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Electron microscopy of the oocyte and granulosa cells in the developing ovarian follicles of the golden hamster (*Mesocricetus auratus*)*

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The golden hamster, *Mesocricetus auratus*, holds the distinction of developing in utero faster than any other placental mammal (Graves, 1945). Although implantation does not occur in this species until 5 d *post coitum* (Graves, 1945; Venable, 1946; Boyer, 1953), its gestation period averages only 15 d 21 h (Ortiz, 1945). The development of this species, studied at the ultrastructural level, should therefore provide information of some importance to the student of developmental embryology. A logical starting-point for a study of this nature is the ovarian oocyte.

Light microscopy of the developing ovarian oocyte in the hamster has been the subject of several papers: Rolle & Charipper (1949); Knigge & Leathem (1950, 1956); Austin (1956); Greenwald (1961); Kent (1962); Blaha (1964); Guraya & Greenwald (1965). They concur that it is generally similar to the ovarian oocyte of the rat, except for its large content of cortical granules. No parallel ultrastructural study has yet appeared in the literature, although Odor (1965) has described the unilaminar follicle, and Austin (1961) has reported some features of the fine structure of tubal ova.

The present paper will describe five stages in the development of the ovarian oocyte in the prepubertal and sexually mature hamster:

(1) Primordial polyovular follicles.
(2) Unilaminar follicles with flattened granulosa cells.
(3) Unilaminar follicles with cuboidal granulosa cells.
(4) Pre-antrum multilaminar follicles.
(5) Follicles with antra, including the mature Graafian follicle.

A later paper will deal with the prenatal and immediately postnatal development of the ovary.

MATERIALS AND METHODS

Ovarian tissue was obtained from hamsters of the following ages:

8 d *post partum* 26 d *post partum*	prepubertal	2 months old 4 months old 6 months old	sexually mature

The tissues from sexually mature females were obtained at all phases of the oestrous cycle, including the peak of oestrus, to ensure obtaining fully mature oocytes.

The tissues were removed under Nembutal anaesthesia, fixed for 4 h in ice-cold

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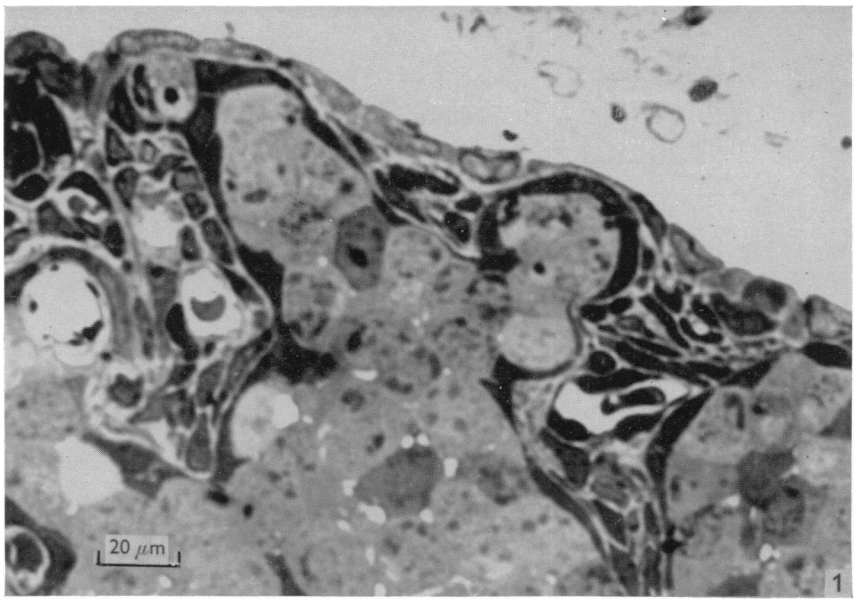
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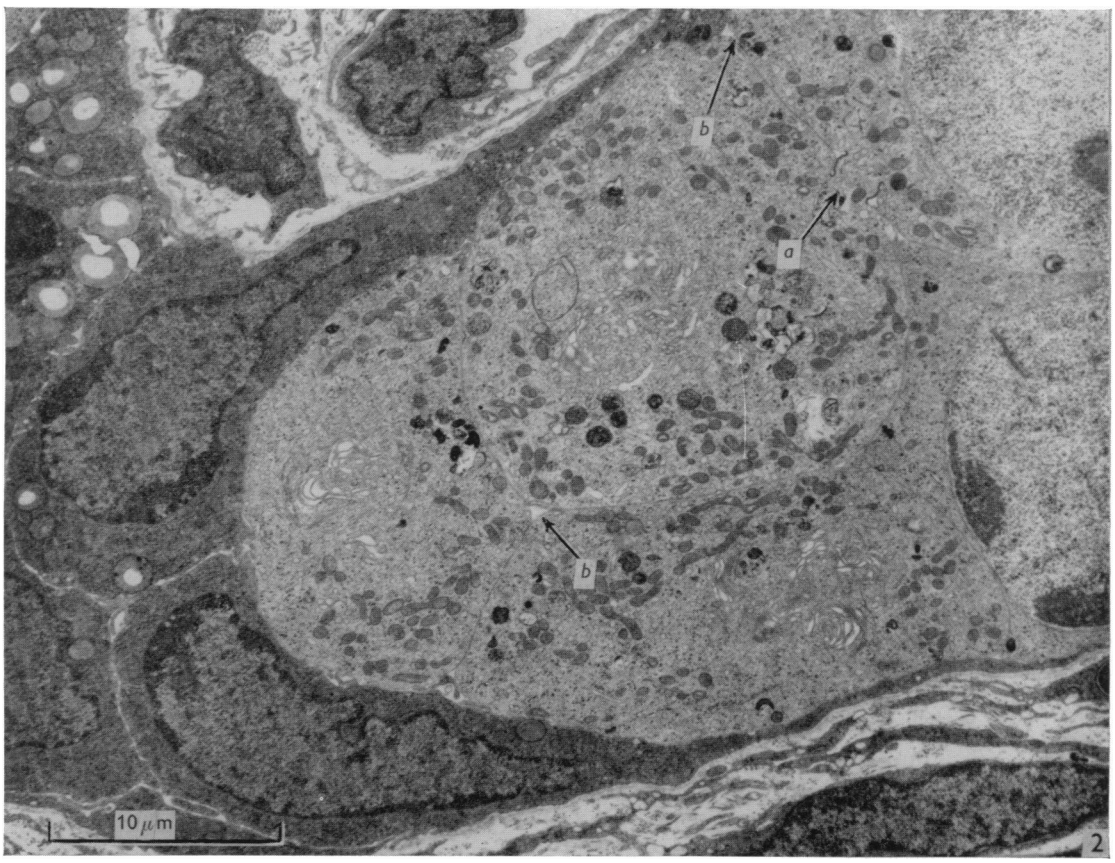
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4% glutaraldehyde buffered at pH 7·4 with 0·1 M cacodylate, washed from 1 h to overnight in ice-cold 0·1 M cacodylate buffer containing 0·13 M sucrose, and then transferred to 1% osmium fixative (Millonig, 1962) for 1 h. The tissues were then dehydrated in ascending ethyl alcohols, rinsed in two changes of propylene oxide for a total of 5 min, and embedded in araldite. Thick (1 μm) sections of the araldite-embedded tissue were cut and stained with methylene blue for the purposes of orientation and study by light microscopy. The blocks were then trimmed and sectioned on a Huxley ultramicrotome. The thin sections were mounted on bare slot grids, stained with uranyl acetate followed by lead citrate (Reynolds, 1963), and viewed with a Metropolitan Vickers EM6 electron microscope.

RESULTS

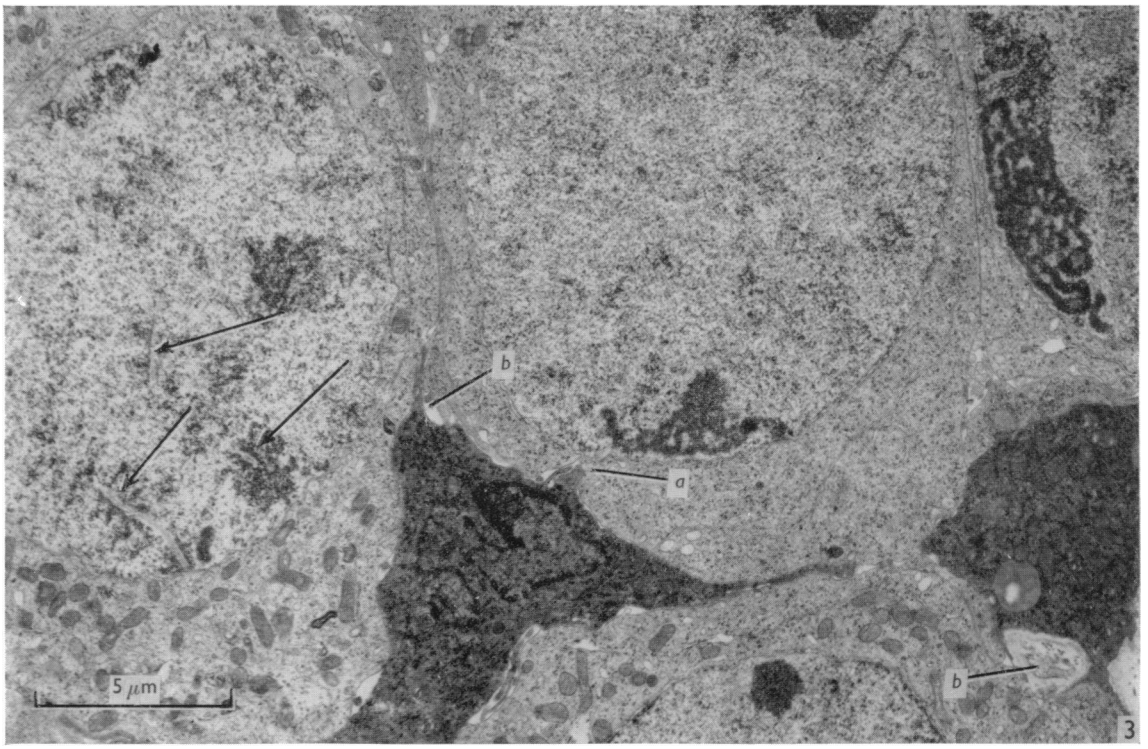
I. *Primordial polyovular follicles*

These were predominant in the 8 d ovary, but small numbers were also seen to occur in older animals.

By light microscopy, the ovary of the 8-d-old animal is seen to be bounded exteriorly by a single layer of cuboidal epithelium. Beneath the epithelium are one to several layers of flattened cells with nuclei which vary in shape from irregularly oval to very thin and elongate. These cells are identical to and continuous with the cells forming connective tissue trabeculae which divide the ovary into compartments. Within the compartments are found large, pale cells with round nuclei. The nuclei contain two to eight chromatin masses which are usually distributed peripherally. Some of the compartments containing the large pale cells are subdivided into clusters of two to five by developing follicle cells. In material viewed with the light microscope, both nucleus and cytoplasm of the follicular cells stain intensely with methylene blue, thus clearly identifying the developing follicles (Fig. 1). The large, pale cells within the developing follicles exhibit a variable affinity for the methylene blue stain, but always stain less intensely than the follicle cells. By electron microscopy the large pale cells were positively identified as oocytes by the presence of typical tripartite chromosome cores of meiotic prophase, as described in the rat by Sotelo (1959). Occasionally a single oocyte may be seen which has become completely, or nearly completely, surrounded by a single layer of flattened follicle cells. These oocytes stain hardly at all with methylene blue, and their nuclei contain far less chromatin than do the nuclei in the polyovular clusters. Careful focusing under oil immersion shows clusters of tiny granules in the cytoplasm.

Fig. 1. Primordial polyovular follicles from ovary of 8-d-old hamster. The flattened follicle cells stain intensely, outlining the clusters of developing oocytes. Methylene blue.

Fig. 2. Primordial polyovular follicle from ovary of 8-d-old hamster. Note high electron density of follicle cells. A massive Golgi apparatus and abundant mitochondria are typical of the developing oocytes. A cytoplasmic bridge connects two oocytes (*a*). Intercellular spaces or channels occur at 3-cell junctions (*b*).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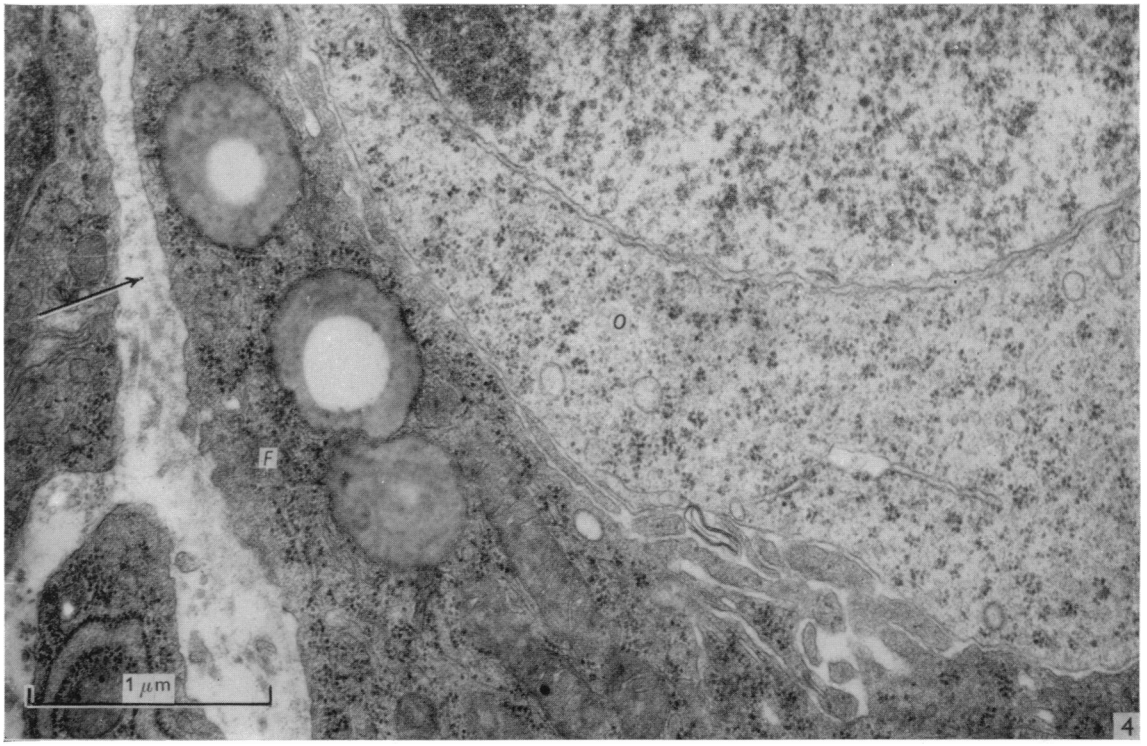
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Granulosa cells

By electron microscopy the granulosa cells which partly invest the polyovular clusters exhibit extremely dark background nucleoplasm and background cytoplasm. The electron density is caused by a fine granulation and is so great as to obscure much cytoplasmic and nuclear detail at low magnification.

