

THE APPEARANCE AND STRUCTURE OF INTERCELLULAR CONNECTIONS DURING THE ONTOGENY OF THE RABBIT OVARIAN FOLLICLE WITH PARTICULAR REFERENCE TO GAP JUNCTIONS

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ABSTRACT

Lanthanum tracer and freeze-fracture electron microscope techniques were used to study junctional complexes between granulosa cells during the differentiation of the rabbit ovarian follicle. For convenience we refer to cells encompassing the oocyte, before antrum and gap junction formation, as follicle cells. After the appearance of an antrum and gap junctions we call the cells granulosa cells. Maculae adherentes are found at the interfaces of oocyte-follicle-granulosa cells throughout folliculogenesis.

Gap junctions are first detected in follicles when the antrum appears. In early antral follicles typical large gap junctions are randomly distributed between granulosa cells. In freeze-fracture replicas, they are characterized by polygonally packed 90-Å particles arranged in rows separated by nonparticulate A-face membrane. A particle-sparse zone surrounds gap junctions and is frequently occupied by small particle aggregates of closely packed intramembranous particles. The gap junctions of granulosa cells appear to increase in size with further differentiation of the follicle. The granulosa cells of large Graafian follicles are adjoined by small and large gap junctions; annular gap junctions are also present. The large gap junctions are rarely surrounded by a particle-free zone on their A-faces, but are further distinguished by particle rows displaying a higher degree of organization.

Gap junctions, or nexuses, are highly differentiated portions of the plasma membrane between adjoining cells, and existing data suggest that they afford little resistance to the transcellular flow of ions and low molecular weight substances (Bennett, 1973). The morphological details of this membrane specialization are best illustrated when an extracellular tracer such as lanthanum is coupled with the freeze-fracture technique (Revel and Kar-

novsky, 1967; Brightman and Reese, 1969; Goodenough and Revel, 1970; McNutt and Weinstein, 1970; Friend and Gilula, 1972). Studies on a variety of vertebrate tissues indicate that the gap junction consists of hexagonally arranged 80–90-Å particles (subunits) in the plane of membranes separated from each other by a 20–40-Å space. The occurrence of this junction in cells surrounding differentiating oocytes has been indicated from

thin section ultrastructural studies on several diverse mammalian species (Bjorkman, 1962; Wimsatt and Parks, 1966; Byskov, 1969; Friedkalns and Weber, 1968; Motta et al., 1971; Espey and Stutts, 1972). However, few studies have employed tracer or freeze-fracture methods to accurately identify this type of membrane specialization (Anderson, 1971; Merk et al., 1973; Fletcher and Everett, 1973; Fletcher, 1973). Anderson (1971) attempted to determine the time of appearance of gap junctions in developing mouse follicles, and a possible hormonal influence on the frequency of occurrence of gap junctions between rat granulosa cells was suggested by the studies of Merk and his co-workers (1972).

This communication describes the ontogeny and fine structural features of various intercellular connections that exist between granulosa cells and oocytes in the developing (preantral, early antral and mature Graafian or preovulatory) ovarian follicle of the rabbit. We have employed both tracer and freeze-fracture methodologies in this investigation and have paid particular attention to the gap junction.

MATERIALS AND METHODS

Ovaries from 6–12-mo old virgin and nonvirgin Dutch Belted rabbits were fixed for 1 h by immersion in a fixative containing 2% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.4), with 2% sucrose. After primary fixation in aldehyde, the tissues were washed several times in buffer containing sucrose, and postfixed for 1 h in 1% OsO₄ in 0.1 M cacodylate buffer at pH 7.5, dehydrated in a graded series of ethanols, and embedded in a mixture of Epon-Araldite (Anderson and Ellis, 1965). In an effort to stage various follicles, semithin sections were stained with toluidine blue, according to Ito and Winchester (1963). Thin sections cut on a diamond knife were collected on 200-mesh grids and stained with uranyl acetate (Watson, 1958) followed by lead as recommended by Sato (1968). Lanthanum was used to outline the extracellular space by treating tissues with a 2% solution of lanthanum nitrate (pH 7.4) during aldehyde fixation and washing in buffer (for another procedure see Revel and Karnovsky, 1967). After overnight washing, the specimens were incubated for 1 h in 0.03 N sodium hydroxide with 2% sucrose, postfixed in 1% OsO₄, and processed as before for electron microscopy.

Freeze Fracture

For freeze fracturing, individual follicles were dissected free from surrounding stromal and interstitial tissue, fixed for 20–30 min in the fixative, and washed in buffer. Follicles measuring 0.3–0.7 mm (early antral) and

0.8–1.2 mm (preovulatory) were pooled and cut into smaller pieces, infiltrated for 1–2 h in 20% glycerol in buffer at room temperature, frozen onto cardboard disks in liquid Freon 22 (chlorodifluoromethane), and stored in liquid nitrogen. 1-mm cubes of cortical ovarian tissue, containing follicles no greater than 150 μ m in diameter, were also prepared. For freeze fracturing and subsequent platinum-carbon shadowing of the specimens, a Balzers apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) was used. Adhering tissue was removed from the replicas with Clorox; the replicas were subsequently picked up on 200-mesh grids and examined in either a Philips 200 or 300 electron microscope. The freeze-fracture process splits cellular membranes through their hydrophobic interior, revealing two aspects of the interior of the unit membrane (Branton, 1966; Pinto da Silva and Branton, 1970). Conventionally, the inner leaflet is designated as the *A*-fracture face and the outer one is referred to as the *B*-fracture face. To obtain proper orientation of the relief on replicas, the direction of shadowing is indicated by an arrow in the lower left corner of the illustrations.

OBSERVATIONS

Preantral Follicles

In an effort to simplify terminology we will refer to those cells surrounding the oocyte in preantral follicles as follicle cells. Once the antrum appears, these cells comprising the follicular epithelium will be called granulosa cells.

In young follicles, the oocyte is surrounded by a single layer of squamous cells having a flattened and indented nucleus (Fig. 1). The oocyte is associated with the follicle cells and the follicle cells are joined to each other by maculae adherentes (Fig. 1, inset). Upon further growth of the follicle the surrounding cells become more cuboidal to columnar in shape and are separated from each other by a 200–300- \AA space (Figs. 2, 3). During this transition the nucleus becomes more irregularly shaped, cytoplasmic volume increases, and numerous cytoplasmic projections appear on the surface of these cells facing the oocyte (Figs. 2, 4). With further growth, the processes of follicle cells freely intermingle with the microvilli of the oocyte and are separated in most places by a regular 200- \AA intercellular space (Fig. 4). However, restricted portions along the oocyte surface become heavily impregnated with lanthanum and show significant variability with respect to the dimensions of the extracellular space. Where projections from either follicle cells or oocyte encroach upon the oolemma, narrowing regions of

