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ORGANIZATION OF INTRAMEMBRANE PARTICLES IN FREEZE-CLEAVED GAP JUNCTIONS OF RAT GRAAFIAN FOLLICLES: OPTICAL-DIFFRACTION ANALYSIS

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SUMMARY

Gap junctions were identified in the membrana granulosa and cumulus oophorus, and between cells of the internal theca, of the preovulatory rat follicle. In replicas of freeze fractured follicles, the A face presented clusters of closely packed intramembrane particles, 7–9 nm in diameter, forming a mosaic pattern, while the B face showed a similar pattern of small pits. Optical diffraction analysis of these electron micrographs revealed that both the intramembrane particles and the corresponding pits were organized in hexagonal lattices with centre-to-centre spacing of 9–10 nm. In small junctions (up to 0·4 μm in diameter), both A and B faces generally consisted of a single lattice, while large junctions (0·5–2 μm) contained multiple lattices differing in orientation. Occasionally intramembrane particles and pits were more loosely arranged throughout the junctional area and failed to show a hexagonal pattern.

Both granulosa and thecal cells often contained intracellular vesicles resembling annular junctions. These contained intramembrane particles whose assembly resembled that of the gap junctions with regard to periodicity and lattice organization. Examination of thin sections suggested that small gap junctions occur also between cytoplasmic processes of coronal cells and the oolemma. No tight junctions were detected between granulosa cells and between thecal cells.

INTRODUCTION

The available information regarding structural characteristics, distribution in mammalian cells, and possible physiological significance of gap junctions or 'nexuses' has recently been reviewed (McNutt & Weinstein, 1973; Gilula, 1974). The existence of such specialized junctions between granulosa cells of the Graafian follicle has been demonstrated by study of thin sections (Merk, Albright & Botticelli, 1973), and was confirmed more recently by freeze-fracture studies (Fletcher & Everett, 1973; Fletcher, 1973; Albertini & Anderson, 1974; Amsterdam, Josephs, Lieberman & Lindner, 1974*a*, *b*). A polygonal, quasihexagonal or strictly hexagonal organization of the intramembrane particles of gap junctions has been recognized by inspection of negatively stained membrane preparations and of images from freeze-fractured or lanthanum-stained tissues (Benedetti & Emmelot, 1965, 1968; Revel & Karnovsky, 1967; Brightman & Reese, 1969; Goodenough & Revel, 1970; McNutt & Weinstein, 1970; Staehelin, 1972), including the granulosa cells of the ovary (Merk *et al.* 1973;

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Fletcher & Everett, 1973; Fletcher, 1973; Albertini & Anderson, 1974; Amsterdam *et al.* 1974*a*, *b*).

We wish to report on the occurrence and appearance of gap junctions in granulosa as well as internal thecal cells of the preovulatory follicle of the rat, as revealed by examination of thin sections and freeze-fractured tissue. In order to obtain quantitative data regarding the organization of the intramembrane particles, electron micrographs of replicas of freeze-fractured follicles were subjected to optical diffraction analysis: diffraction patterns from restricted areas or from the whole image of the junction were studied and related to the size and location of the junction. A summary of some of the results has been presented (Amsterdam *et al.* 1974*a*, *b*).

MATERIALS AND METHODS

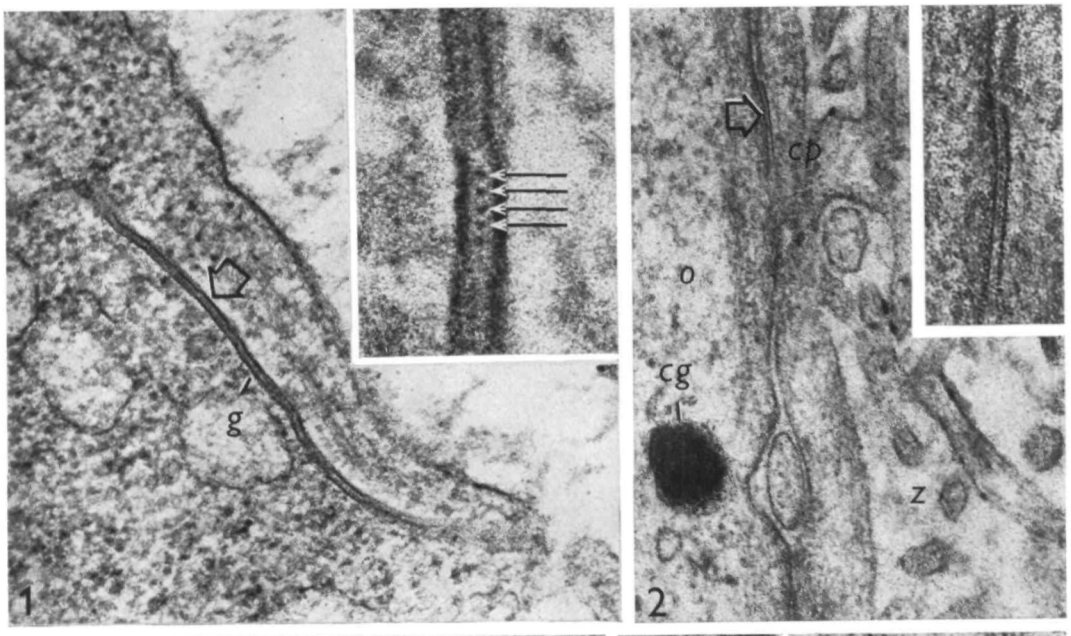
Wistar-derived female rats of the departmental colony, which had shown 2 consecutive 4-day cycles, were used on the morning of pro-oestrus, i.e. before the preovulatory gonadotrophin surge (Ayalon, Tsafriri, Lindner, Cordova & Harell, 1972). Animals were killed by cervical dislocation. Ovaries were excised and placed in phosphate-buffered saline (PBS) and enlarged Graafian follicles protruding from the surface of the ovary were removed by microdissection under a binocular microscope. The isolated follicles were immersed in diluted Karnovsky (1965) fixative (3 % glutaraldehyde and 1·6 % formaldehyde in 0·1 M cacodylate buffer, pH 7·4) for 2–4 h at 24 °C. The follicles were postfixed with osmium tetroxide in the same buffer at 4 °C for 2–6 h. Tissue was stained *en bloc* for 2 h with 0·5 % uranyl acetate in veronal acetate buffer, at pH 5 approximately (Farquhar & Palade, 1965), dehydrated in a graded series of ethanol and propylene oxide and embedded in Epon (Luft, 1961). Thin sections were cut with a glass knife, collected on 300-mesh grids and stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1965). The sections were strengthened by carbon deposition.