The nuclei are typically elongate and irregular in shape (Fig. 2) and may be deeply invaginated (Fig. 3). Small clumps of chromatin lie beneath the inner nuclear membrane and are also scattered throughout the dark nucleoplasm. One or two large chromatin masses are usually present beneath the nuclear membrane, and probably represent nucleoli.

The cytoplasmic matrix is highly electron dense due to the presence of fine granulation. This granulation is *not* to be confused with free ribosomes, which by comparison are much larger and stand out distinctly against the granular background in high-magnification photographs (Fig. 4). Free ribosomes are numerous and may be seen singly, in clusters, or in chains.

Both rough and smooth endoplasmic reticulum are randomly dispersed throughout the cytoplasm. The smooth membranes vary in morphology from round vesicles to elongate cisternae. The Golgi apparatus is typically composed of long, stacked cisternae.

The mitochondria vary in shape from round to elongate (Fig. 4). Their matrix is even more electron-dense than the background cytoplasm. The cristae are considerably paler than the mitochondrial matrix and run at right angles to the long axis of the mitochondrion. One or several cristae within a mitochondrion may be enlarged and contain a fine granular substance.

The granulosa cell cytoplasm typically contains a number of large rounded lipoid inclusions (Fig. 4).

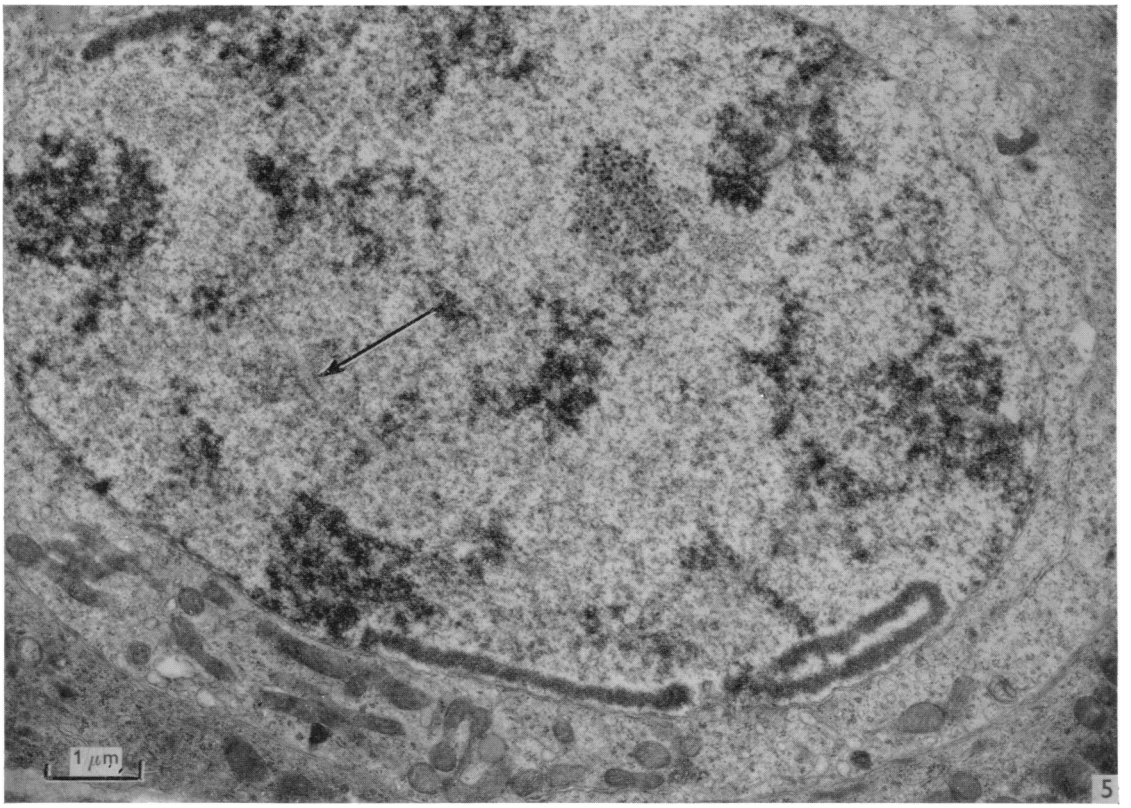
The granulosa cells are in close association with the oocytes, their plasma membranes being separated from each other by a gap of about 250 Å. At frequent intervals, however, a larger space occurs between follicle cell and oocyte which contains microvillous extensions of the follicle cell cytoplasm (Fig. 4). These processes may be seen at times to indent the oocyte cytoplasm and to approach the nuclear membrane (Fig. 3*a*).

Oocytes

The oocyte nuclei are large, round and usually eccentric. The background nucleoplasm is typically as pale or paler than the background cytoplasm. The nuclei

Fig. 3. Polyovular follicle from ovary of 8-d-old hamster. Electron-dense follicle cell at lower centre has a deeply invaginated nucleus. Pseudopodia from this cell indent cytoplasm of oocyte and approach nuclear membrane (*a*). Follicle cell at lower right contains lipoid droplet. Intercellular spaces occur between follicle cells and oocytes (*b*). Tripartite chromosome cores are visible in the nuclei of two oocytes (arrows). Nucleolus in ovum at right is elongate.

Fig. 4. Oocyte (*O*) and follicle cell (*F*) from polyovular follicle, 8-d-old hamster. Cytoplasm of follicle cell contains abundant free ribosomes, rough and smooth endoplasmic reticulum, and lipoid droplets. Mitochondrial matrix is highly electron-dense; some cristae are swollen. Processes from follicle cell invade gap between follicle cell and oocyte. Follicle cell rests on basement membrane (arrow).



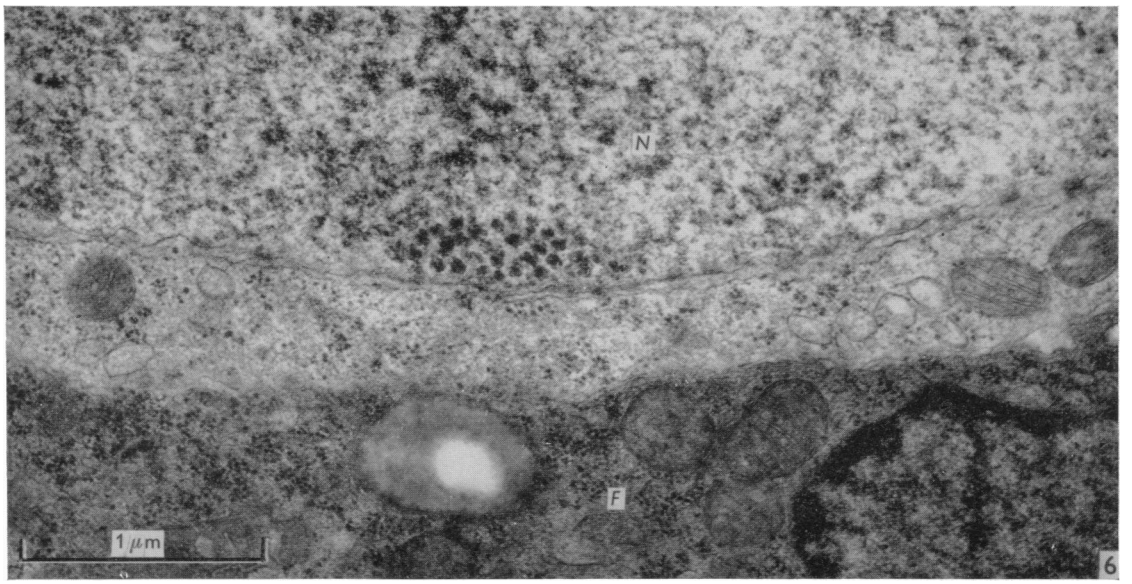
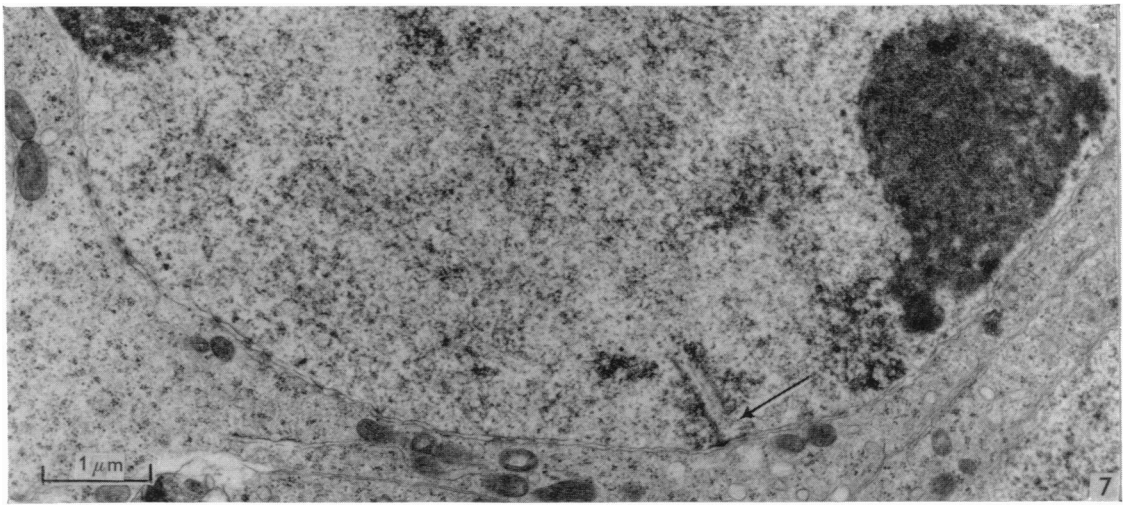


Fig. 5. Oocyte from primordial polyovular follicle, 8-d-old hamster. Amorphous electron-dense material and a cluster of discrete granules are seen in the nucleoplasm. Nucleolus is linearly dispersed beneath nuclear membrane. A tripartite chromosome core is present (arrow). Mitochondria are numerous, of variable morphology, and exhibit an electron-dense matrix.

Fig. 6. Oocyte and follicle cell from primordial polyovular follicle, 8-d-old hamster. A cluster of electron-dense granules appears beneath nuclear membrane of oocyte. *N*, Nucleus of oocyte, *F*, follicle cell.

contain varying amounts of electron-dense material which may appear amorphous, or which may be in the form of distinct granules (Figs. 5, 6). One or two nucleoli are present and invariably lie directly beneath the nuclear membrane. They may be rounded, electron-dense reticular meshworks (Figs. 3, 7) or may be linearly