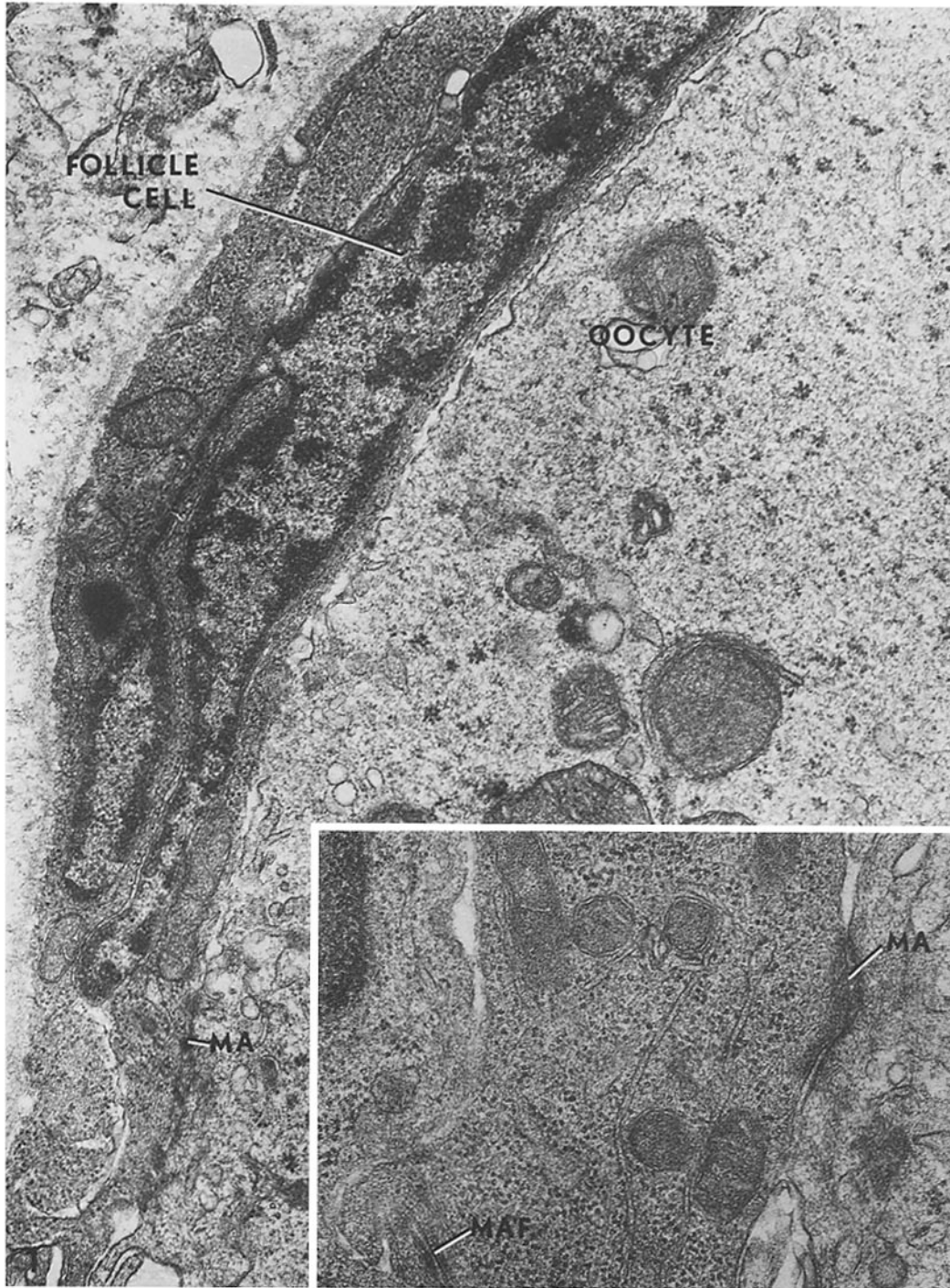
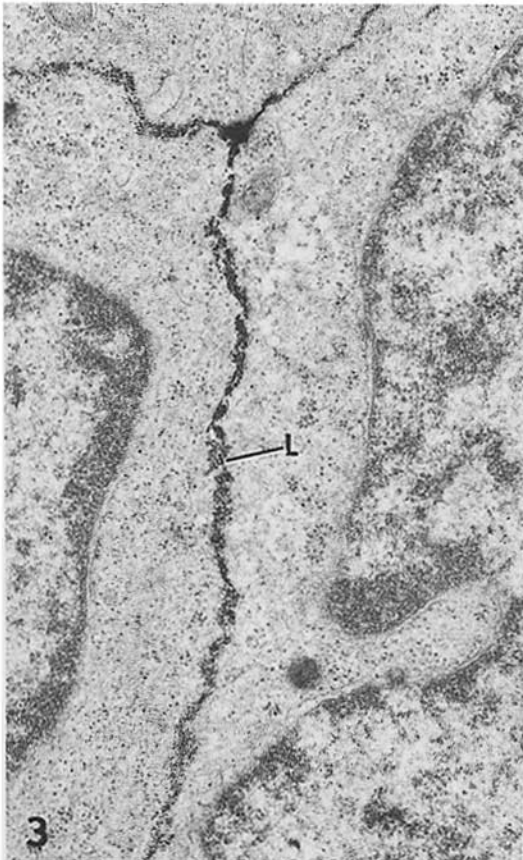


FIGURE 1 Young preantral follicle illustrating oocyte-follicle cell relation. Note the maculae adherentes (*MA*) between oocyte-follicle cell and between follicle cells (*MAF*; see inset). $\times 20,000$; inset, $\times 40,000$.



FIGURES 2-4 Preantral follicles treated with lanthanum. Lanthanum outlines the follicle cell border (*L*, Figs. 2 and 3) and microvilli of the oocyte (*MV*, Fig. 4). Fig. 2, $\times 12,900$; Fig. 3, $\times 21,000$; Fig. 4, $\times 21,000$.

cell contact are apparent and certain of the follicle cell processes may invaginate the egg cortex (Fig. 4).

In replicas of preantral follicles, the cytoplasmic *A*-face is characterized by many randomly distributed particles varying in size from 60 to 100 Å. Surface protuberances and pits of different dimensions (300–900 Å) were present on both fracture faces, and no evidence of junctional membrane was detected in follicle cell membranes from preantral follicles.

Early Antral Follicles

Close pentilaminar membrane contacts and maculae adherentes are commonly observed along straight and undulating borders of the rather polyhedral to stellate shaped granulosa cells of follicles forming an antral cavity (Figs. 5, 6). In sections, lanthanum is seen to readily percolate through the intercellular space in regions of intimate membrane apposition and accentuates a space about 30–40 Å which often displays a striated pattern having a periodicity of approximately 100 Å (Fig. 7). The gap junction is not always of uniform dimensions and can be seen to dilate to a width of 80–90 Å at irregular intervals (Fig. 7). These junctions are most prominent between those granulosa cells resting on the thick basement membrane (*membrana limitans*), which separates these cells from those of the theca interna. *En face* views of this region reveal the typical array of 90-Å subunits seen in gap junctions with roughly a 100-Å periodicity (Fig. 7, inset). A 15-Å lanthanum-stained dot is centrally located on certain of the particulate subunits. Pinocytotic pits engorged with lanthanum tracer are not uncommon along areas of extrajunctional membrane.