Fig. 1. Gap junction (*g*) between granulosa cells of the cumulus oophorus in the preovulatory follicle. At the centre of the junction, cut perpendicular to the plane of the membrane, the central lamina appears as a dotted line (wide arrow) composed of dense globules 4–5 nm in diameter and separated by 3–4 nm translucent gaps (see arrows in inset). The outer leaflets of the junction are heavily stained. × 52 800. Inset × 280 000.

Fig. 2. Gap junction (indicated by arrow) in area of contact between the oocyte (*o*) and cytoplasmic process (*cp*) of corona cell. At higher magnification (inset), the junction appears as a pentalaminar structure, 18 nm in overall thickness. The central dense lamina of the junction appears as an interrupted line. *cg*, cortical granule; *z*, zona pellucida. The photograph was taken at a tilting angle of 18° to the optical axis on the goniometer stage of the Philips 300 electron microscope. × 46 000. Inset × 115 000.

Fig. 3. A pentalaminar gap junction (*g*) between thecal cells. Note the appearance of large lipid droplet (*l*) and large mitochondria (*m*) with well developed tubular cristae. *gc*, Golgi cisternae. × 57 000.

Fig. 4. Annular junction in granulosa cell (*ag*). The area enclosed by the junction is filled with small vesicles and some dense material. The central lamina of the junction appears as a dotted line (see area between arrows in enlarged inset). In order to visualize this junction, the specimen was tilted 31° to the optical axis. Without tilting, details of the junctional membrane could not be detected. × 56 000. Inset × 240 000.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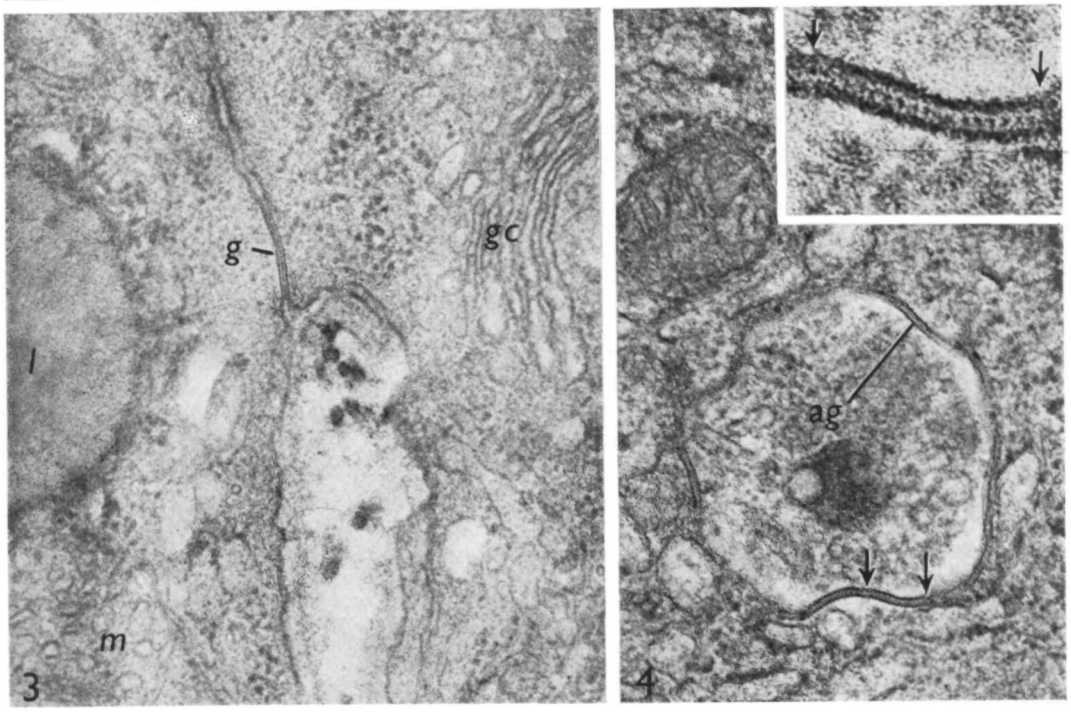
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Freeze-fracture