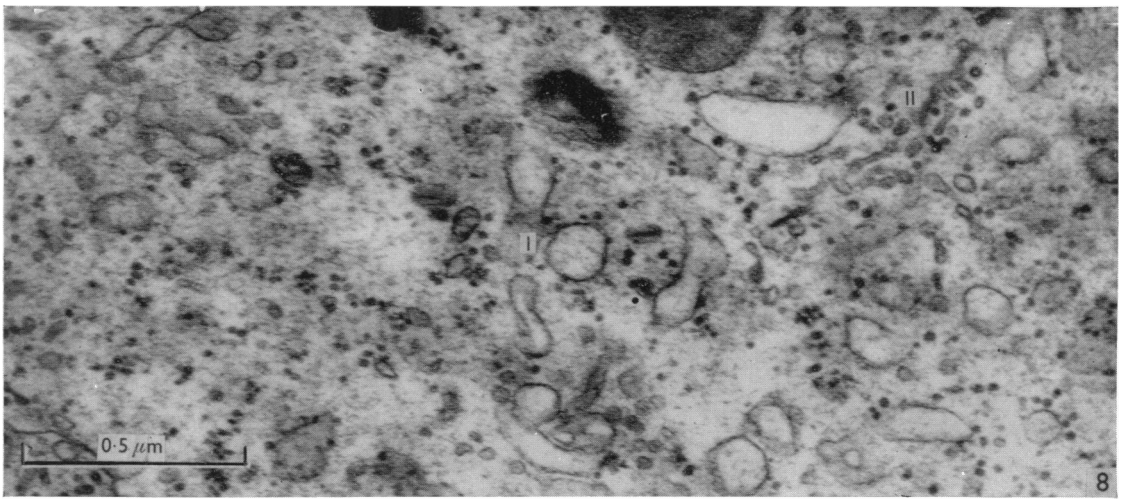
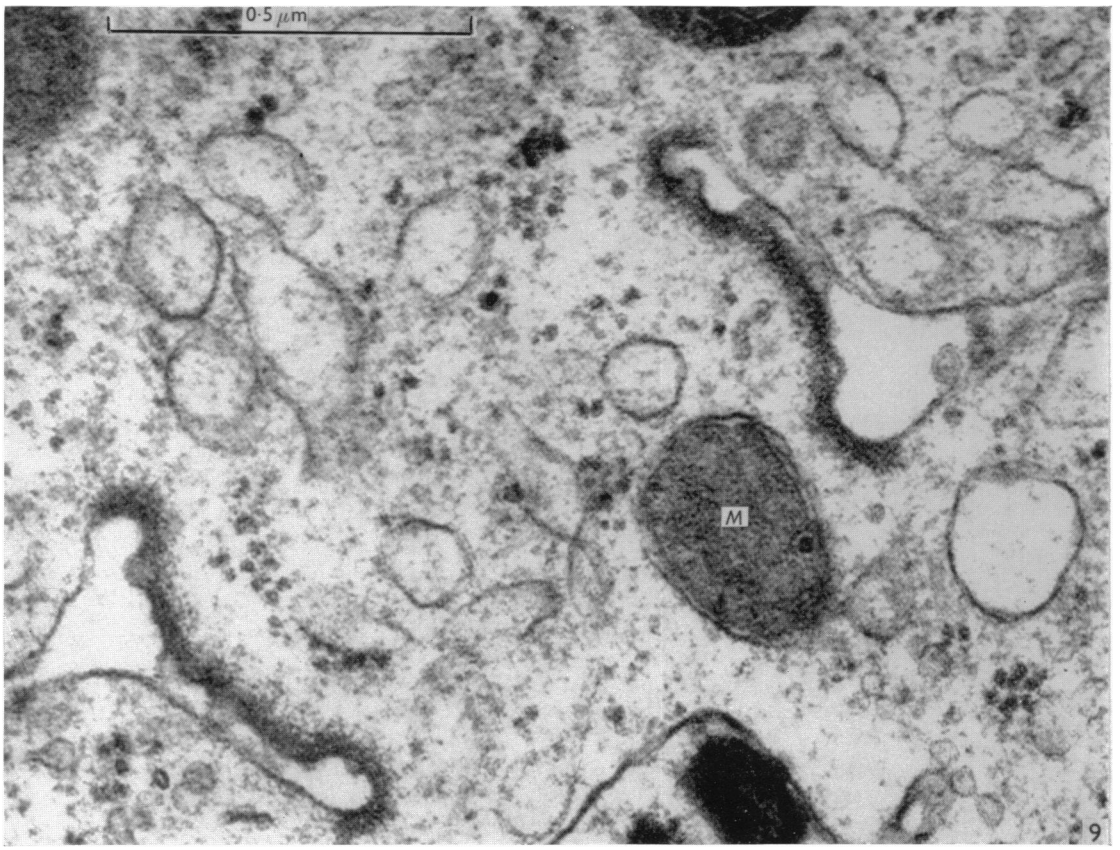


Fig. 7. Oocyte from primordial polyovular follicle, 8-d-old hamster. Nucleolus is a rounded reticular meshwork. A tripartite chromosome core in nucleus terminates at nuclear membrane (arrow). Nuclear pores are closed by diaphragms and are associated with electron-dense amorphous material.

Fig. 8. Cytoplasm of oocyte in unilaminar follicle from ovary of 26-d-old hamster. Two distinct categories of smooth endoplasmic reticulum are present: I. Moderately elongate cisternae and large vesicles measuring at least 1200 Å in diameter. II. Slender, short tubules and tiny vesicles with average diameter of 300 Å.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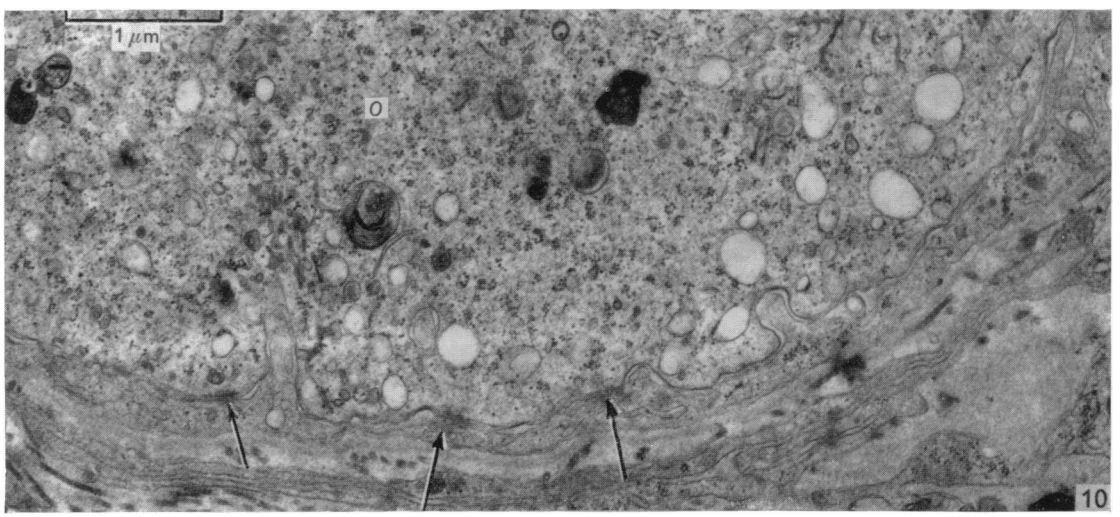
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dispersed beneath the nuclear membrane (Fig. 5). Most of the oocytes seen at this stage contain typical 3-filament cores of meiotic chromosomes as described by Sotelo (1959) (Figs. 3, 5, 7). Sotelo found these cores to be present in the rat oocyte during a circumscribed period about the time of birth.

The 3-filament cores follow a winding path through the cytoplasm and are frequently seen to terminate at the nuclear membrane (Fig. 7). They are associated with varying amounts of granular chromosome material.

Nuclear pores occur at irregular intervals and appear to be closed by a diaphragm. Small accumulations of dense material are seen on both sides of the diaphragm at these points (Fig. 7).

The cytoplasm of the oocyte is not extensive. Its most prominent features are a large number of randomly scattered mitochondria and a single massive Golgi apparatus (Fig. 2). A considerable number of free ribosomes averaging 170 Å in diameter are present. These particles occur singly or in clusters, and are seldom seen associated with endoplasmic reticulum. Many profiles of smooth endoplasmic reticulum are scattered throughout the cytoplasm. At this stage of oocyte development and at all later stages the smooth endoplasmic reticulum falls into two distinct categories (Fig. 8):

(1) The predominant type: large vesicles and moderately elongate cisternae. The vesicles vary greatly in size, but seldom have a diameter less than 1200 Å.

(2) Tiny vesicles of fairly uniform size (approximately 300 Å in diameter), and slender, short tubules.

The Golgi apparatus is typically composed of curved stacks of smooth lamellae with associated vacuoles and vesicles. The cisternae may be greatly swollen, although no formed material is observed within.

The mitochondria vary in shape from round to filamentous. The matrix is electron dense; the cristae are pale and frequently run parallel to the long axis of the mitochondrion. Mitochondria do not occur within the Golgi region, but otherwise appear randomly scattered throughout the cytoplasm.

In some of the oocytes a variety of cytoplasmic inclusions are seen. These include electron-dense bodies with vesicular or particulate contents, and vacuoles containing amorphous or membranous material (Fig. 2). It is not known whether these phenomena are developmental or degenerative in nature.

Distinct cytoplasmic bridges have been seen between developing oocytes (Fig. 2*a*). The bridges contain cytoplasmic organelles such as mitochondria, smooth endoplasmic reticulum and ribosomes. Directly beneath the plasma membrane

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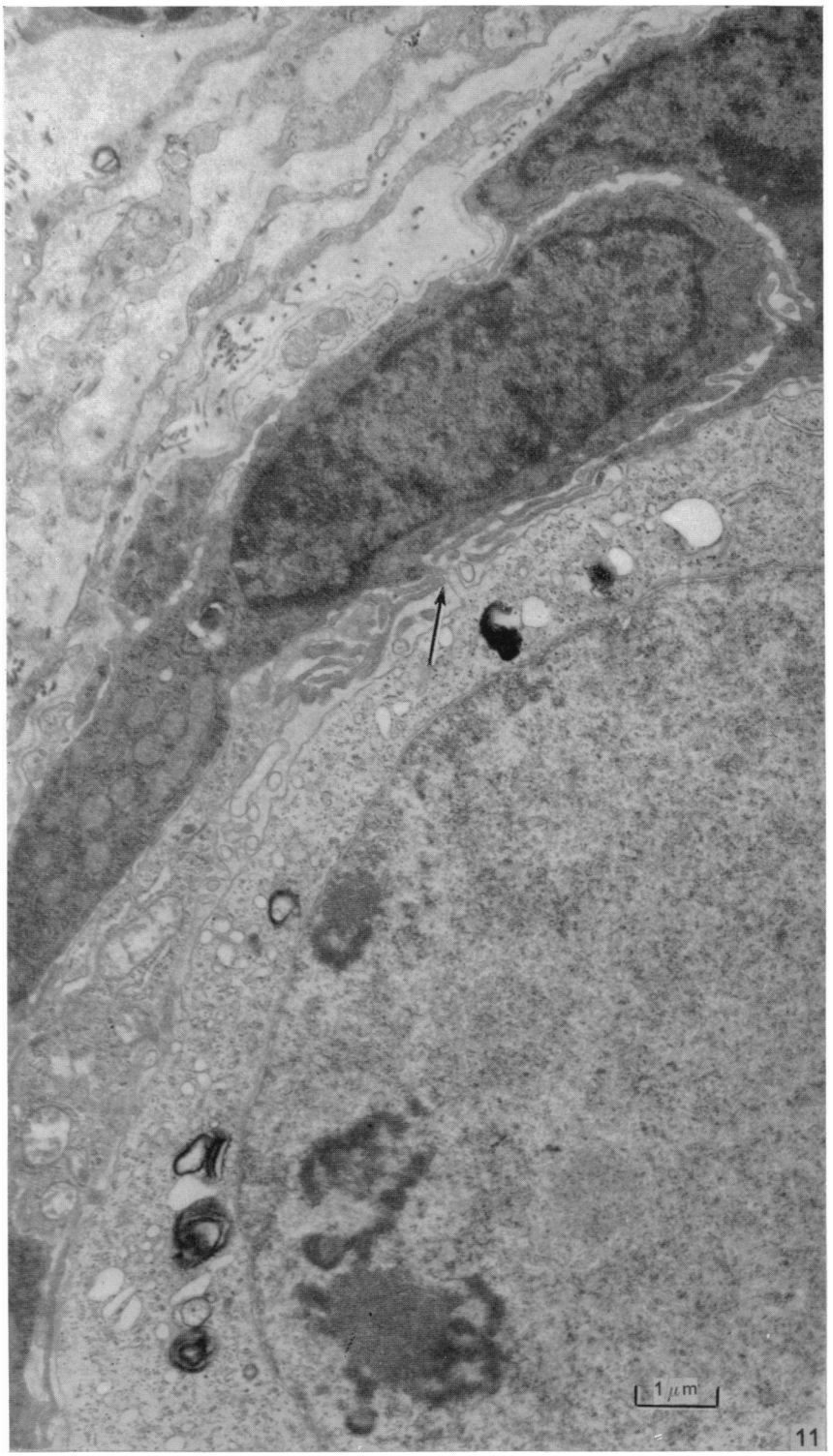
Fig. 9. Cytoplasmic bridge between developing oocytes in polyovular follicle. The bridge contains free ribosomes, smooth endoplasmic reticulum and a mitochondrion (*M*). At the lateral limits of the bridge, directly beneath the plasma membrane, is a band of electron-dense material approximately 365 Å wide.

Fig. 10. Oocyte (*O*) with one layer of flattened granulosa cells, 26-d-old hamster. The granulosa cells in this particular specimen do not exhibit high electron density. They rest on a distinct basement membrane. Indentations of oocyte cytoplasm by granulosa cell pseudopodia occur at frequent intervals. Zones of attachment between granulosa cells and oocyte are indicated by arrows. Two categories of smooth endoplasmic reticulum are present within cytoplasm of oocyte.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which forms the lateral limit of a bridge, an electron-dense band measuring approximately 365 Å in width is present (Fig. 9).

The plasma membrane of the oocyte is smooth and without microvillous projections. There is evidence of pinocytotic activity.

At junctions where three oocytes meet (or two oocytes and a follicle cell) intercellular spaces are seen (Figs. 2, 3).

II. *Unilaminar follicles (flattened granulosa cells)*

A few unilaminar follicles are seen in the 8 d animal. At 26 d they are the predominant type present.

Granulosa cells

The smaller unilaminar follicles possess one row of flattened granulosa cells around the oocyte. These flattened cells rest upon a distinct basement membrane. The cytoplasm may be greatly attenuated. The association between oocyte and granulosa cell is exceedingly close and at frequent intervals zones of attachment resembling desmosomes are seen. Also at frequent intervals short extensions of granulosa cell cytoplasm indent the cytoplasm of the oocyte (Fig. 10). The morphology of granulosa cells at this stage is not appreciably different from that in the primordial polyovular follicle.