Replicas made from early antral follicles exhibit a great pleomorphism of gap junctions. These junctions are composed of quasihexagonally ordered particulate aggregates, not unlike the *A*-fracture face appearance of gap junctions in tissues of other vertebrates (Figs. 8, 9) (Goodenough and

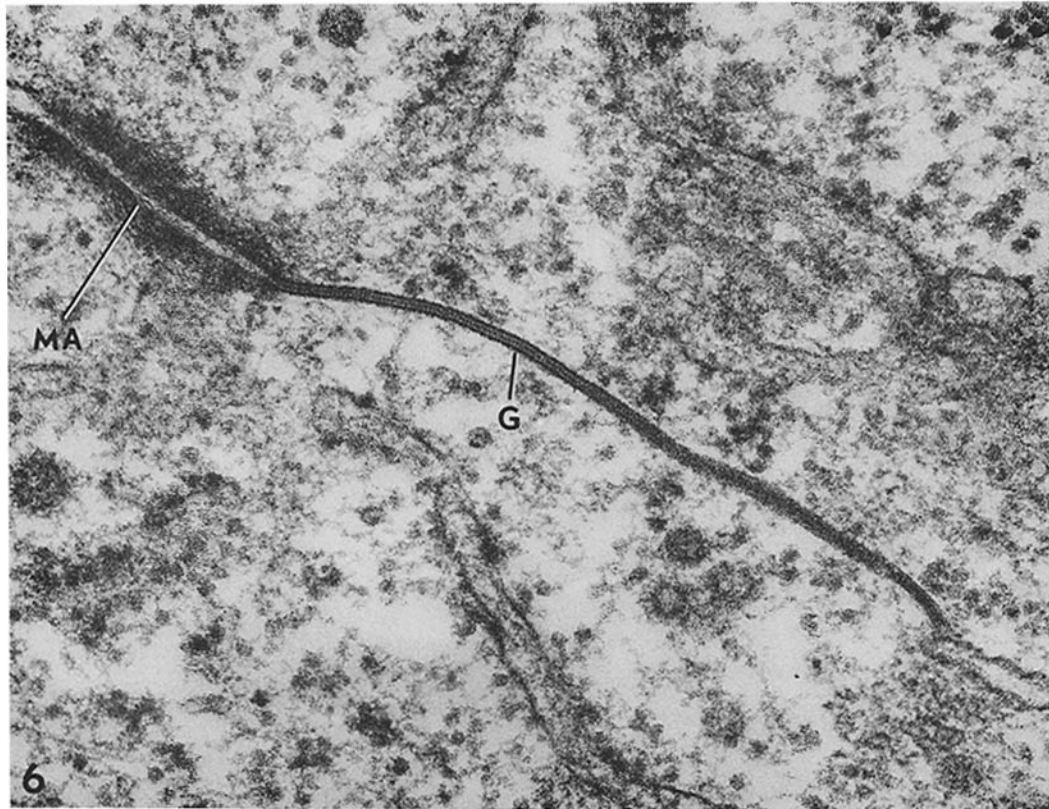
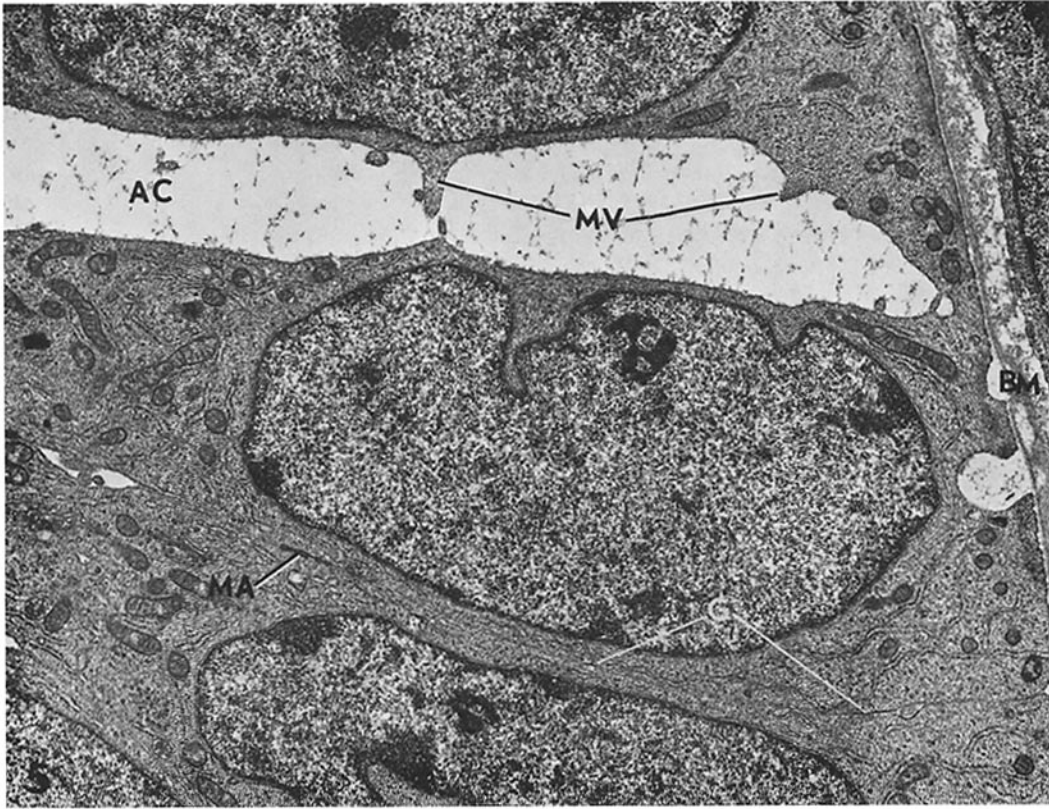
Revel, 1970; McNutt and Weinstein, 1970; Friend and Gilula, 1972). A centrally positioned 15-Å depression is visible on some *A*-face junctional particles (Fig. 11). The 90-Å membrane particles are generally arranged as small domains of close hexagonal packing showing a 100-Å repeat and which are frequently separated by particle-poor areas (Fig. 11). These features are consistent with those observed on regions of *B*-fracture face membrane, frequently found in association with large gap junctions (Fig. 10). Large gap junctions, with a well demarcated boundary, vary in width (0.3–2.0 μm) and are located on smooth contoured regions of the cell membrane as well as on plateaus and rounded elevations (Fig. 10). A particle-sparse zone surrounds larger *A*-face junctional domains in which smaller particle aggregates are frequently situated (Fig. 10). Aggregates of smaller, polygonally packed particles are often seen to be in association with each other on particle-poor areas of the *A*-face; these do not always associate with large gap junctions (Figs. 8, 9, 12). The particles of the gap junctions are, for the most part, uniform in size, in contrast to particles on the extrajunctional region of the membrane. Spherical surface depressions and excrescences are randomly distributed on nonjunctional *A*- and *B*-faces of the granulosa cell membrane.