For freeze-fracturing, follicles were fixed for 30 min in 2·5 % glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4, at 24 °C, and washed 3 times in the same buffer. The fixed tissue was immersed in 25 % glycerol buffered in 0·1 M cacodylate (pH 7·4) at 4 °C overnight. The follicles, frozen on to gold disks in liquid Freon 22 and cooled in liquid nitrogen, were fractured and platinum-carbon shadowed in a Balzers apparatus (BA 360, Balzers High Vacuum Corp.). Adhering tissue was removed from replicas with 12 % sodium hypochlorite. The replicas were subsequently picked up on 300-mesh grids. Thin sections and replicas of freeze-fractured tissue were examined in either a Jeolco 100B or Philips 300 electron microscope at 80 kV. Some thin sections were examined with the aid of the goniometer stage of the Philips 300 electron microscope.

Optical diffraction

Optical diffraction patterns of micrographs were obtained by use of an optical diffractometer constructed essentially according to the design proposed by Taylor & Lipson (1964). Patterns were recorded on Microfile film (Eastman Kodak), using a 70-mm reflex camera back with its camera lens removed. Two features were incorporated into the diffractometer design to facilitate operation of the instrument. Just behind the main diffraction lens (f = 1185 mm), a field lens (f = 300 mm) on a swing in-out mount was installed. With the lens in the optical path, a focused image of the diffracting object was formed at the recording plane and was viewed through the reflex camera. When the field lens was swung out of the optical path, the optical diffraction pattern was immediately visible in the same plane. Thus it was possible to switch rapidly from viewing the object to viewing its diffraction pattern. The second feature consisted of 2 sets of sliding slits oriented perpendicularly to each other. The region within the slits could be varied to enclose an area up to 5 × 5 cm. The entire region within the slits was visible with the field lens in place. (An optical diffractometer incorporating these characteristics is now available from the Lansing Corporation, Ithaca, New York.)

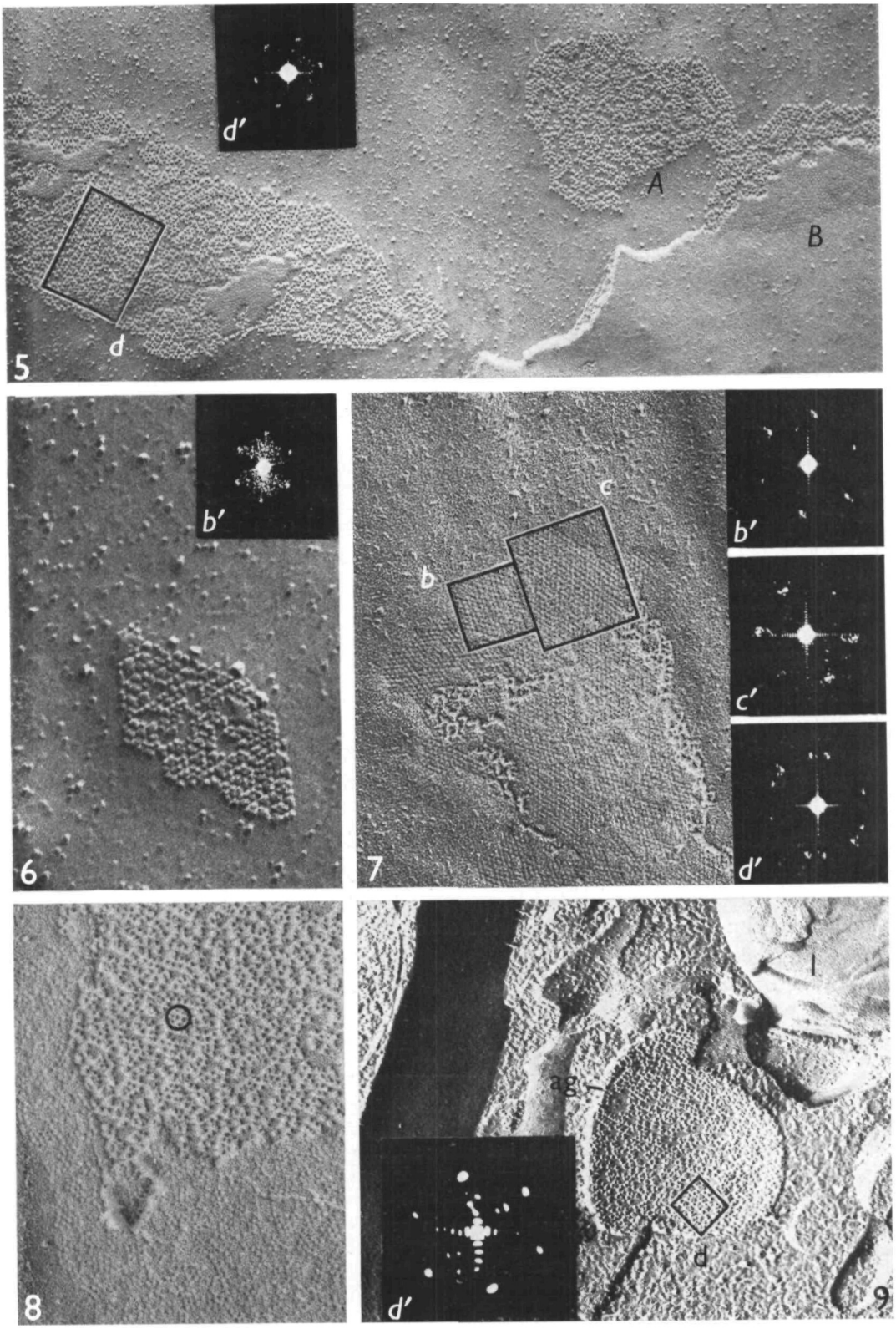
Fig. 5. Gap junctions between mural granulosa cells. Medium-sized gap junctions can be recognized where the fracture plane passes through the plasma membrane. These show clusters of intramembrane particles on the A face (*A*) and small pits on the B face (*B*). The diffractogram (*d'*) obtained from a restricted junctional area (rectangle marked *d*), shows a clear hexagonal pattern. × 64 700.