As the follicle develops, however, the flattened cells at one side of the oocyte start to enlarge and assume columnar shape, pushing outward from the oocyte and stretching around the ends of neighbouring flattened granulosa cells (Fig. 11). The enlarging cells send out long, thin, complexly branching processes which may contact the oocyte on the one hand and a neighbouring granulosa cell on the other. The contact between the granulosa cell processes and the plasma membrane of the oocyte is intimate, and zones of attachment are frequently observed at these points. In the space between the ramifying granulosa cell processes, a pale amorphous material is beginning to accumulate. An occasional microvillus from the oocyte is seen to traverse this material, and contact a granulosa cell pseudopodium (Fig. 11, arrow).

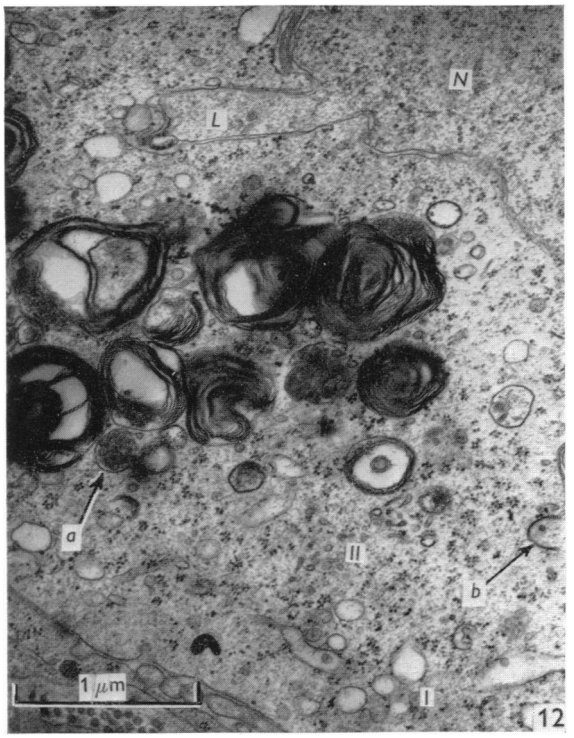
The flattened cells of the unilaminar follicle may all be of the highly electron-dense type, or a variable number may lack this electron density. The pale cells appear to resemble the dark cells in all respects except electron density.

Oocytes

The nuclei tend to be slightly eccentric. The ground nucleoplasm is very pale, and fine dark granules are scattered throughout. No condensations of chromosomal material or 3-filament chromosome cores are seen. In general, the larger the oocyte the paler the nucleus.

One or two large nucleoli may be present and are usually located peripherally.

Fig. 11. Unilaminar follicle from ovary of sexually mature hamster. Granulosa cells in this specimen exhibit high background electron density. Those at upper right are assuming cuboidal form and are sending long ramifications between oocyte and neighbouring follicle cells. Microvillus from oocyte is seen in contact with ramifications of granulosa cell (arrow). Nucleolus of oocyte consists of a central mass surrounded by spidery arms of denser material. Nuclear pores appear to be closed by diaphragms. Concentric lamellar membrane systems are present in cytoplasm of oocyte.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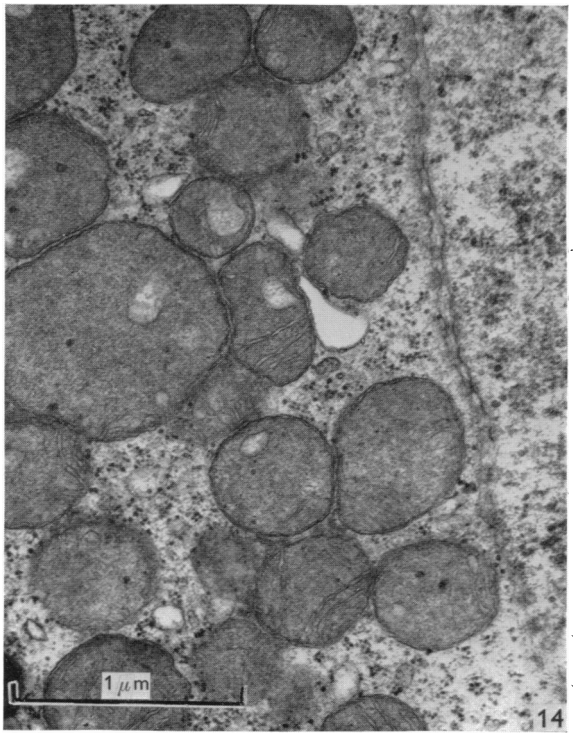
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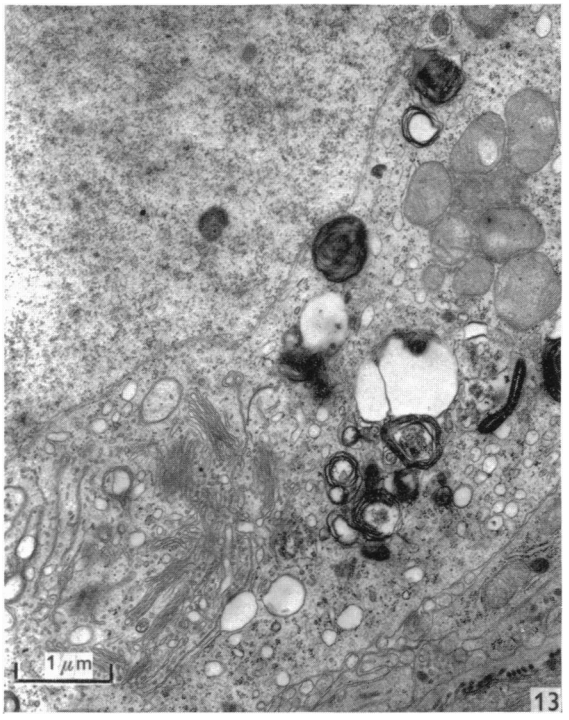
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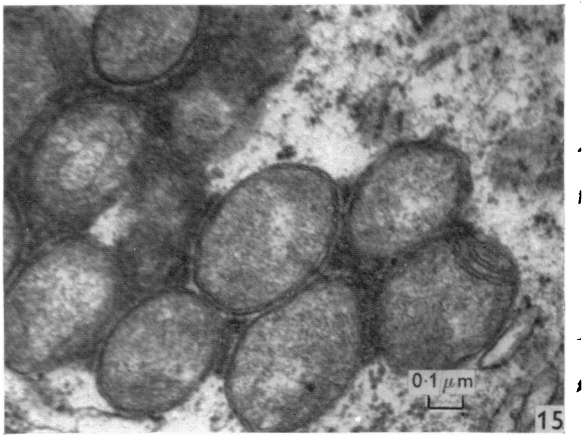
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They differ greatly from those of oocytes in primordial polyovular follicles. They typically have a central mass of a uniform, moderately electron-dense consistency, which is surrounded by spidery arms of a denser material. Smaller accumulations of the less dense material may occur at the end of the spidery arms and are capped half-moon fashion with the darker material. Sometimes the dense arms of the nucleolus turn back toward the central mass and may appear to rejoin it (Fig. 11).

Pores in the nuclear membrane are closed by diaphragms (Fig. 11). Long nuclear lobes are occasionally seen to extend outward into the cytoplasm and may be associated with cytoplasmic membranes (Fig. 12).

The cytoplasmic ground substance is pale. Free ribosomes are fairly abundant and are arranged singly, in clusters of two to five, or occasionally in a helix. Scattered profiles of rough endoplasmic reticulum may be either tubular or vesicular. The arrangement of ribosomes is much more regular along the tubular strands. On the vesicular type the particles are irregularly spaced and areas of the membrane may be void of them. Vesicles of smooth endoplasmic reticulum are much more numerous than those of rough endoplasmic reticulum, and again tend to fall into two distinct morphological categories (Fig. 12).

The Golgi apparatus is confined to an area on one side of the nucleus and may appear as one large mass of lamellae and vesicles (Fig. 13) or may be divided into two or more smaller masses.

Mitochondria occur mostly in a single area at one pole of the oocyte containing up to fifty profiles. They are round to oval, no filamentous forms being present. The mitochondrial matrix has become somewhat less electron dense, and may contain one to eight pale areas. Some of the pale areas have indistinct edges; others appear to be partially or completely bounded by a unit membrane (Figs. 13, 14). Cristae are few in number, paler than the surrounding matrix, and are usually confined to one side of the mitochondrion. Clusters of mitochondria within the main mass of mitochondrial profiles appear to be embedded in a heavy granular material (Fig. 15). The outer aspects of the cluster are usually free of this material.

A number of highly electron-dense lamellar bodies are present in the perinuclear region (Figs. 11, 13), and are more numerous in the larger oocytes. They may form a solid concentric system, or may consist of a ring of several lamellae surrounding a vacuole. The vacuole may appear optically empty, or may contain granular or

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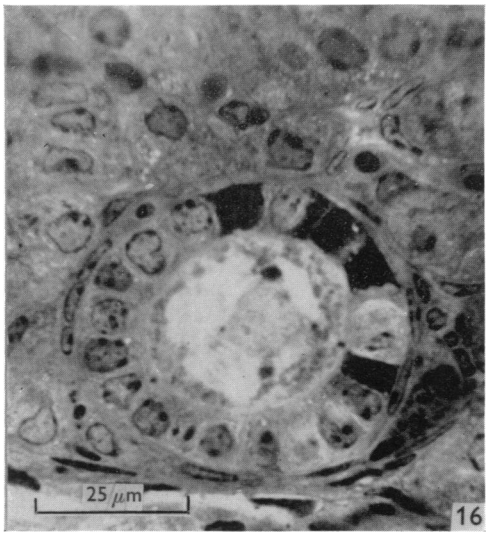
Fig. 12. Oocyte in unilaminar follicle from ovary of sexually mature hamster. A lobe (*L*) protrudes from nucleus (*N*) into cytoplasm. A large group of lamellar membrane systems lies in mid-cytoplasm. A multivesicular body is seen (*a*). Two categories of smooth endoplasmic reticulum (I and II) and rods with bulbous ends (*b*) are in evidence.

Fig. 13. Oocyte in unilaminar follicle from ovary of sexually mature hamster. The Golgi apparatus is in the form of a single mass lying near the nucleus. Lamellar membrane systems may be solid concentric bodies or may enclose a vacuole with granular contents. Mitochondria are embedded in a heavy, granular material and exhibit vacuoles and peripheral cristae.

Fig. 14. Oocyte in unilaminar follicle from ovary of sexually mature hamster. Mitochondria exhibit pale areas which may or may not be bounded by a membrane. Cristae are lacking or confined to lateral areas.

Fig. 15. Mitochondria in oocyte from unilaminar follicle. The mitochondria are embedded in a heavy, electron-dense granular material. Cristae are few in number or absent.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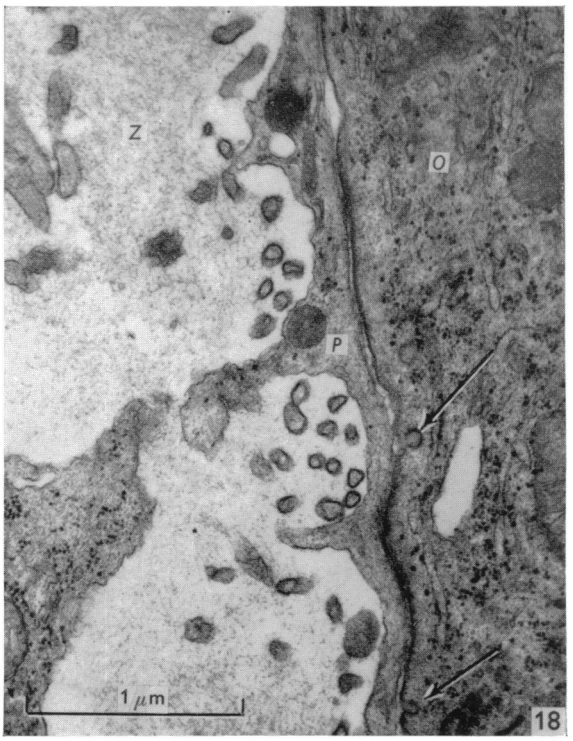
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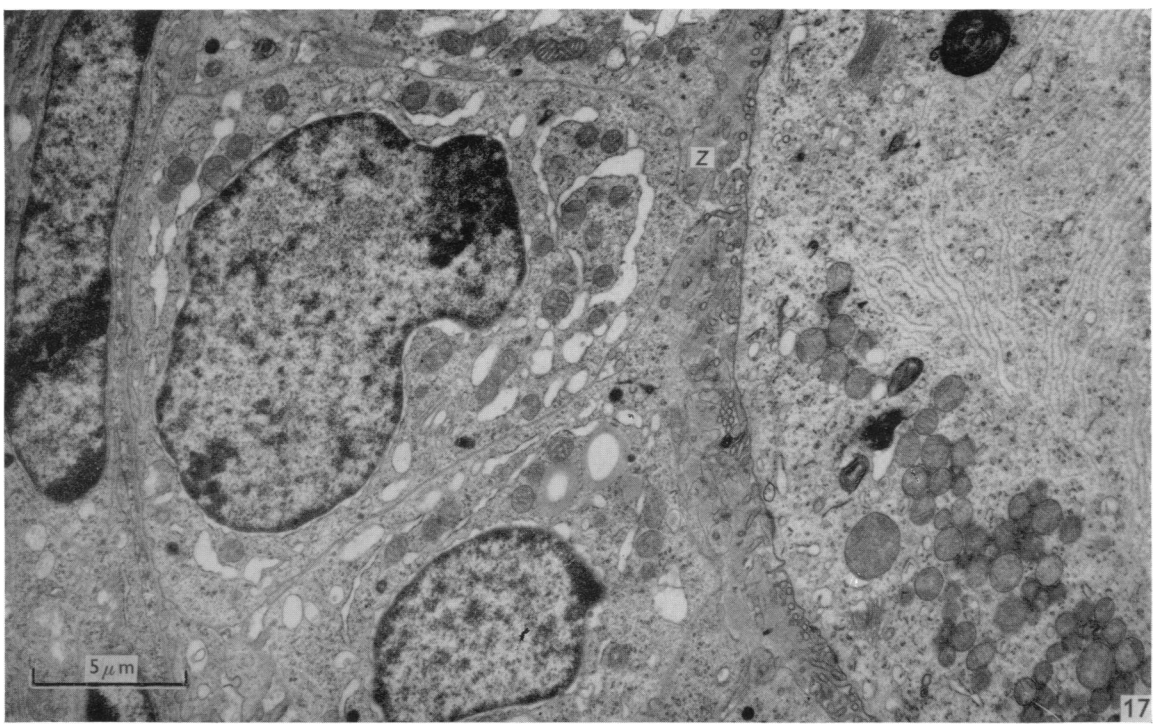
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amorphous material (Fig. 13). The lamellar bodies are often associated with rounded profiles of rough endoplasmic reticulum and multivesicular bodies (Fig. 12*a*).