Graafian Follicle or Preovulatory Follicle

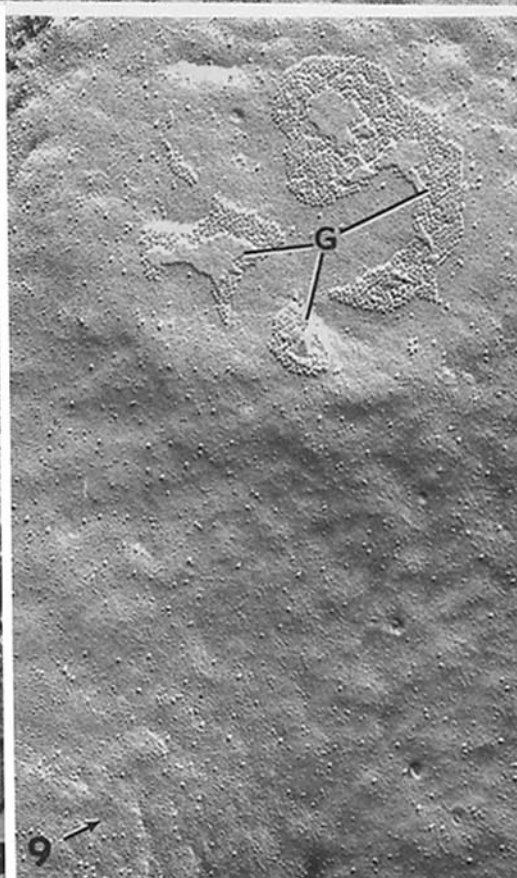
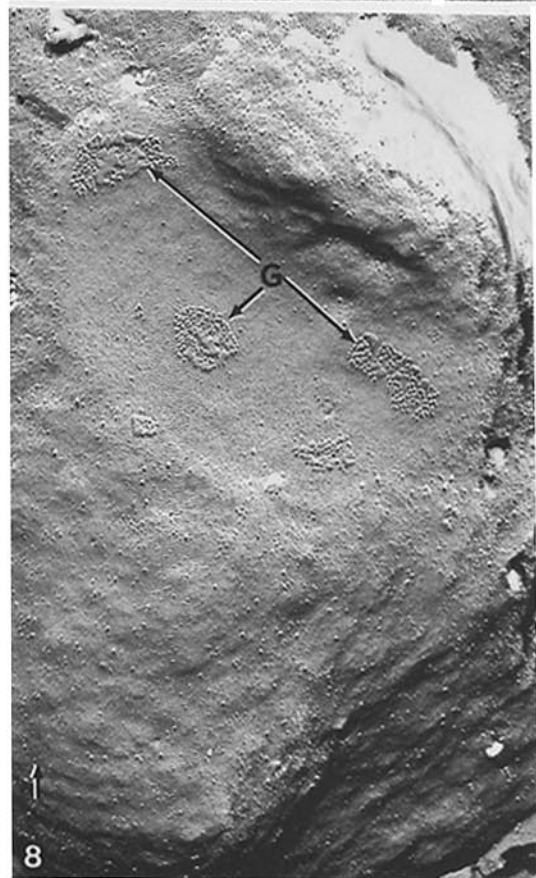
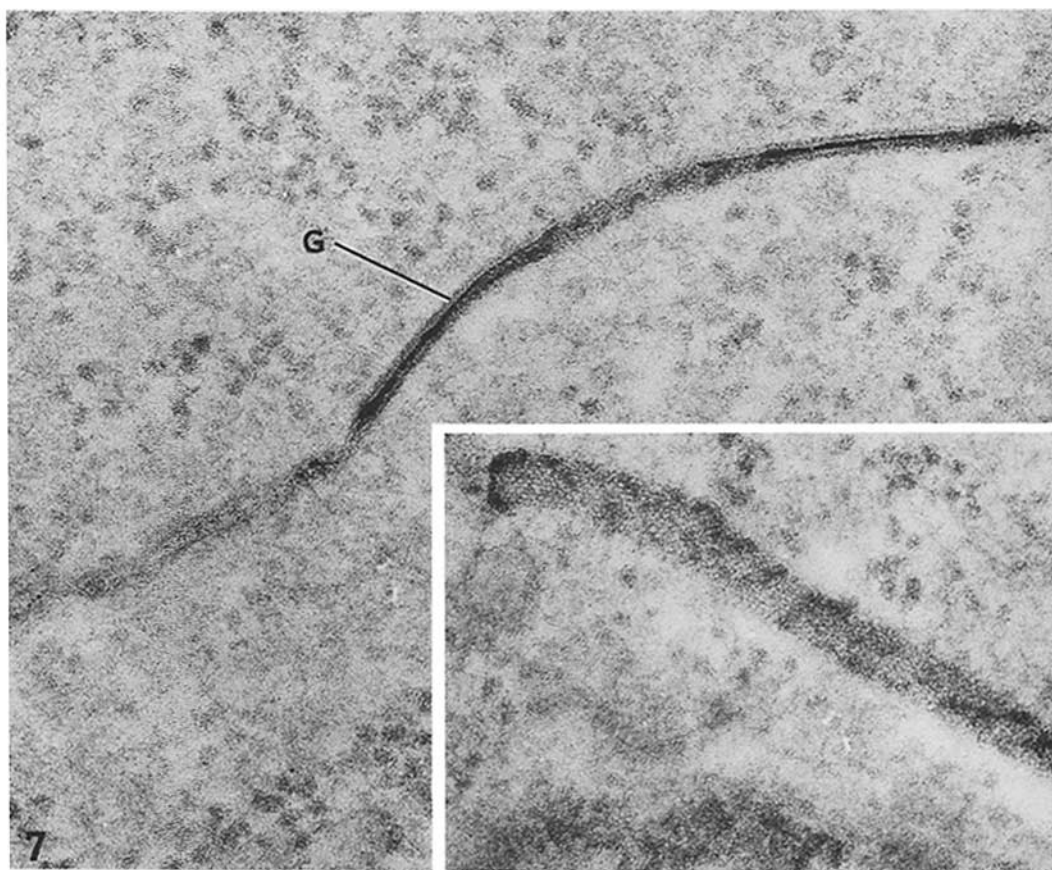
Extensive gap junctional contacts are made between granulosa cells of large Graafian (0.9 mm or greater) follicles. This surface specification is prominent along convoluted areas of intercellular apposition and along deeply penetrating processes between cells. These are the so-called annular gap junctions (Fig. 18). Freeze-fracture profiles of the annular junctions display ordered, pitted, and particle arrays as seen in Fig. 18. It is difficult to determine from these images whether the gap junction is situated on the end of a process or belongs to an intracellular vesicle. Most freeze-fracture features of gap junctions were similar to those described above, with the notable exception

FIGURE 5 Early antral follicle. A portion of the antral cavity (*AC*) is depicted into which microvilli (*MV*) from the granulosa cells project. The regular intercellular space between granulosa cells is interrupted by a macula adherens (*MA*) and two more basally located gap junctions (*G*). *BM*, basement membrane. × 20,000.

FIGURE 6 Higher magnification of a macula adherens and gap junction conjoining two granulosa cells. × 180,000.



6



that particle rows on the *A*-face appeared more ordered (Fig. 13). Most of the larger junctions were characterized by small rectangular domains of a highly ordered hexagonal packing (Figs. 15, 16, and 17). Particle-depleted margins were uncommon and rarely were smaller *A*-face particle aggregates observed marginally to gap junction domains (Figs. 13, 16, 17). Granulosa cell gap junctions at this stage of follicular development were larger and more symmetrical in shape (spherical to oval) (Figs. 13, 16, 17). Convexities of the cell surface were frequently occupied by gap junctional membrane. No peculiar morphological features of the extrajunctional *A*- or *B*-fracture faces were detected other than those previously described.

In Fig. 14, it is interesting to note the heavy staining of the gap junction with lanthanum in contrast to the fine precipitate of tracer in the extracellular space. Similar dense deposits of the tracer were found at discrete foci along the oocyte surface (Fig. 4) of follicles of all stages, suggesting that the tracer is avidly bound to select areas of the plasma membrane (see also Martinez-Palomo et al., 1973).

Microvilli are found on the lateral basal aspect of the granulosa cells, particularly those granulosa cells resting on the basement membrane (Fig. 5). The microvilli delineate a region between adjacent cells reminiscent of the sectioned profile of a bile canaliculus.