Fig. 6. Small junction of granulosa cell (A face). The optical diffractogram of the total junctional area gives a single hexagonal pattern, although parts of the junctional areas are devoid of intramembrane particles. Note that the hexagon is slightly elongated in diffractogram *b'* (see Discussion). × 111 000.

Fig. 7. The B face of fractured granulosa cell membrane showing a medium-sized junction. At the junctional area small pits are arranged in hexagonal lattices as revealed by the diffractograms *b'* and *c'*, diffraction patterns derived from areas *b* and *c* within the junction; *d'*, diffractogram obtained from the combined areas *b* and *c*. (Rigorously, the diffraction pattern of a sum of objects is the *vector* sum of the intensities of the Fourier transforms of the individual objects, referred to a common origin. However, for weakly diffracting objects such as we encounter here, the diffraction pattern of a sum of objects is closely approximated by the sum of the individual patterns.) × 91 800.

Fig. 8. Part of large junction of granulosa cell that does not show any hexagonal order upon diffraction on both A and B faces. Occasionally, however, single hexagonal cells can be discerned (e.g. area enclosed by small circle). × 96 000.

Fig. 9. Freeze-fractured annular junction (*ag*) in a thecal cell. Note part of large lipid droplet (*l*) characteristic of thecal cell. Optical diffraction from a restricted area of the junction (*d*) yields a clear hexagonal pattern (*d'*). × 64 000.


d′
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RESULTS

Appearance of gap junctions in thin sections

Gap junctions between granulosa cells were found to be abundant in the cumulus cells, including cells of the corona radiata, as well as in mural granulosa cells and the theca interna (Figs. 1, 3). After staining *en bloc* with uranyl acetate, these junctions appeared either as a heptalaminar structure, or more often, as a pentalaminar structure in which the central dense line could at times be resolved into a file of dots; the 'dots' being dense globular particles of about 5 nm in diameter, separated by translucent gaps of 3–4 nm (Fig. 1). The total thickness of the junction was about 20 nm (Figs. 1, 3). Small junctions of similar appearance, 18–20 nm in thickness, were also discerned between cytoplasmic processes of corona radiata cells and the oolemma in thin sections when examined on a tilting stage (Fig. 2).

Intracellular vesicles bounded by pentalaminar structures could be recognized within granulosa cells and within thecal cells (Fig. 4). Frequently these annular junctions contained small vesicles (Fig. 4).

Anatomy of gap junctions as revealed by freeze-fracture and optical diffraction techniques

The plasma membrane of both granulosa and thecal cells presented patches of closely associated particles on the A face and of small pits on the B face, characteristic of gap junctions (Figs. 5, 14). These gap junctions varied in size (from about 0·2 to 2·0 μm across). The intramembrane particles in the junctional areas are 7–9 nm in diameter. These particles do not completely cover the junctional area (Figs. 5, 6, 10, 14), but rather are organized in clusters with narrow spaces in between, so that they form mosaic patterns. The small depressions found on the B face show a similar mode of organization (Figs. 7, 12, 13). Optical diffraction analysis of the junctional area reveals that the particles are organized as hexagonal lattices with a particle-to-particle spacing of 9–10 nm for both A and B faces. In small junctions (up to 0·4 μm in diameter), only a single hexagonal lattice is observed on both A and B faces (Figs. 6, 12). Even such small junctions occasionally show bald patches not completely occupied by intramembrane particles (Fig. 6). Frequently the reflections obtained in the optical diffractograms, which are characteristic of an hexagonal array, are not of equal intensity (e.g. Figs. 7, 13). This is a consequence of the unidirectional nature of the shadow casting which results in a reduction of the intensity in the diffractogram in the direction perpendicular to that of the shadow.