In the cytoplasm of most oocytes a few tiny rods with bulbous ends are present (Fig. 12*b*). The rods may be short and straight (average length 0·2 μm), or longer and curved (up to 0·6 μm in length). The body of the rod is highly electron dense, the bulbous ends are pale. For illustrations of this phenomenon at later stages, see Figs. 20, 25 and 26. Other inclusions resembling lysosomes are occasionally seen. Pinocytotic vesicles are common at the oocyte periphery.

III. *Unilaminar follicles (cuboidal granulosa cells)*

Unilaminar follicles of both types, as well as all the later stages of oocyte development, are seen in the ovary of the sexually mature hamster.

By the time all of the granulosa cells of the unilaminar follicle have assumed cuboidal shape, great changes have taken place in the oocyte. This is obvious even by light microscopy (Fig. 16). Conspicuous granules (1–2 μm in diameter) which stain metachromatically with methylene blue are scattered throughout the peripheral cytoplasm. Between this cortical region and the nucleus, delicate threadlike structures are visible upon careful focusing. The ratio of light granulosa cells to dark granulosa cells has increased.

Granulosa cells

The nuclei are of highly irregular contour. The inner and outer nuclear membranes of the light cells are widely separated except at points where annular nuclear pores occur. The outer nuclear membrane has a scalloped appearance and may extend outward into the cytoplasm as much as 600 mμm (Fig. 17). This separation of the nuclear membranes has not been noted in the dark cells.

The cytoplasm of the light cells contains abundant clusters of free ribosomes, often grouped in tetrads. The most prominent feature of the cytoplasm is the large quantity of swollen rough endoplasmic reticulum, which may be seen at higher magnifications to contain fine strands of fibrillar or granular material. The swollen cisternae are entwined among the numerous mitochondria and are found in all parts of the cytoplasm. The mitochondria are round to moderately elongate and the electron density of their matrix varies from moderate to heavy. The cristae are pale, irregular and enlarged.

Fig. 16. Unilaminar follicle with cuboidal granulosa cells from ovary of sexually mature hamster. About one in four of the granulosa cells have stained intensely. Note granules in peripheral cytoplasm of oocyte and filamentous material in inner cytoplasm. Methylene blue.

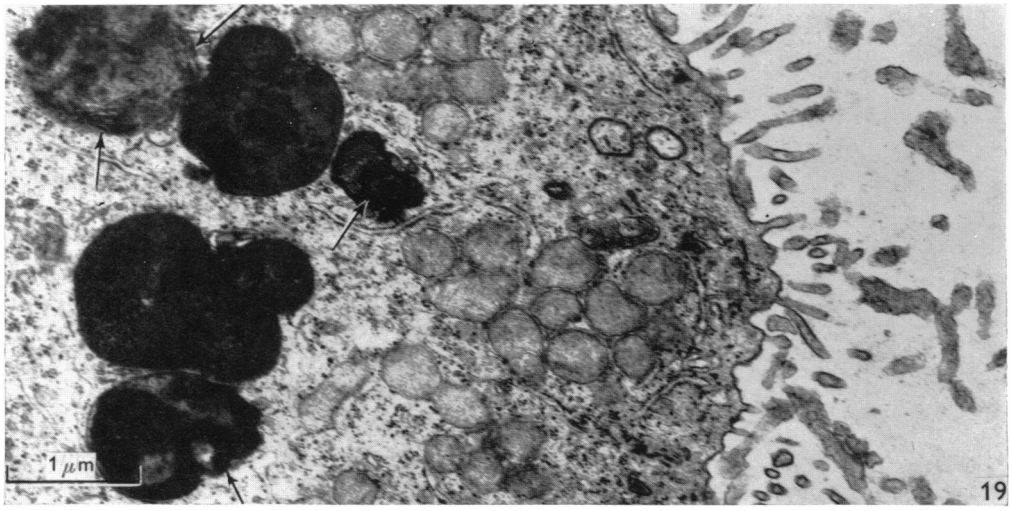
Fig. 17. Unilaminar follicle with cuboidal granulosa cells from ovary of sexually mature hamster. Nuclear membranes in the granulosa cells are widely separated at intervals. The endoplasmic reticulum is greatly swollen. Zona pellucida (*Z*) is forming. In oocyte, mitochondria, Golgi apparatus and lamellar membrane systems are peripheral; the inner cytoplasm is filled with filaments.

Fig. 18. Peripheral cytoplasm of ovum (*O*) and zona pellucida (*Z*) in single-layer (cuboidal) follicle from sexually mature hamster. Pseudopodium (*P*) from granulosa cell is closely apposed to surface of oocyte and contains two dense bodies. Pinocytotic vesicles arise at oocyte periphery (arrows).

Profiles of smooth endoplasmic reticulum are rare. The Golgi apparatus is moderately well developed and typically lies between the nucleus and the zona pellucida.

The endoplasmic reticulum in the dark cells is not swollen. Both light and dark cells may contain lipoid droplets.

A definite zona pellucida has formed into which extend processes from the granulosa cells and microvilli from the oocyte (Figs. 17, 18). The zona contains a



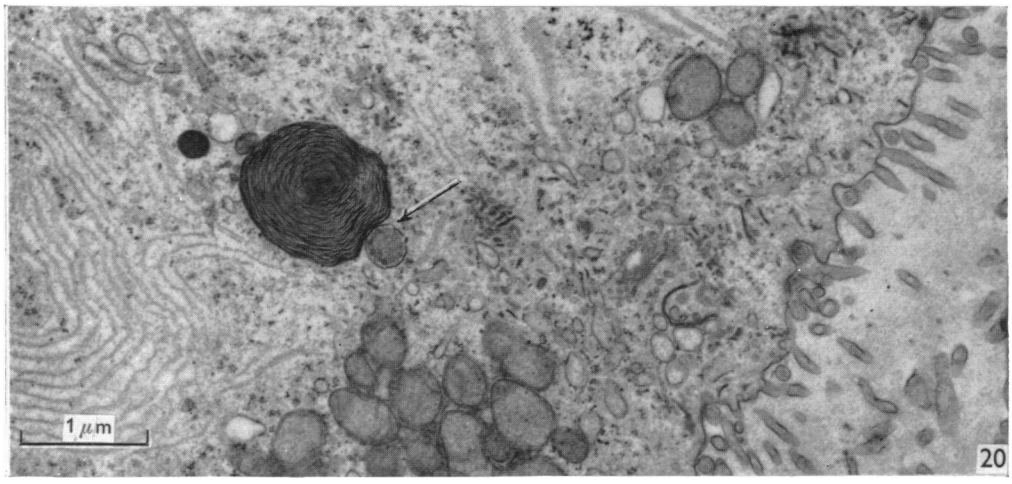


Fig. 19. Electron-dense bodies in peripheral cytoplasm of oocyte in unilaminar follicle with cuboidal granulosa cells. Lamellar membranes can be seen in three of the bodies (arrows).

Fig. 20. Peripheral cytoplasm of oocyte in follicle with one layer of cuboidal granulosa cells. Groups of mitochondria appear to be embedded in an amorphous matrix. Two categories of smooth endoplasmic reticulum and a number of rods with bulbous ends are present. A large lamellar body is closely associated with a multivesicular body (arrow).

loose, fibrillar material which is less dense or absent at the surface of the oocyte and is also less dense near the surface of the granulosa cells. The granulosa cell processes penetrate the zona and may contact the plasma membrane of the oocyte for distances up to 4 μm. They may contain round electron-dense bodies (Fig. 18). Areas of attachment resembling desmosomes are seen along the areas of contact.

Oocytes

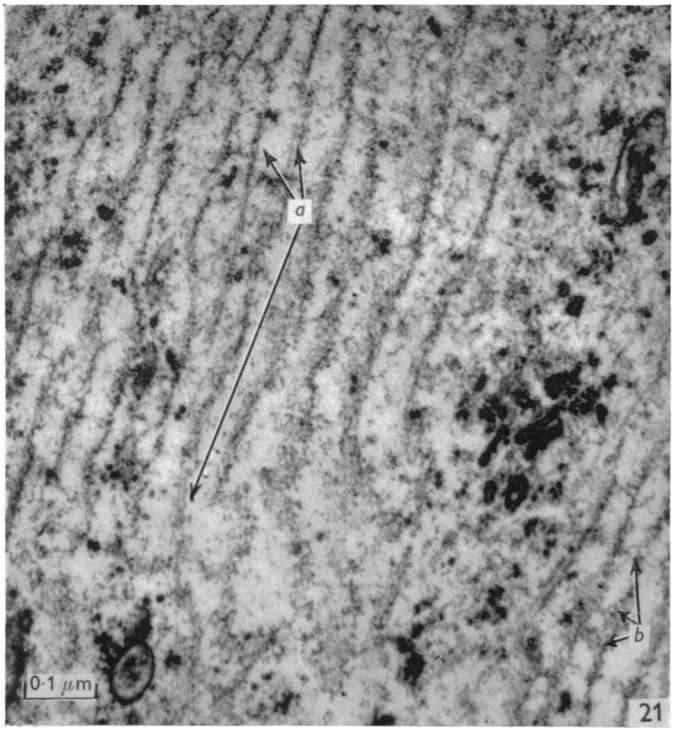
Mitochondria, Golgi apparatus, rough endoplasmic reticulum, free ribosomes and lamellar bodies are largely confined to the peripheral third of the cytoplasm. The mitochondria are round to oval and occur in groups of 15 to 50 about the periphery. Again the groups appear to be embedded in an electron-dense substance. The mitochondrial matrix is of moderate electron density, and seldom contains pale vacuoles. Cristae are few in number or lacking. When present they are typically in the form of half moons at one end of the mitochondrion. Curved stacks of Golgi membranes are seen in the intervals between masses of mitochondria. Numerous electron-dense rods with bulbous ends are seen throughout the peripheral area. They sometimes assume a crescent or horse-shoe shape, and in some instances appear to close upon themselves, forming a ring.

Strands of rough endoplasmic reticulum are scattered throughout the peripheral cytoplasm. Numerous smooth-surfaced vesicles are also seen, falling again into two morphological categories. Free ribosomes averaging 170 Å in diameter are abundant in the peripheral area and tend to occur singly more frequently than in clusters.

Large electron-dense bodies measuring up to 1·8 μm in diameter (Fig. 19) and lamellar bodies of about the same size (Fig. 20) occur singly or in groups in the peripheral zone, usually internal to the masses of mitochondria and Golgi material. They apparently correspond to the large metachromatic granules seen by light microscopy. The concentric lamellae of the lamellar bodies may be distinct and stand out clearly against an electron-lucent background (Fig. 20) or may appear indistinct as though embedded in a matrix of moderately electron-dense material. Close inspection of certain of the dense bodies (Fig. 19) shows what appears to be the beginnings of lamellar formation within them, so that the two types conceivably transform one into the other. Again, multivesicular bodies are seen in association with lamellar bodies (Fig. 20).

The inner portion of the cytoplasm of the oocyte is filled with arrays of fine filaments (Fig. 17). The filaments are grouped in stacks numbering from two to twelve, and run roughly parallel to the oocyte periphery. At high magnification the filaments appear to consist of fine fibrils or of tiny granules, depending upon plane of section. Occasionally a filament appears as two parallel lines with a space between (Fig. 21). In favourable sections a few filaments exhibit periodicity at intervals of approximately 150 Å (Fig. 21*a*). Accurate measurement of filament width is not possible, as the edges of the filaments are indistinct, but best estimates from measurements at magnifications from $\times$ 96000 to 300000 fall between 111 and 208 Å. The distance between the filaments in a stack is roughly constant and averages 1140 Å, with a range of 555–1950 Å. The filaments in a stack sometimes appear to be interconnected at intervals by fine fibrillar material (Fig. 21*b*).