DISCUSSION

This study has been primarily concerned with the development of gap junctions between rabbit ovarian granulosa cells and suggests that this membrane specialization arises coincident with formation of an antrum in the investigated species. Two types of granulosa cell gap junctions in the rat ovary have been described by Merk et al. (1973). The abutment form occupies planar portions of apposing membranes, while the so-called annular

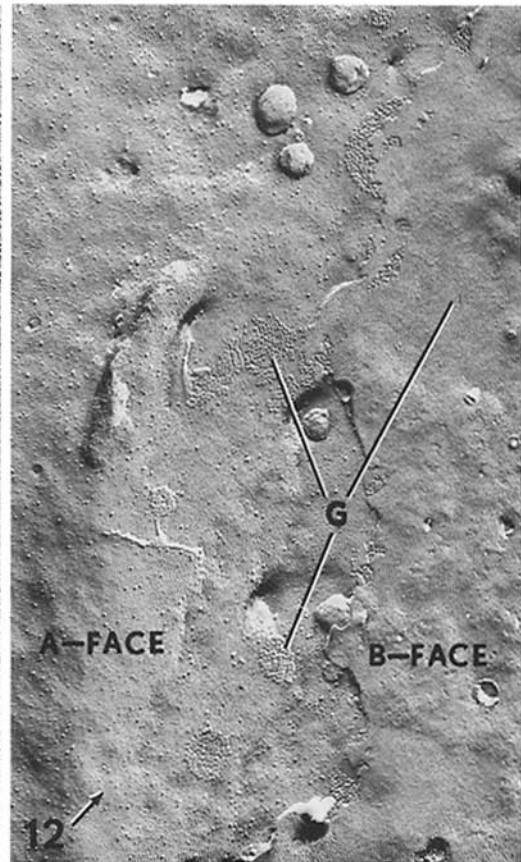
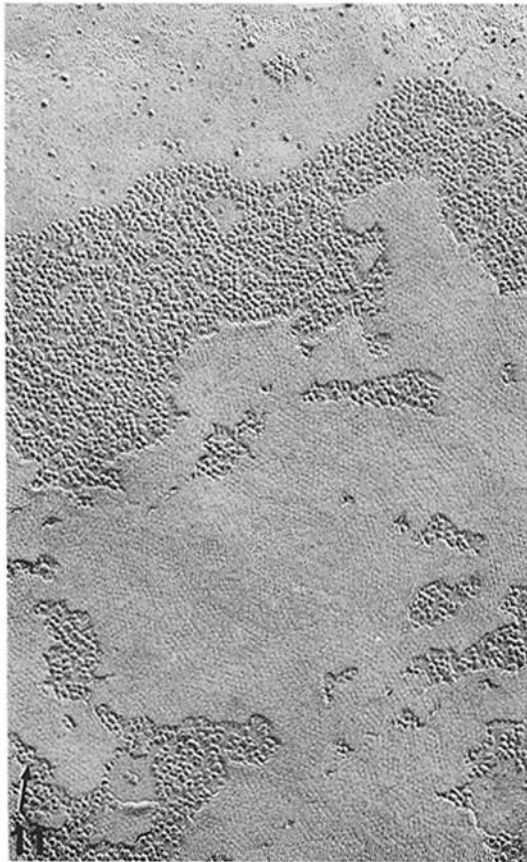
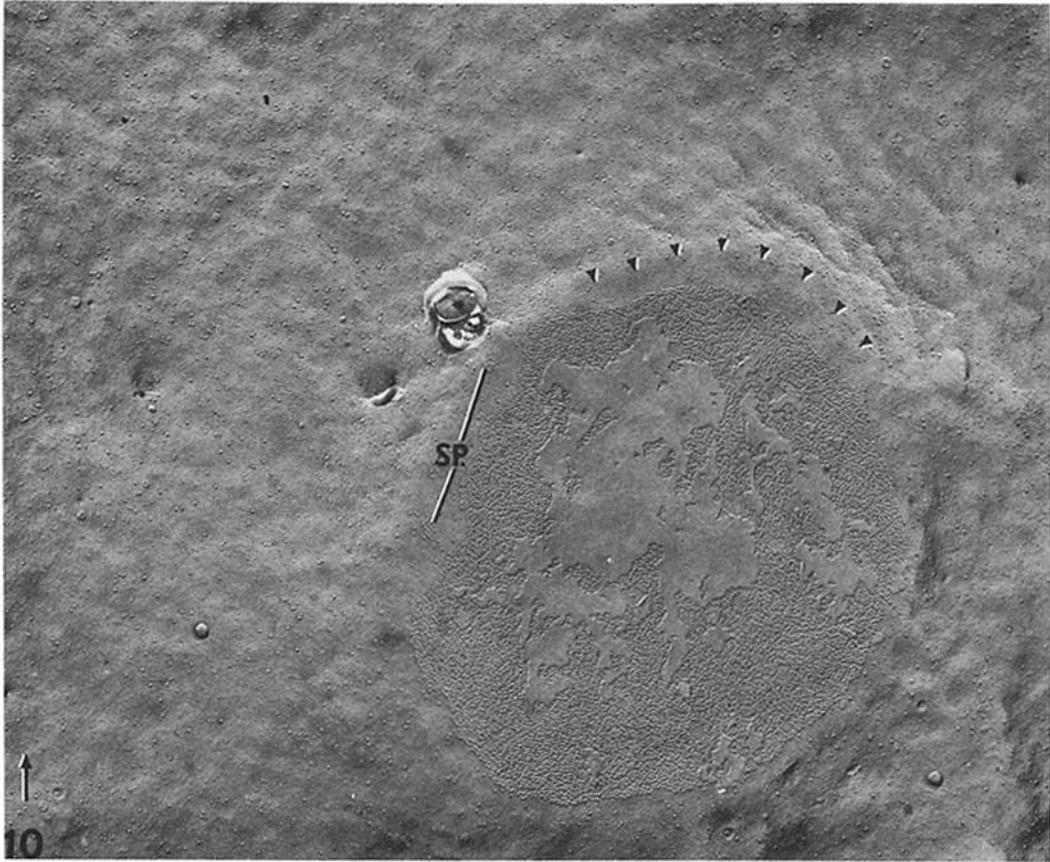
variety line invading projections between granulosa cells which, in cross section, are spherical gap junction profiles lacking apparent continuity with the limiting plasma membrane. Electron microscope analysis of serial sections of rat and rabbit granulosa cells supports the notion that some of the annular gap junctions may represent true intracellular inclusions (Merk et al., 1973; Espey and Stutts, 1972). Various nomenclatures have been applied to these structures, such as annular desmosomes in the cow (Priedkalns and Weber, 1968), thick-walled bodies in the mouse (Byсков, 1969), and sphaerae oclusae in the rabbit (Espey and Stutts, 1972). The annular junction is not unique to granulosa tissue and has been described in the enamel-forming organ of the rat (Garant, 1972) and the keratinizing wool follicle of the sheep (Orwin et al., 1973).

As indicated previously, correlative morphological and physiological findings implicate the gap junctions as the site of intercellular communication (Gilula et al., 1972; Bennett, 1973; McNutt and Weinstein, 1973). Although no direct physiological evidence is available, we feel that the potential role of the granulosa cell gap junction in cell-to-cell communication becomes apparent if certain physiological parameters of follicular growth are taken into consideration. In connection with this, cell kinetics studies on the mouse ovaries by Pedersen and Hartman (1971) indicate that the granulosa cell population enters a phase of rapid proliferation at the time of antrum formation (see also Moore et al., 1974). Recently Goldenberg et al. (1972) have shown in the hypophysectomized immature female rat that diethylstilbesterol (DES) stimulates proliferation of granulosa cells (determined by [³H]thymidine incorporation) as well as their ability to bind [³H]follicle-stimulating hormone (FSH). FSH alone acted as a poor mitogenic agent but was necessary for sculpturing of the antrum when administered in combination with DES. Treatment of immature hypophysectomized

FIGURE 7 An early antral follicle depicting lanthanum outlining a gap junction (*G*). Inset is an *en face* section of the junction revealing the quasihexagonal ordering of subunits. Fig. 7, $\times 139,800$; Inset, $\times 139,800$.