Medium-sized junctions, about 0·5–0·7 μm in diameter, contain a small number (2–10) of hexagonal lattices differing in orientation (Fig. 7). Optical diffraction

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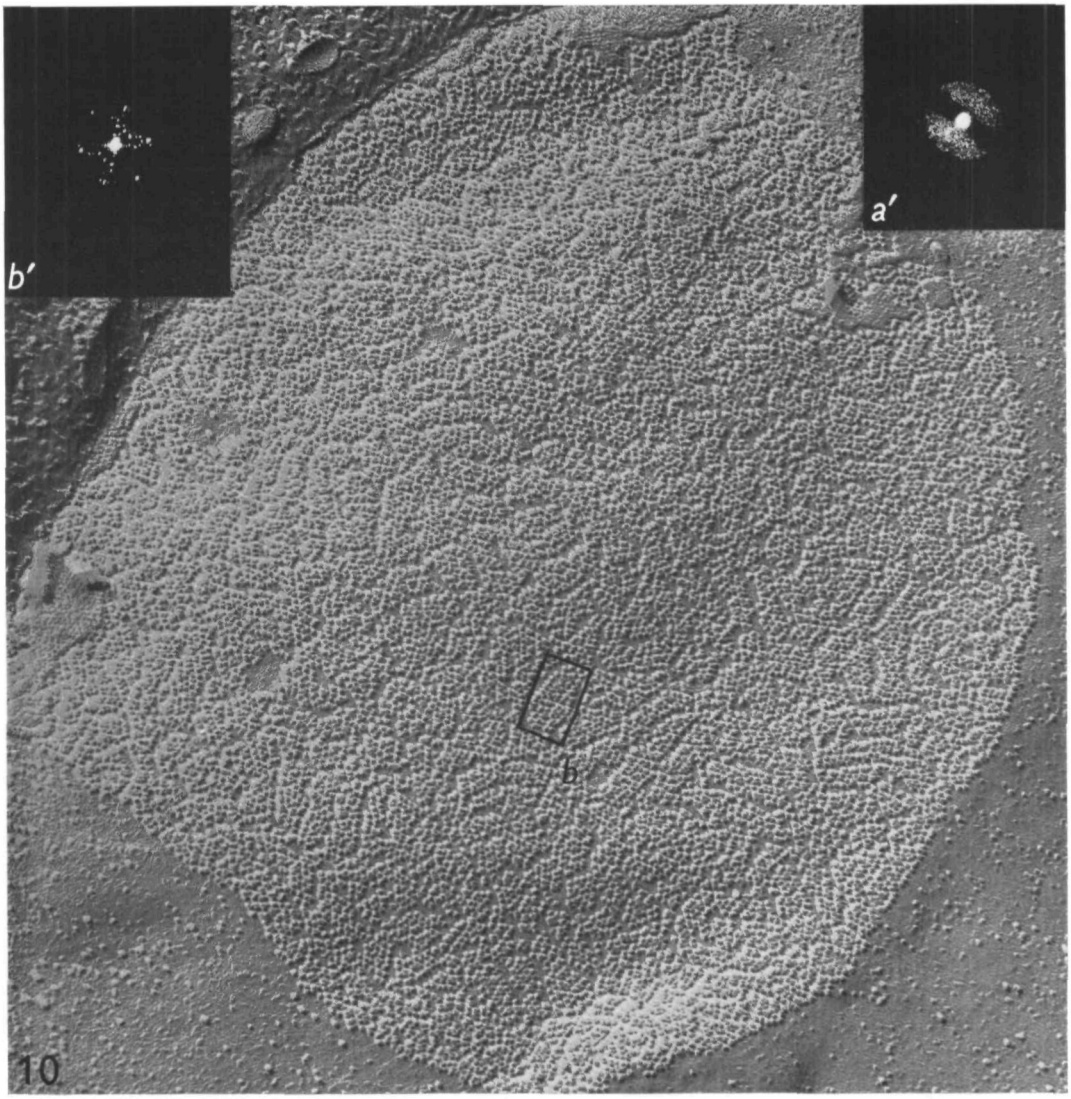
Fig. 10. Large junction of granulosa cell showing the A face. Intramembrane particles are organized in many small clusters. The diffraction pattern (*a'*) derived from the total area is ring shaped, indicating random orientation of the component lattices. Many of the individual clusters yield single hexagonal patterns: a representative diffractogram obtained from one such cluster (*b*) is shown (*b'*). × 79 800.

Fig. 11. Image of annular junction (*ag*) in granulosa cell. The intramembrane particles are assembled in clusters or elongated bands. A hexagonal pattern (*c'*) was obtained from the area indicated (*c*). × 64 000.



