

Fig. 24. Stratum granulosum of a post-ovulatory follicle 72 hours after ovulation. Granulosa cells undergo marked vesicular changes and become a mass of fragments and shrunk vesicles. x2,300.

Plate IV
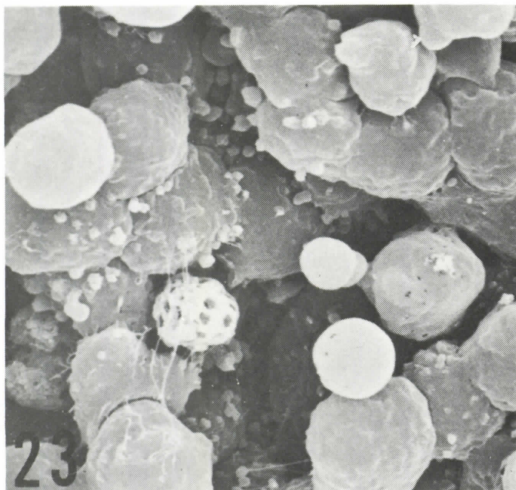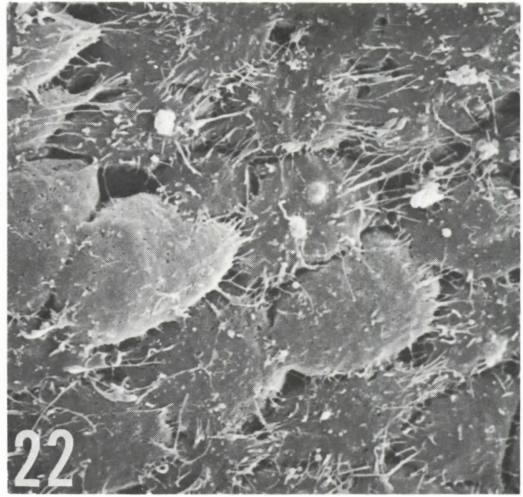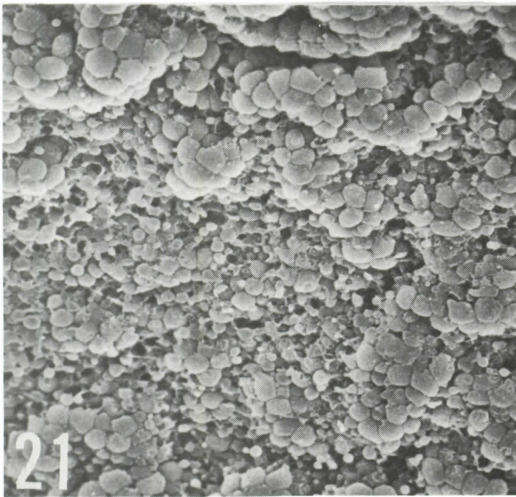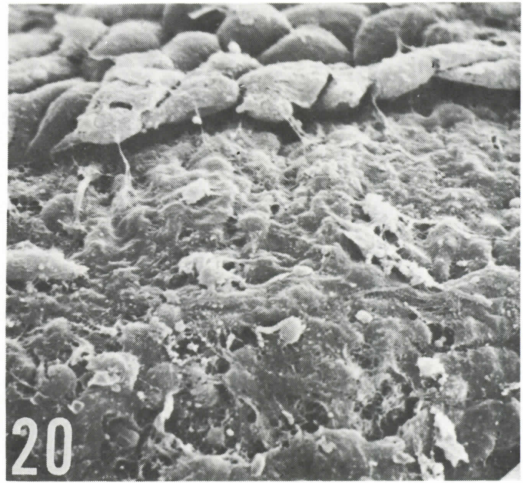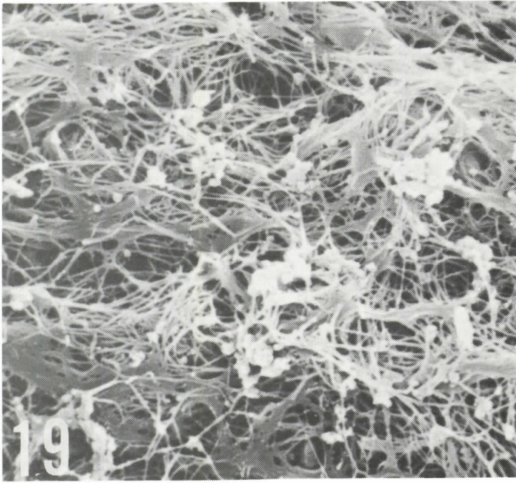
鶏の排卵に伴う卵胞スチグマ組織の
構造的変化の知見補遺

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鶏の排卵は卵胞スチグマ部の破裂によってもたらされる。本研究は形態学的観点から，スチグマの破裂機構を明らかにするため，排卵に伴うスチグマ組織の構造的変化を調べた。所見は次のとおりであった。

1. 成熟卵胞のスチグマ部は，本来的に特徴的構造を具えていた。この部は，卵胞壁の厚さの大部分が外卵胞膜層によって占められ，また，血管の分布が著しく劣っていた。外卵胞膜層は線維結合組織であり，層状に配列するシート状の膠原線維束によって緻密に構築されていた。

2. 排卵直前にはスチグマ部の顆粒層と外卵胞膜層に大きな構造的変化が現われた。スチグマの顆粒細胞は空胞様に変性し，破裂部位では扁平化して消失した。一方，外卵胞膜層の膠原線維束は単線維または細線維に解疎し，薄い線維層となった。

3. 上記のようなスチグマ組織の構造的変化は，その変化像から判断して単なるスチグマの拡張に伴って二次的に生じたものではなく，むしろ何らかの酵素的作用によってもたらされたものと推察された。

4. 排卵後に卵胞は急に収縮した。これに伴って顆粒層は基底膜とともに卵胞膜層から剥離し，顆粒細胞は空胞様変性を伴う退行過程に入った。

5. 以上の所見から，スチグマ破裂は，先行的にスチグマ組織の崩壊，脆弱化がもたらされ，これに卵胞膜の張力が加わることによって引きおこされるものと考えられた。