Cytoplasmic organelles are not present between the filaments within a stack.


a
b
0·1 μm
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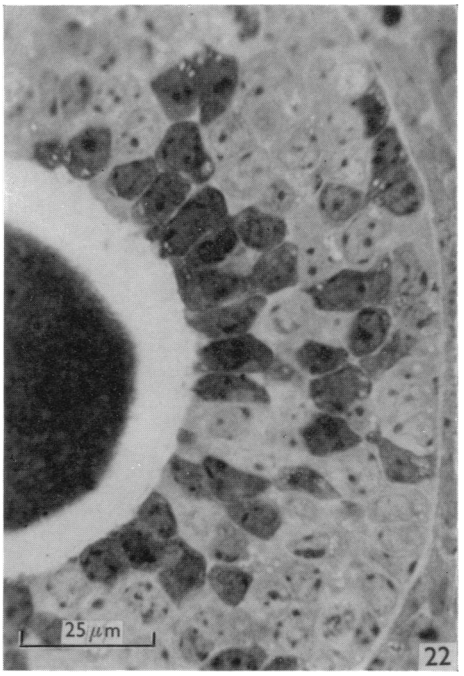
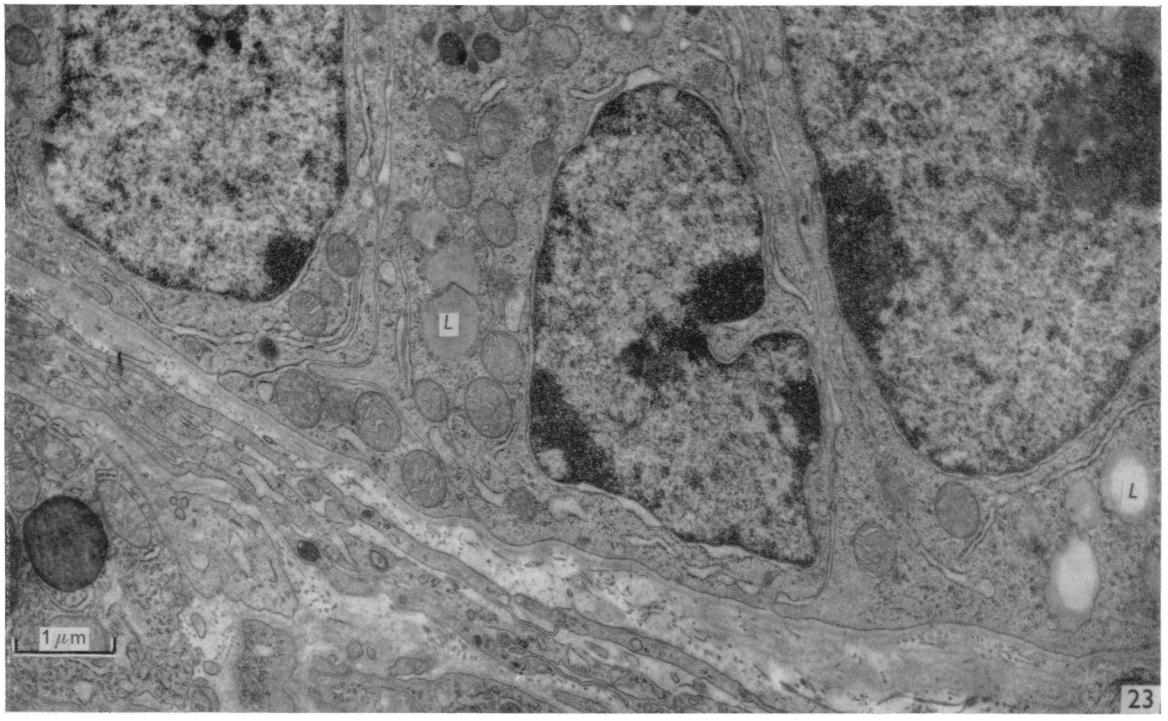

25 μm
22


L
L
1 μm
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In areas between stacks, however, one occasionally sees a group of Golgi membranes, rods with bulbous ends, and strands of rough endoplasmic reticulum. Mitochondria, lamellar bodies and multivesicular bodies are apparently excluded from the filament-containing part of the cytoplasm at this stage. Both within and between the stacks of filaments are seen round electron-dense particles measuring 250–300 Å in diameter. They are first encountered at this stage, and at later stages of oocyte development they are scattered throughout the entire cytoplasm.

IV. *Multilaminar follicles*

Light microscopy shows that the ratio of light to dark cells in the granulosa varies considerably from follicle to follicle at the multilaminar stage. The dark cells tend to run radially outwards from the zona pellucida, forming columns, as though they had divided transversely several times (Fig. 22). The cytoplasm of the oocytes at this stage stains more deeply with methylene blue than in earlier stages. Large numbers of granules in the peripheral cytoplasm of the oocytes are still seen.

Granulosa cells

The granulosa cells have not changed appreciably from those in unilaminar (cuboidal) follicles except for the following points:

(1) Relatively fewer granulosa cells have greatly swollen rough endoplasmic reticulum.

(2) A larger number of lipoid inclusions is present in the cytoplasm.

(3) A number of round electron-dense bodies may be present within the cytoplasm (Fig. 23).

Figure 23 shows the outer layer of granulosa cells resting upon a distinct basement membrane and separated from the underlying theca cells by a connective tissue space containing collagen fibres.

Oocytes

The diameter of the oocyte has greatly increased and the peripheral band containing the organelles is proportionately smaller. Many of the mitochondria are still round to oval and disposed in large groups about the periphery. As the size of the oocyte increases, however, more of the mitochondria occur singly, and these tend to be elongate.

Masses of Golgi material occur between mitochondrial masses, and are frequently associated with a lattice-work of interconnected smooth membranes which contain

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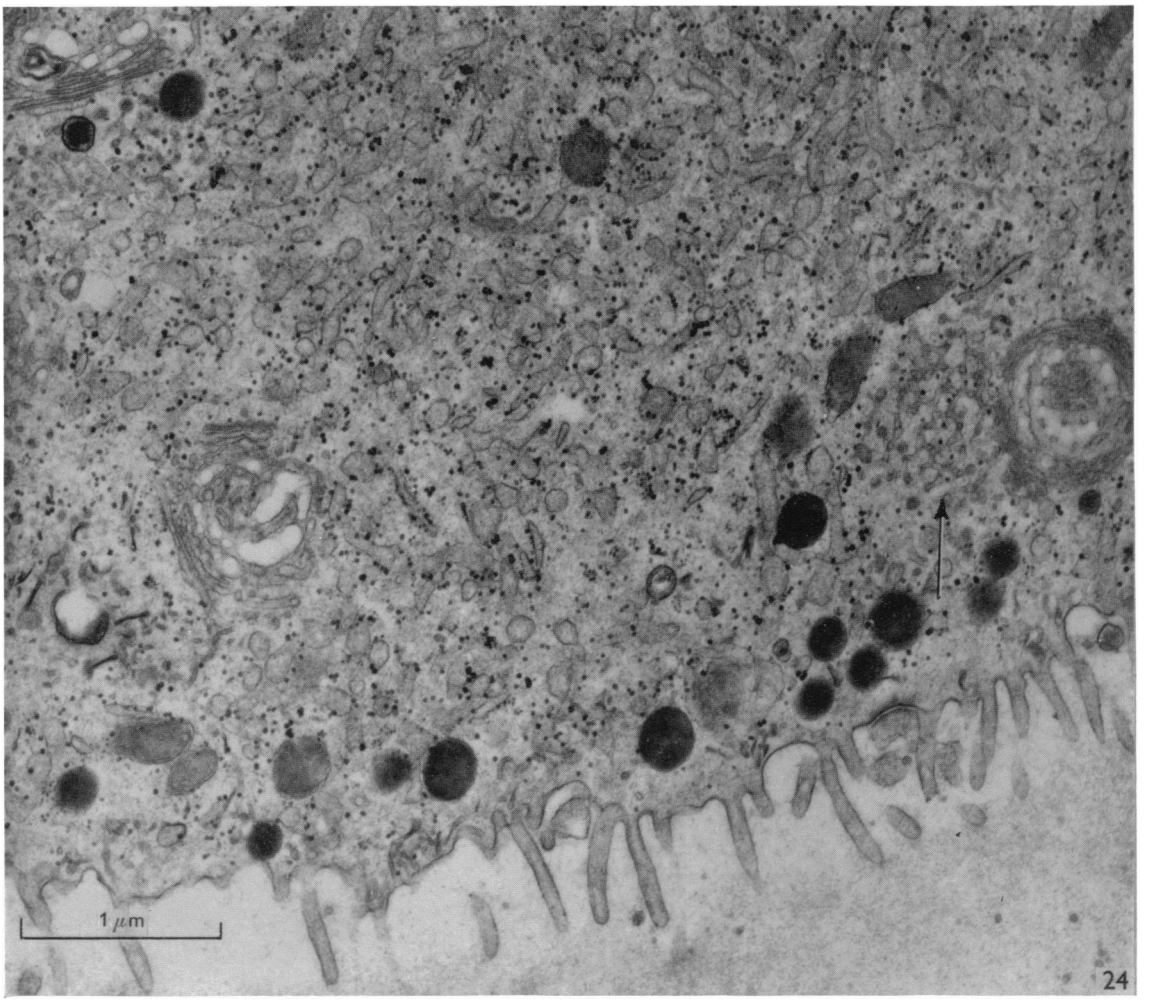
Fig. 21. Filaments in cytoplasm of oocyte in follicle with one layer of cuboidal granulosa cells. Note double nature of filament and periodicity (*a*). Fine fibrillar material appears to connect some filaments (*b*).

Fig. 22. Multilaminar follicle from ovary of sexually mature hamster. Cytoplasm of oocyte is granular and has stained deeply. Dark granulosa cells are arranged in columns running radially outward from zona pellucida. Methylene blue.

Fig. 23. Outer layer of granulosa cells in multilaminar follicle. The cells rest on a distinct basement membrane and are separated from the underlying theca cells by a connective tissue space containing collagen fibres. Several electron-dense bodies and lipoid droplets (*L*) are present within the cytoplasm.



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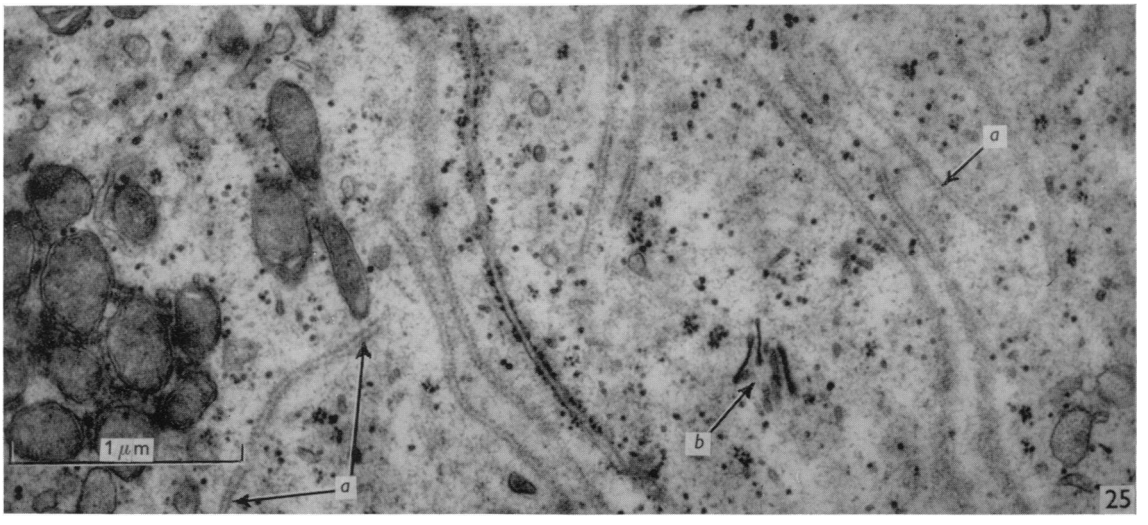

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electron-dense material (Fig. 24). Electron-dense rods with bulbous ends are very numerous (Figs. 25, 26), and may assume bizarre forms (Fig. 26). They are often associated with the smaller type of smooth endoplasmic reticulum.

Large lamellar bodies are present in the inner part of the peripheral zone and again may be associated with multivesicular bodies. Inclusions resembling lysosomes are aligned directly beneath the plasma membrane (Fig. 24).

Rough endoplasmic reticulum is infrequently seen and tends to lie interior to mitochondria and Golgi material. Smooth endoplasmic reticulum (Figs. 24, 27) is abundant, particularly in the peripheral cytoplasm and two morphological categories are again present. Pinocytotic vesicles form between the microvilli at the surface of the oocyte (Fig. 26).

A marked change has occurred in the arrangement of the filaments in the inner cytoplasm. Aggregates of mitochondria and vesicles of smooth endoplasmic reticulum now occur between (but not within) the stacks of filaments. Instead of running parallel to the oocyte periphery, the filaments now show a tendency to run at right angles to the cortical zone. The stacks are often horse-shoe shaped or circular, especially near the peripheral zone of organelles (Fig. 27). The filaments have more than doubled in width. Again, accurate measurement is impossible, but measurements taken lie between 312 and 458 Å. The filaments are comparable in width to neighbouring strands of rough endoplasmic reticulum (Fig. 25). Each filament appears to consist of two parallel lines of granular material separated by a pale core. The core may be seen in suitable sections to be divided into compartments, the wall between compartments occurring at intervals of about 455 Å (Fig. 27). The usual spacing between filaments in a stack is about 700–1100 Å. However, at times two or more filaments may be seen in intimate contact, appearing to share a common inner boundary (Fig. 28). One gets the impression that one filament is forming upon another in template fashion. A population of round electron-dense profiles measuring 250–300 Å in diameter is again present (Fig. 27).