FIGURE 8 Replica of granulosa cell fracture face (early antral follicle) surface showing cluster of small gap junctions (*G*) in an area relatively low in particle density when compared to surrounding nonjunctional *A*-face membrane. $\times 61,500$.

FIGURE 9 Similar view of cell surface membrane as in Fig. 8 showing several gap junctions (*G*) of varying size on particle-poor region of *A*-face. $\times 61,500$.



rats with DES was also reported to stimulate cellular growth but not antrum formation (Merk, et al., 1972). Merk et al. (1972) also noted that after stimulation with DES there was an apparent increase in the number of gap junctions connecting granulosa cells. In intact control animals, Merk and his associates also observed a relationship between the extent of thecal development in a given follicle and the number of gap junctions present. These results appear to be in agreement with the contention of Hisaw (1947) that at least the proliferative aspect of follicular growth was directed by estrogens presumably derived from the thecal investment. In addition to their inferred mitogenic role, Merk et al. (1972) suggested that estrogens directly influence the production of granulosa cell gap junctions.

It is generally believed that preantral follicular growth is independent of pituitary support and involves cellular proliferation only; formation of the antrum and its enlargement as well as continued granulosa cell proliferation are aspects of follicular growth that do depend on an intact pituitary (Hisaw, 1947; Schwartz and Hoffman, 1972). Channing (1970) reported a differential sensitivity to gonadotropins by granulosa cells of different size rhesus monkey follicles. Granulosa cells obtained from large preovulatory follicles on days 12–14 of the menstrual cycle luteinize (secrete progesterone) "spontaneously." Cells derived from small 1-mm follicles at any stage of the cycle luteinize only if both human luteinizing hormone (LH) and FSH are provided in the culture medium. Either of these hormones alone, when added to cell cultures from medium sized follicles of days 7–11 of the cycle, induced luteinization. All granulosa cells used in these studies showed responsiveness to gonadotropins to some degree and were obtained from antral follicles. More recently Erickson et al.¹ evaluated the ability of cultured

rabbit granulosa cells to secrete progesterone in response to gonadotropic hormones. Granulosa cells from preantral follicles were found to lack the capacity to elaborate progesterone into the medium in the presence of human chronic gonadotrophin (HCG) or a combination of LH/FSH, whereas cells from early and later antral follicles secreted high levels of progesterone under the same in vitro conditions. The observations of Erickson et al.¹ coupled with the data presented in this communication, illustrate structural and physiological differences between preantral and antral rabbit granulosa cells. Whether a correlation exists between gonadotropic hormone sensitivity and gap junctions remains to be demonstrated. Might it be that particle organizations in the plane of the membrane reflect yet another functional aspect of the ongoing differentiation of this epithelium?

Various roles for granulosa cell gap junctions have been postulated and include coordination of cellular differentiation during follicle development (Anderson, 1971), and/or reorganization of the follicle during luteinization (Merk et al., 1972, 1973; Fletcher and Everett, 1973). Luteinization is an extremely rapid process that may require wide-spread and expedient interchange of intracellular substances such as cyclic (c) AMP. Hillard (1973) reported that as early as 3 days after ovulation the rabbit corpus luteum is secreting substantial amounts of progesterone, and Channing (1970) observed that granulosa cells of the rhesus monkey secrete high levels of progestins by day 2 in culture. In accordance with the second messenger hypothesis (Butcher et al., 1972), it is thought that protein hormone-receptor interactions at the cell periphery elicit changes in endogenous cyclic nucleotide levels, activating the steroidogenic machinery of these cells. Substances of molecular dimensions comparable to cAMP have been shown to pass through gap junctions in other tissues (Sheridan, 1971). The role of this nucleotide in controlling cell cycle duration in vitro has recently been alluded to by Abell and Monahan (1973). Furthermore, changes in follicu-

¹Erickson, G. F., J. R. G. Challis, and K. J. Ryan. The control of the differentiation of rabbit granulosa cells. Manuscript submitted for publication.

FIGURE 10 This electron micrograph demonstrates the symmetrical nature of certain gap junctions (antral follicle). Small particle aggregates (*SP*) located in a low particle density rim or halo (see small arrows) of *A*-face membrane can be seen adjacent to this large macular gap junction. $\times 39,000$.

FIGURE 11 Higher magnification of the same junction as in Fig. 10. The ordering of *A*-face membrane-intercalated particles into polygons of various sizes roughly corresponds to the distribution of pits on the *B*-face. $\times 105,900$.

FIGURE 12 Region of intercellular apposition having many small gap (*G*) junctions where the fracturing process reveals *A*-face on the left and *B*-face on the right. Antral follicle. $\times 45,000$.