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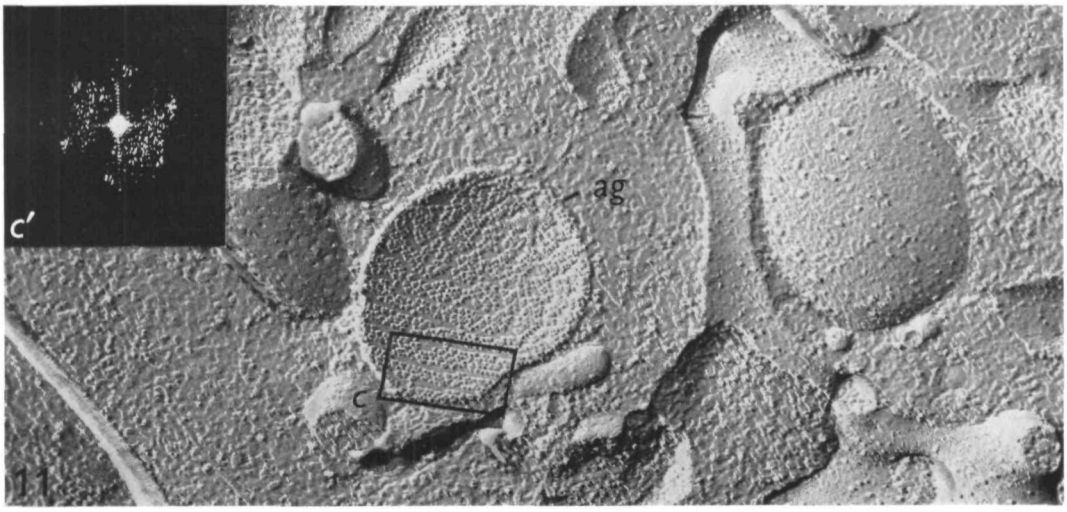

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patterns derived from larger junctions (up to 2 μm in diameter) do not show a set of reflections characteristic of a single hexagonal lattice (Figs. 10, 13). Rather such patterns produce a ring whose radius corresponds to the same reciprocal spacing (8·0–8·5 nm) as the reflections obtained from single lattices. However, if the diffracting area is restricted to a small region of such a large junction, a characteristic hexagonal pattern is obtained (Figs. 10, 13). Large junctions thus are composed of many small lattices whose relative orientation is random, so that the diffraction pattern derived from the whole junction appears as a ring. On rare occasions, junctions were observed in which no hexagonal lattice could be demonstrated by optical diffraction, and the intramembrane particles appeared more loosely packed, though scattered single hexagonal cells could be recognized by visual inspection (Fig. 8). The individual lattices that constitute the largest junctions were in general smaller in area than those found in junctions consisting of only one or a few such elements (compare Figs. 10, 13 with 6, 7, 12).

Junctional elements with similar characteristics to those described above were also abundant in the theca interna (Fig. 14). Tight junctions were not evident in any granulosa or internal theca cells that were screened.

In both granulosa and thecal cells, fractured membranes of intracellular vesicles were observed which were studded with particles associated in the same manner as those found in junctional areas of the plasma membrane. These fractured vesicle walls included areas in which a clear hexagonal pattern could be revealed by the diffractograms. The size of the intramembrane particles was 7–9 nm, while centre-to-centre spacing was 9–10 nm (Figs. 9, 11).