V. *Follicles with antra and mature Graafian follicles*

Light microscopy of fully mature Graafian follicles fixed 2 h before expected ovulation reveals the oocyte within the antrum surrounded by the corona radiata. The majority of the granulosa cells comprising the follicle wall (parietal granulosa cells) stain lightly with methylene blue, but a varying number take a moderately dark to dark stain. The cells attached to the basement membrane tend to be columnar but the ones nearer the antrum become progressively more rounded and contain

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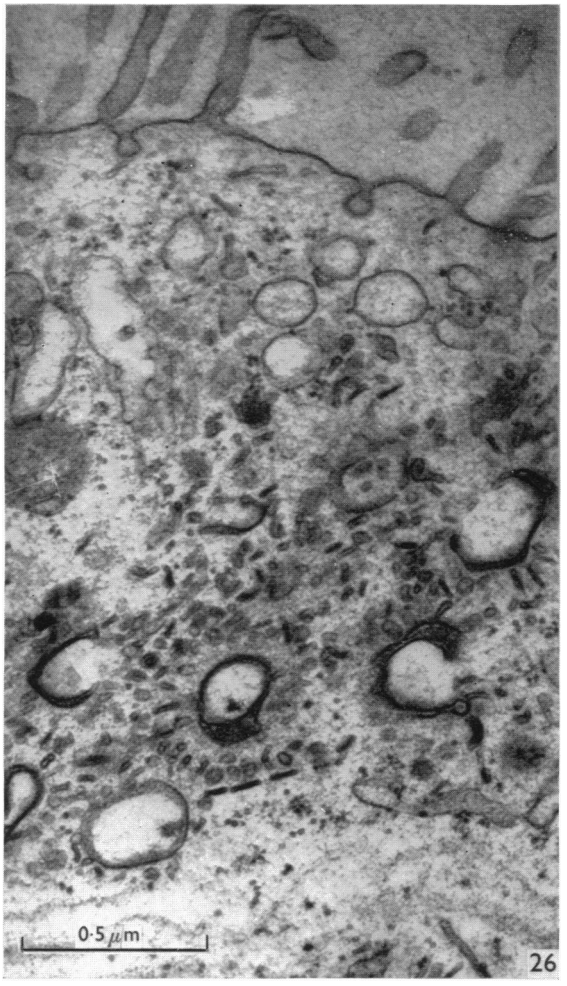
Fig. 24. Peripheral cytoplasm of oocyte in multilaminar follicle. Electron-dense bodies resembling lyosomes are aligned at oocyte periphery. An interconnecting system of small, smooth tubules is seen near Golgi apparatus (arrow). A second Golgi apparatus is seen at right, but is not associated with a system of tubules. The larger type of smooth endoplasmic reticulum is also present.

Fig. 25. Filaments in cytoplasm of oocyte in multilaminar follicle. Double nature of filaments is apparent, and some show a compartmented nature (*a*). Filament width is comparable to that of neighbouring rough endoplasmic reticulum. A population of round electron-dense bodies 250–300 Å in diameter is present between filaments. Electron-dense rods with bulbous ends are associated with small vesicles of smooth endoplasmic reticulum (*b*).



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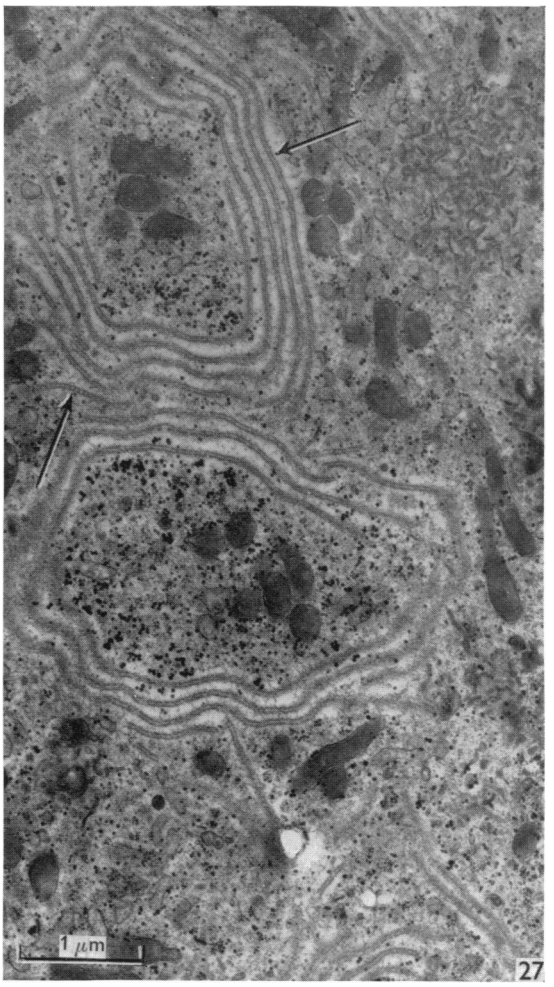

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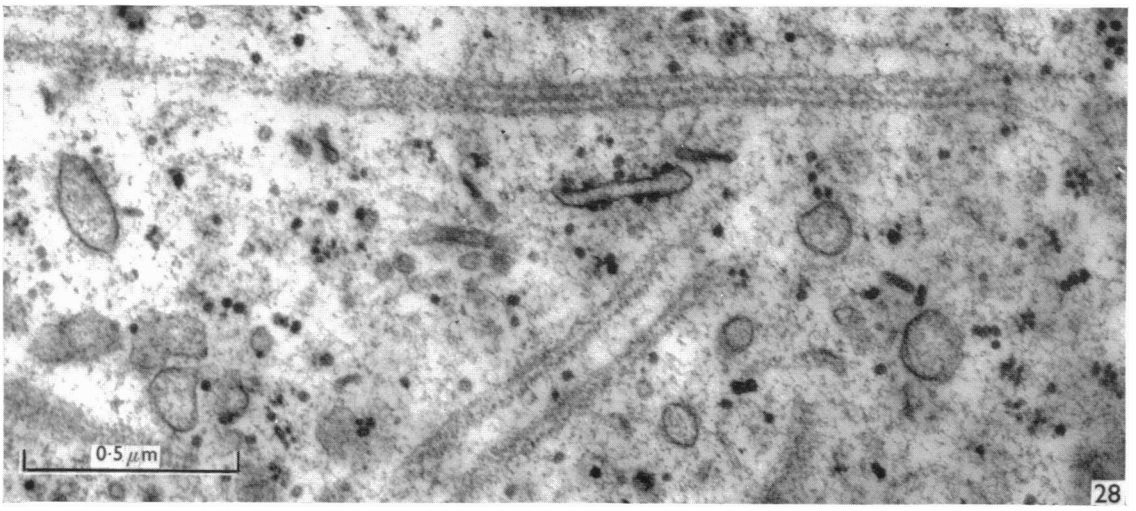

28

progressively more lipoid droplets. Three to four nucleoli are common. Dark cells are predominant in the corona radiata and free in the antrum. They tend to be irregular in shape and two or more are frequently seen attached to one another.

As the follicles increase in size, sectioning of the tissue for electron microscopy becomes increasingly difficult. In no instance was it possible to obtain sections of a fully mature oocyte and corona cells which were not badly affected by chatter. Although the sections were far from optimal, sufficient information could be obtained from them to compare the fully mature oocyte with earlier stages.

Granulosa cells

By electron microscopy the parietal granulosa cells are seen to rest on a distinct continuous basement membrane. Many intercellular spaces and channels run between the granulosa cells (Fig. 29) and are filled with amorphous moderately electron-dense material (Figs. 29, 30). The channels never open directly at the base of the granulosa cells, but are always bounded by basement membrane (Fig. 30).

The nuclei of the granulosa cells have not changed appreciably since earlier stages. Based on cytoplasmic morphology, two principal types of cell appear to be present, with considerable intergrading between them. The extremes are shown in Figs. 31 and 32. Figure 31 depicts a cell floating freely in the antrum near a mature oocyte. The background cytoplasm is dark and contains abundant rough endoplasmic reticulum as well as free ribosomes. The endoplasmic reticulum is not swollen and the nuclear membranes are not widely separated. Figure 22 depicts parietal granulosa cells near the antrum. The background cytoplasm of the cell at the centre is lighter than that of the cell in Fig. 31. The nuclear membranes are widely separated and the endoplasmic reticulum is greatly swollen. Abundant free ribosomes are present.

'Light' and 'dark' cells are not confined to either type, and there is so much intergrading that many cells cannot be typed as one or the other. Lipoid inclusions and round electron-dense bodies are common (Fig. 29). A typcal condition of the endoplasmic reticulum is seen in Fig. 30 where many of the membranes are free of ribosomes, while portions of others are heavily studded with them.

Free surfaces of the corona cells bear long, slender processes (Fig. 33) which may contact the outer surface of the zona pellucida but are not seen to extend into it. Other processes extend into the liquor folliculi, which appears as a uniform scattering of granular and fibrillar material.

The zona pellucida has enlarged and now forms a homogeneous, well-defined,

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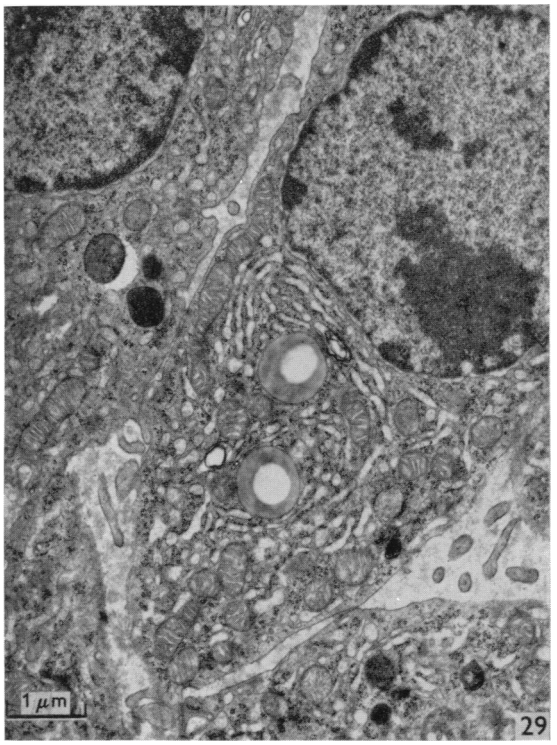
Fig. 26. Peripheral cytoplasm of oocyte in multilaminar follicle. Bizarre forms are assumed by electron-dense rods with bulbous ends. The rods are closely associated with small vesicles of smooth endoplasmic reticulum. A pinocytotic vesicle is forming at oocyte periphery.

Fig. 27. Circular arrays of filaments in cytoplasm of oocyte in multilaminar follicle. Electron-dense particles 250–300 Å in diameter are particularly abundant within the circular arrays. The double nature of the filaments is clearly apparent, and in some profiles compartments can be seen (arrows). A large mass of small smooth tubules is seen at upper right.

Fig. 28. Inner cytoplasm of oocyte in multilaminar follicle. Two cytofilaments appear to share an interior wall.


1 μm
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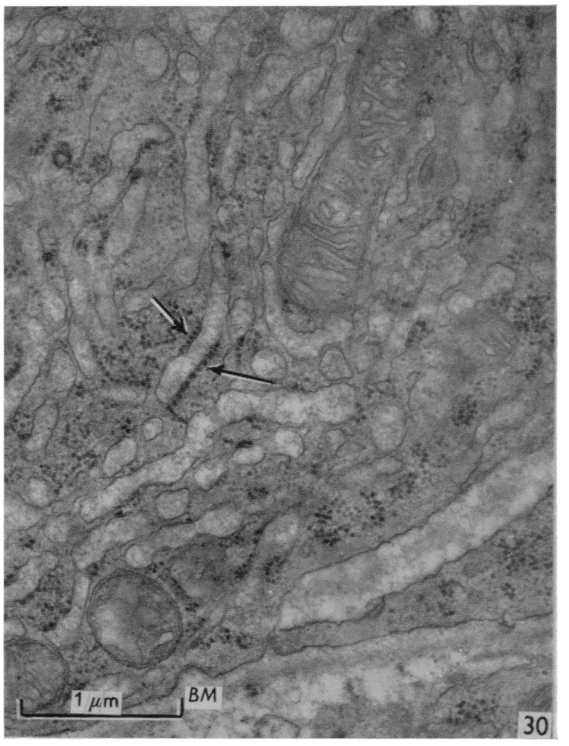
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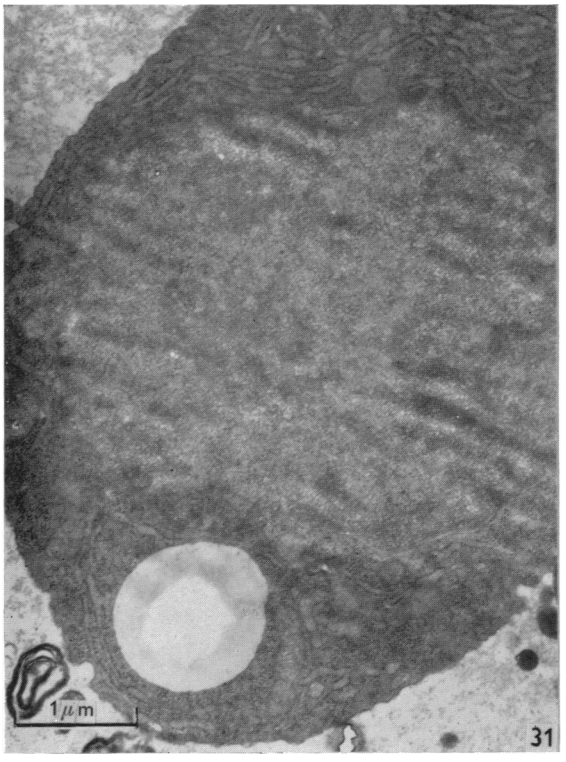
1 μm
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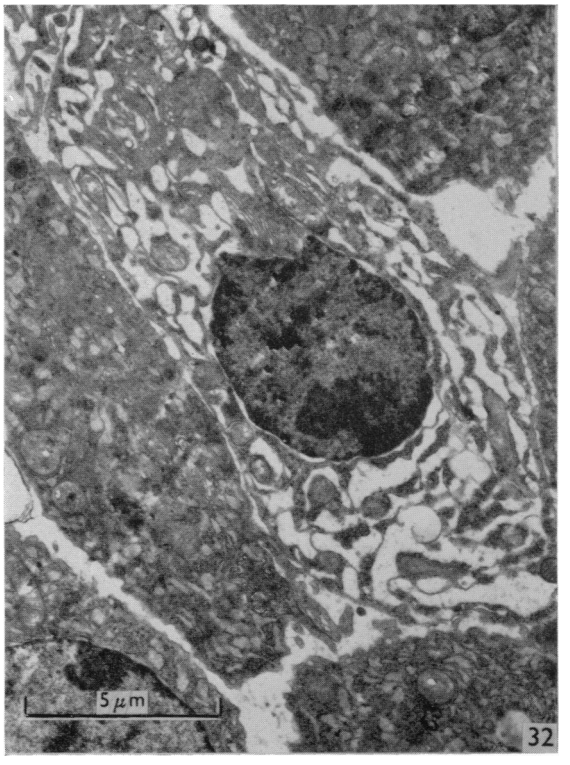

5 μm
32

moderately electron-dense band approximately 13 μm in width around the oocyte (Fig. 33). It is separated from the oocyte by a space approximately 1·5 μm wide. Within this space is a scattering of fine granules and fibrils which resemble those of the liquor folliculi. Microvilli extend from the surface of the oocyte into this space but do not penetrate the zona pellucida. They are not as numerous as in the oocyte of the pre-antrum follicle.