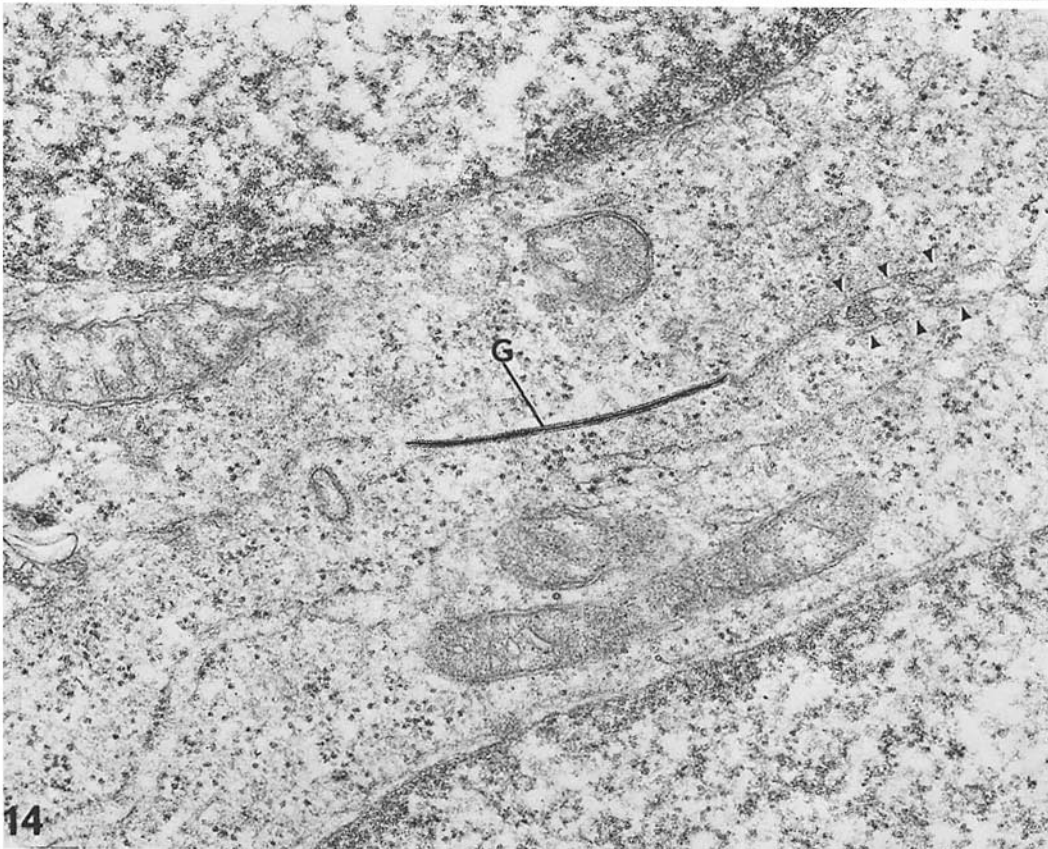
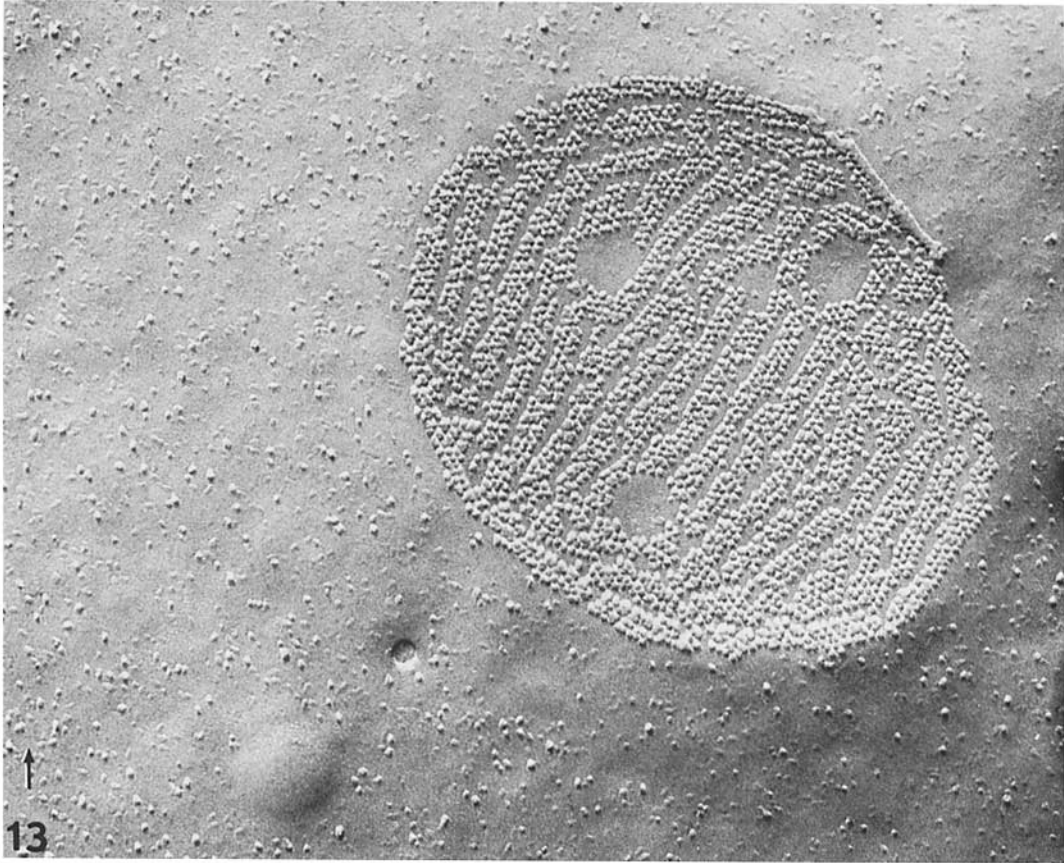
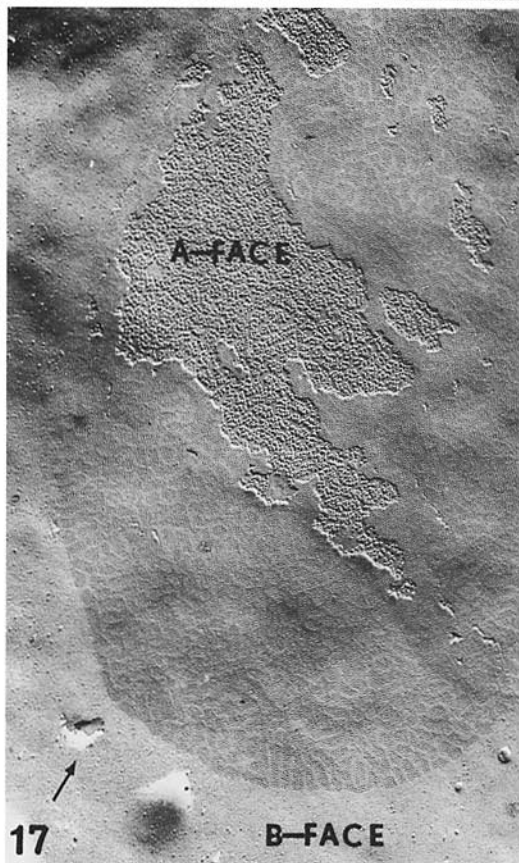
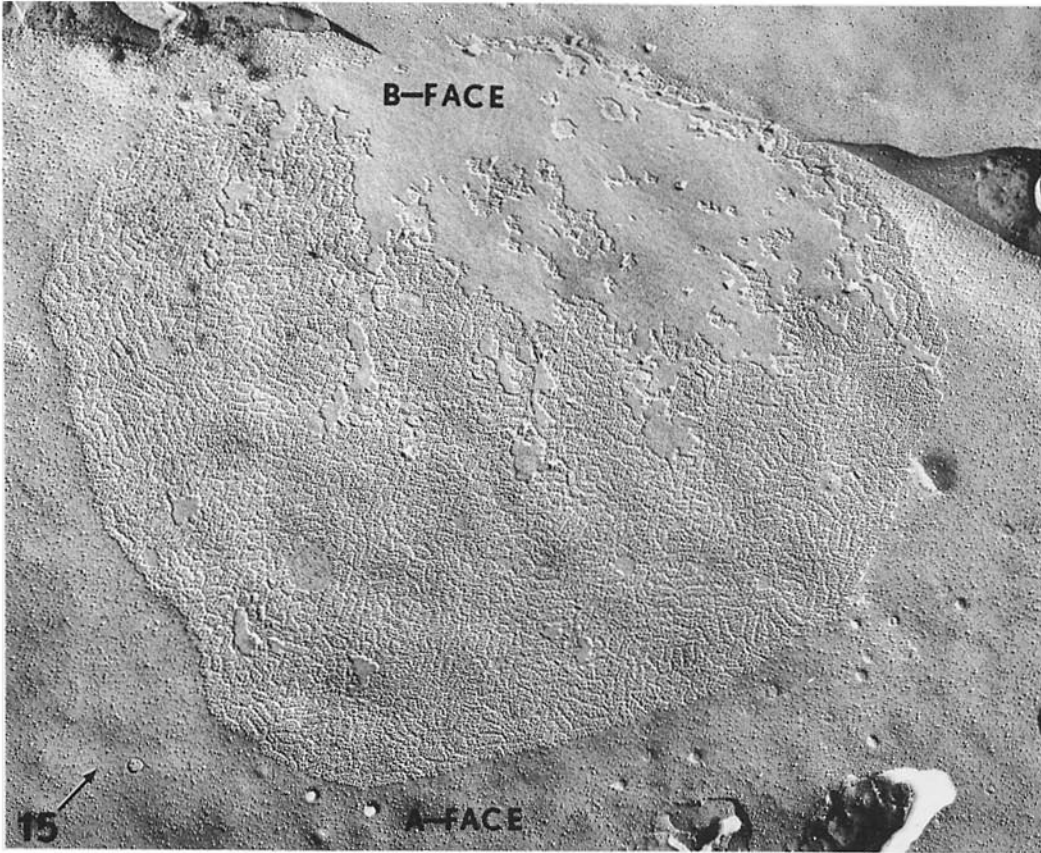


FIGURE 13 Large Graafian follicle. This replica shows a gap junction of oval shape and is distinguished by the high degree of order of its particles into rows separated by parallel particle-free isles and three spherical nonparticulate regions. $\times 139,800$

FIGURE 14 Large Graafian follicle. Lanthanum penetrated gap junction (G) between two granulosa cells. The extracellular space (arrows) is occupied by a fine lanthanum precipitate. $\times 40,000$.