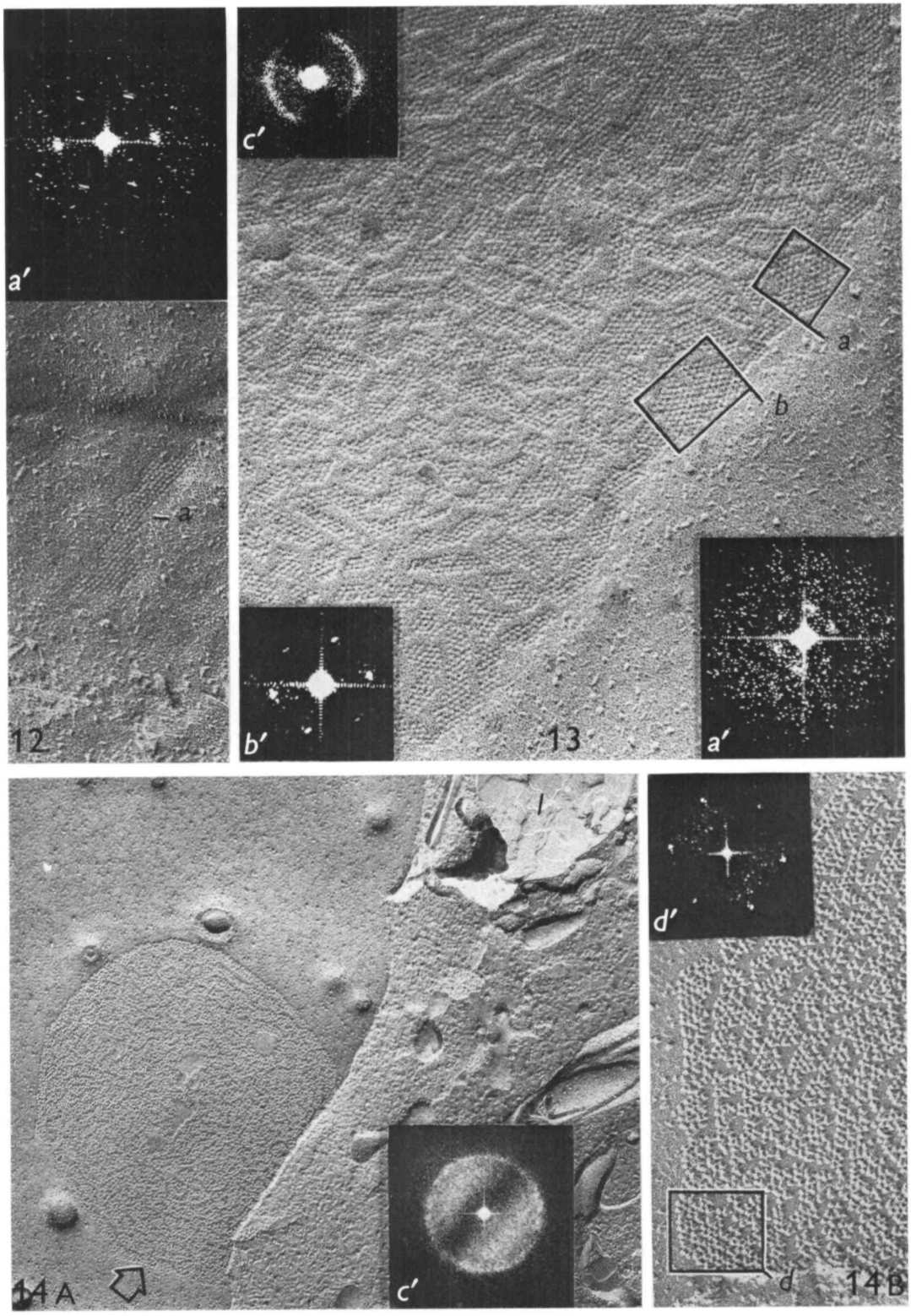
DISCUSSION

The freeze-fracture technique lends itself to study of the fine structure of junctional elements, since the fracture plane often lies within the lipid bilayer (Branton, 1966; Pinto da Silva & Branton, 1970) and thus exposes the inner faces of the membrane.

Fig. 12. The B face of the granulosa cell membrane showing small junction (*a*). At the junctional area small pits are arranged in a single hexagonal lattice, as revealed by the diffractogram (*a'*) obtained from the entire area of the junction. × 91 800.

Fig. 13. Portion of large junction (2 μm in diameter) of granulosa cell showing the B face. Organization of the pits in small clusters is similar to the assembly of the intramembrane particles on the A face (cf. Fig. 10). Diffraction patterns derived from individual clusters (e.g. *a* and *b*) are characteristic of single hexagonal lattices (*a'*, *b'*), while the diffraction pattern from the whole junctional area has a ring shape (*c'*) characteristic of an array of many small clusters with different orientations. × 103 500.

Fig. 14. Large gap junction in the theca interna (Fig. 14A). Diffractogram obtained from the total area of the junction shows a complete ring (*c'*). *l*, part of large lipid droplet. Higher magnification of part (indicated by arrow) of the large junction (Fig. 14B) reveals a mosaic pattern of aggregation of intramembrane particles similar to that found for granulosa cell gap junctions (cf. Fig. 10). A restricted area (*d*) of the large junction gave a distinct hexagonal diffraction pattern (*d'*). Fig. 14A, × 43 500; Fig. 14B, × 110 000.


a′
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12
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13
a′
d′
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14B
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In the present study, optical diffraction analysis was adopted as a complementary tool, to permit quantitative examination of the organization of the intramembrane particles in specialized cell junctions as they appear in replicas of freeze-fractured material. This approach should be equally applicable to the evaluation of images of negatively stained or lanthanum-infiltrated junctions.

Merk *et al.* (1973) described gap junctions between granulosa cells as 5- or 7-layered structures of 19 nm overall thickness which, upon oblique section, exhibited cross-striated patterns of 4·5-nm periodicity. These striations were interpreted as possibly representing points of continuity between cells. In our sectioned material, gap junctions usually appeared as pentalaminar structures, 20 nm across, whose central lamina was frequently broken up so that it presented a dotted line composed of globular particles, with a periodicity of 8·0–8·5 nm. The particles were 4–5 nm in diameter and were separated by electron-translucent gaps 3–4 nm in width. Similar periodicities (about 7 and 8 nm, respectively) were apparent in the central lamina of gap junctions of mouse ependymal cells (Brightman & Reese, 1969) and human Lesch-Nyham fibroblasts (Azarnia, Larsen & Loewenstein, 1974). Occasionally, the gap junctions in our material appeared as heptalaminar structures. The variable appearance of gap junctions as penta- or heptalaminar structures as a result of modifications in the staining procedure has been previously discussed in detail (Brightman & Reese, 1969; Merk *et al.* 1973; McNutt & Weinstein, 1973; Gilula, 1974).