FIGURES 15-17 Graafian follicle. Large gap junctions showing high degree of organization on particulate and pitted *A*- and *B*-faces respectively. Fig. 17 illustrates partitioning of particles and pits into polygonal aggregates by unspecialized channels extending throughout the junctional domain. Fig. 15, $\times 39,000$; Fig. 16, $\times 80,700$; Fig. 17, $\times 45,000$.

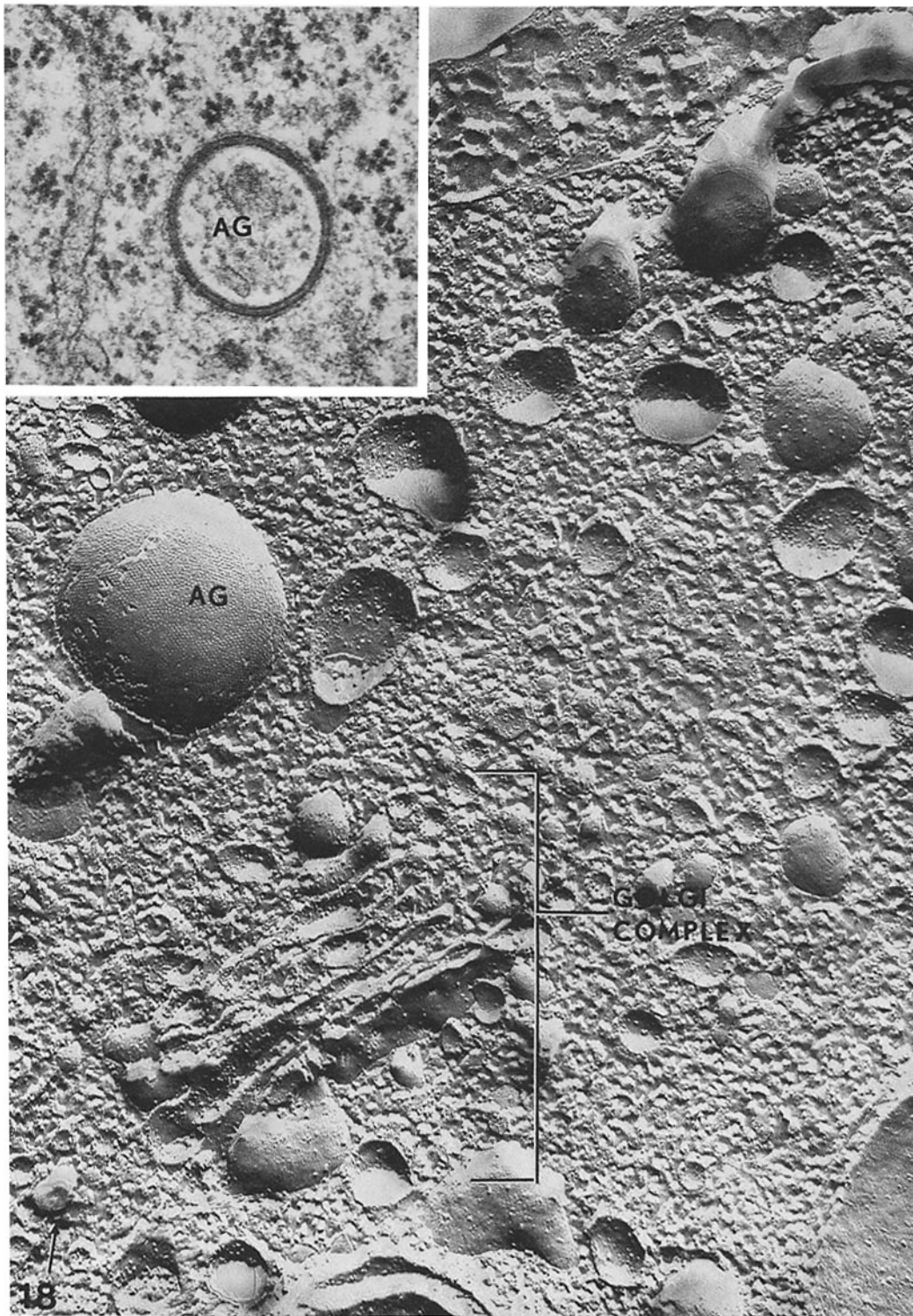


FIGURE 18 Large Graafian follicle. A small portion of the cytoplasm showing Golgi complex and annular gap (AG; also see inset) junction. Fig. 18, $\times 80,700$; inset, $\times 60,000$.

lar cAMP levels have been implicated in the ovulatory process of the rabbit (Marsh et al., 1973).

Little information is available relating the size of gap junctions to the extent of electrical coupling (coupling coefficient), but it is clear that very minute gap junction plaques exist between coupled cells of the adult vertebrate retina (Raviola and Gilula, 1973). In addition, in cultured fibroblasts there is evidence that metabolic cooperation may be achieved through such specializations (Gilula et al., 1972). In this study we observed small gap junctions on particle-depleted areas of the A-face during early antrum formation. At later times in development of the follicle, large gap junctional domains were surrounded by a particle-depleted zone which often contained small particle clusters. These marginal features are absent in the gap junctions of mature follicles which, in addition, display a higher degree of particle organization. We interpret these characteristics to reflect the ongoing differentiation of the gap junction during folliculogenesis. The development of gap junctions and other intercellular connections has been alluded to by Revel et al. (1973) in the chick embryo, by Gilula (1973) in the sea urchin embryo and by Yee (1973) in the regenerating mouse liver.

Based on serial section electron microscope analysis, Merk et al. (1973) and Espey and Stutts (1971) suggested that some of the spherical or annular gap junctions are intracellular. The suggestion has been made that these may represent portions of gap junction that are removed from the cell surface by a process akin to endocytosis (Merk et al. 1973). It is interesting to note that Overton (1968) and Staehelin (1973) made a similar suggestion for desmosomes and tight junctions, respectively. These authors speculate that the internalization of this specialized portion of the plasma membrane may represent a step in its degradation.

As shown in this study substantial areas of the granulosa cell surface of antral follicles are occupied by gap junctions, and whether this feature reflects a compensatory response to intercellular communication during proliferative preovulatory differentiation and a structural requirement during luteinization remains enigmatic. Differences between reflex versus spontaneously ovulating species may exist, and a strict correlation between antrum formation and the emergence of gap junctions remains to be established. Merk et al. (1972) have shown that, in the rat ovary stimulated with DES, gap junctions appear between granulosa

cells of preantral follicles. While gap junctions may arise concomitantly with the acquisition of protein hormone receptor function, their potential interrelationship is unresolved. In any event, we feel that the gap junction between granulosa cells presumably imparts, at least, a structural substrate by which controlled, coordinated, morphological, and chemical differentiation of the follicle may proceed uninterrupted.

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