In freeze-fractured material, we observed intramembrane particles arranged in a distinct hexagonal pattern on the A face, and similarly arranged pits on the B face. Diffractograms indicated a centre-to-centre spacing of 9–10 nm for both pits and particles. The slight variability in spacing may be attributable to the fact that some of the hexagonal patterns were not perfectly symmetrical, possibly due to the dished shape of the membrane or to slight artifactual distortion during fracturing.

The number of hexagonal lattices distinguishable by their divergent orientation increased with the overall size of the junction. It is tempting to speculate that each uniformly oriented lattice is formed around a single focus of assembly of membrane particles, and that the number of such lattices composing a larger junction is indicative of the number of initiation points from which it arose. Indeed, studies on the ontogeny of gap junctions during neurulation in *Rana pipiens* (Decker & Friend, 1974) provide some evidence for a multifocal origin of large gap junctions: these appear to result from the growth of small clusters of intramembrane particles at sites of close contact between cells, and their eventual coalescence and arrangement in polygonally packed aggregates. This mode of genesis would explain our observation that the average size of the individual lattices included in a junction tends to be inversely related to junctional size, since the growth of each lattice might be limited by the proximity of neighbouring initiation points.

At times we observed junctions devoid of a clear hexagonal diffraction pattern on either the A or B face, although a few hexagonal unit cells could be discerned by the eye. These areas may represent young junctions in which the ordered assembly of intramembrane particles has not been completed (cf. Albertini & Anderson, 1974). Alternatively, there may be dynamic transitions between the most tightly packed

state, represented by hexagonal stacking, to a more disperse mode of assembly showing no lattice order, possibly in response to metabolic changes (Peracchia & Dulhunty, 1974). Preliminary observations in this laboratory (unpublished) indicate that close to the time of LH-induced ovulation, gap junctions between granulosa cells are reduced in size and number and the clusters of intramembrane particles observed in freeze-fractured material lack hexagonal stacking. These points deserve further study.

Intracellular vesicles exhibiting ultrastructural features similar to those of gap junctions, including 8·0–8·5 nm periodicity in the central lamina and a hexagonal diffraction pattern, were observed in both granulosa and thecal cells. Annular junctions were encountered in granulosa cells of rat (Merk *et al.* 1973) and rabbit (Albertini & Anderson, 1974), and appeared to increase in number in rabbit follicles after treatment with human chorionic gonadotrophin (Bjersing & Cajander, 1974). Some of these inclusions may represent internalized junctional elements, or else fingerlike projections of one cell pushing into a neighbouring one.

Gap junctions were numerous in all layers of the membrana granulosa as well as in the theca interna. It should be emphasized that we examined a uniform population of preovulatory Graafian follicles, not including pre-antral and early antral follicles. The structure of the thecal junctions was similar, in all respects, to those found between granulosa cells. Small gap junctions were also seen in thin sections at points of contact between cytoplasmic extensions of corona radiata cells and the oolemma. Tilting of the preparations on a goniometer stage was essential for demonstrating these junctions in thin-sectioned material. It is desirable to confirm these observations by the freeze-fracture technique, but this presents formidable technical difficulties.

The presence of desmosomes between the oocyte and granulosa cells has been reported (Zamboni, 1974; Albertini & Anderson, 1974). Our present demonstration of gap junctions between the oocyte and corona cells in the mature Graafian follicle suggests that desmosomes and gap junctions may appear sequentially during follicular development, not only between granulosa cells themselves (Albertini & Anderson, 1974), but also between the oocyte and the cells surrounding it.

The precise function of the gap junctions awaits elucidation. It is possible that follicular gap junctions serve to maintain the structural integrity of the distended, fluid-filled follicle. More intriguing is the possibility that they subserve intercellular communication. Similar specialized junctions in other systems are thought to be highly permeable to small molecules such as fluorescein (mol. wt. 322) and to facilitate electrical coupling between cells (Sheridan, 1971; Azarnia *et al.* 1974). We recently found that receptors to human chorionic gonadotrophin are confined to the theca interna and the peripheral three to four layers of the mural granulosa cells of preovulatory follicles (Amsterdam, Koch, Lieberman & Lindner, 1975). No significant binding of radiolabelled ovulatory hormone to the oocyte or cumulus cells could be demonstrated by radioautography, although the oocyte of such follicles responds to the hormone by resuming its meiotic division (Lindner *et al.* 1974). It is thus conceivable that gap junctions facilitate the passage of an electrical or chemical signal from the site of hormone-cell interaction in the periphery of the follicle toward the oocyte. Cyclic AMP formation in follicular tissue increases rapidly in response to

gonadotrophins (Lindner *et al.* 1974) and this nucleotide (mol. wt. 329) has been tentatively implicated in the hormonal induction of ovum maturation. The abundance of gap junctions throughout the follicular wall may thus provide a structural basis for the remote action of gonadotrophins on ovum maturation.

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