Oocytes

The peripheral band of organelles has become much narrower and has been invaded in places by filaments from the inner cytoplasm. Mitochondria are smaller and many are moderately elongate. They are dispersed singly or in small groups along the periphery rather than occurring in masses, and may also be seen singly or in groups between stacks of filaments in the inner cytoplasm (Fig. 33). Golgi material is seldom encountered. Many vesicles of smooth endoplasmic reticulum are in close association with the mitochondria. Rough endoplasmic reticulum is not seen, and clusters of free ribosomes are uncommon. However, masses of granules averaging 170 Å in diameter embedded in an amorphous matrix are seen at intervals in the peripheral cytoplasm (Fig. 34). Round electron-dense particles measuring 250–300 Å in diameter are scattered throughout the entire cytoplasm (Fig. 34). Rods with bulbous ends are numerous. Lamellar bodies are no longer present, but smaller electron-dense bodies resembling lysosomes are aligned beneath the plasma membrane (Fig. 33).

The cytofilaments have not changed appreciably, although their compartmented nature is rather more evident (Fig. 34).

DISCUSSION

Discussion of results in the light of the methods used

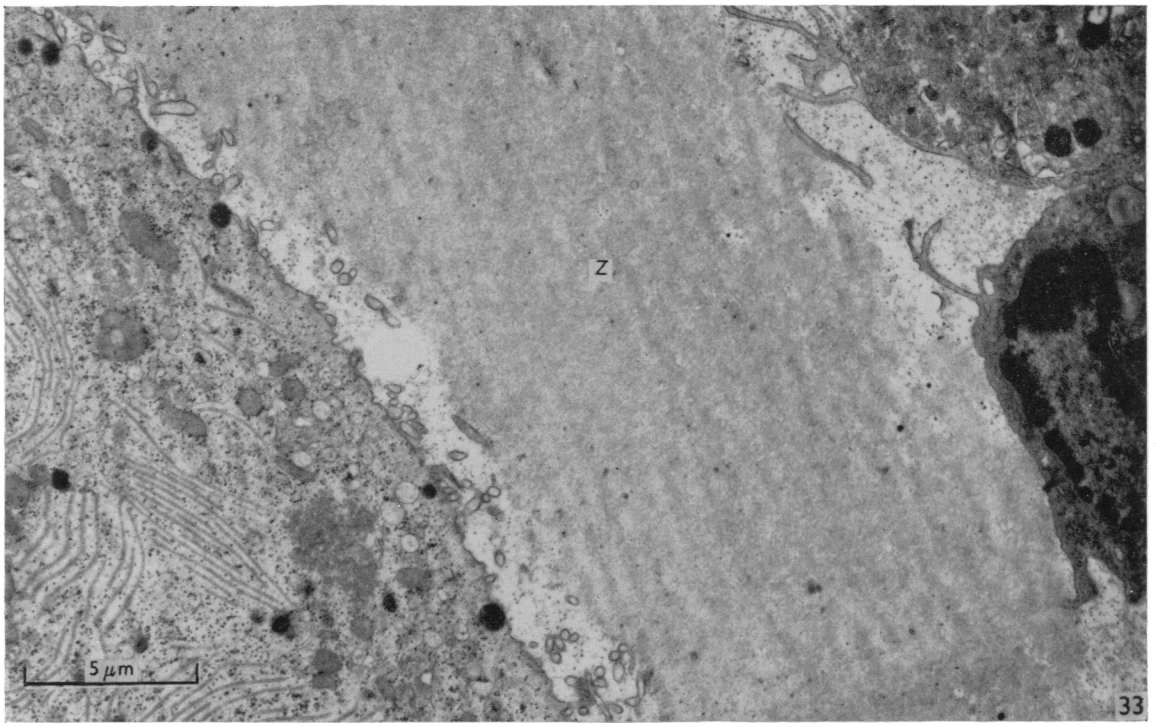
Several of the findings in both oocyte and follicle cells of the hamster may be attributable to fixation of the tissue in glutaraldehyde followed by osmium tetroxide. As reported by Sabatini, Bensch & Barrnett (1963): 'The dialdehydes are excellent cross-linking agents that react rapidly, especially with active hydrogen, amino and imino groups in protein and hydroxyl groups of polyalcohols.'

Fig. 29. Parietal granulosa cells in mature Graafian follicle. The cytoplasm is abundantly supplied with endoplasmic reticulum of both rough and smooth varieties. Lipoid droplets and dense bodies are numerous. Channels containing amorphous material occur between cells.

Fig. 30. Cytoplasm of parietal granulosa cells in mature Graafian follicle. The cytoplasm is laden with endoplasmic reticulum. Some cisternae are entirely smooth; portions of others are heavily studded with ribosomes (arrows). Channels between cells contain amorphous material. The channels are not in direct communication with the underlying connective tissue space, being separated from it by a distinct basement membrane (*BM*).

Fig. 31. Detached granulosa cell floating in antrum of mature Graafian follicle. Background cytoplasm is highly electron dense and is heavily laden with rough endoplasmic reticulum. A large lipoid droplet is present.

Fig. 32. Parietal granulosa cells in mature Graafian follicle. Cell at centre has grossly swollen endoplasmic reticulum and widely separated nuclear membranes. Endoplasmic reticulum of neighbouring cells is less swollen and nuclear membranes appear more normal.



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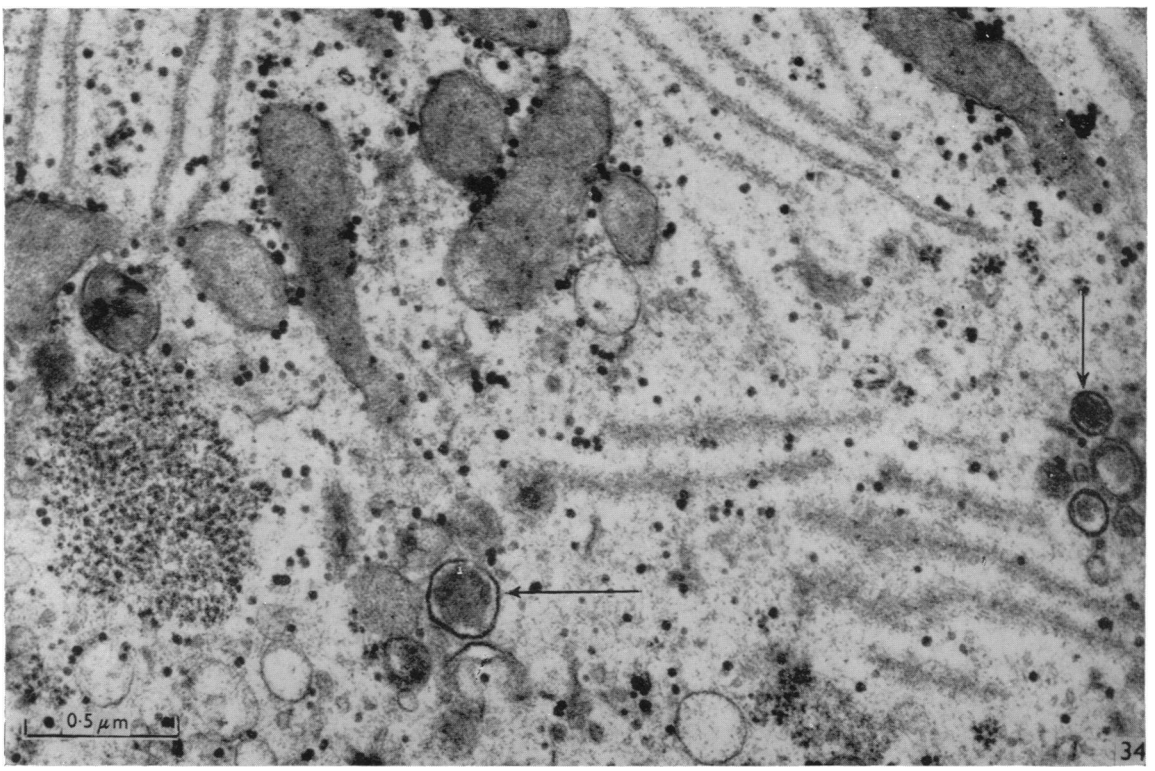

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A striking feature of glutaraldehyde–osmium-fixed araldite-embedded material can be seen by light microscopy after methylene-blue staining. In the primordial polyovular follicles the flattened follicle cells associated with the oocytes can be clearly identified by their intense staining reaction. This reaction appears to occur in all the follicle cells in the primordial polyovular follicle, and in the great majority of the follicle cells in the youngest single-egg follicles. By the time the granulosa cells of the single-layer follicle have become cuboidal, the ratio of light to dark cells has increased, and varies considerably from follicle to follicle during the maturation process. This might reflect differentiation into light cells, or merely a change in a complex secretory cycle.

Those follicle cells which exhibit an intense affinity for methylene blue are seen at the ultrastructural level to exhibit high electron density of both background nucleoplasm and background cytoplasm. It has been found in this laboratory (Weakley & Coupland, unpublished) that certain cells in foetal and neonatal rabbit adrenal medulla also display a strong affinity for methylene blue and show electron-dense background nucleoplasm and cytoplasm at the ultrastructural level. This phenomenon may prove, therefore, to be common to developing endocrine tissue.

The oocytes of the primordial polyovular follicle, although staining much less intensely than the follicle cells, do exhibit a range of affinity for methylene blue which varies inversely with the degree of maturity of the oocyte. This range of affinity does not appear to correlate with differences at the ultrastructural level. Such variations of electron density as are seen are so slight as to be attributable to variations in section thickness. It appears, therefore, that the strong methylene-blue affinity of the dark follicle cells cannot be ascribed to the same causes as the lesser affinity of the oocyte for this stain.

The oocytes of single- and double-layer follicles have little or no affinity for methylene blue. In older multilaminar follicles, however, a variable affinity of the egg for methylene blue is exhibited and persists until ovulation. Again this is not reflected at the ultrastructural level.

The nature of the methylene-blue positive material in the follicle cells and oocytes cannot be determined from the present data. Many different substances will stain with methylene blue, the principal mechanism involved being attraction of the positively charged stain for negatively charged groups in the substrate (Pearse, 1961).

Another phenomenon which may reflect chemical changes in the maturing oocyte is seen during thin sectioning. The cutting of ultra-thin sections becomes progressively difficult at the later stages of oocyte development. This difficulty may result from the interaction of the fixatives employed with substances present in the liquor folliculi and oocyte in the immediate pre-ovulation period.

Fig. 33. Zona pellucida (Z) in follicle with antrum. Zona material appears as a highly condensed band which is separated from ovum by a gap of 1·5 μm. Microvilli of oocyte and pseudopodia of corona cells contact but do not penetrate zona. Dense bodies resembling lysosomes are seen directly beneath plasma membrane of oocyte.

Fig. 34. Cytoplasm of oocyte from follicle with antrum. A mass of granules the size of small ribosomes is present at lower centre. Granules 250–300 Å in diameter are scattered throughout the cytoplasm. Multivesicular bodies are present (arrows). Note elongation of mitochondria.

Other cytological phenomena such as cytofilaments and electron-dense rods with bulbous ends may prove on subsequent investigation to reflect not species differences but rather the superiority of fixation with glutaraldehyde followed by osmium tetroxide over fixation with osmium alone. Since the advent of glutaraldehyde fixation, 'cytotubules' and 'cytofilaments' have been reported in a variety of cells (e.g. De Thé, 1964; Slautterback, 1963; Battig & Clevenger, 1961). These vary considerably in morphology, diameter and location, and thus do not appear to have a single function.

Granulosa cell types

Several functions have been attributed to the granulosa cells, and it is therefore hardly surprising to find at least two types of cell present. It has long been accepted that the granulosa cells act as 'nurse cells' for the developing oocyte. Those granulosa cells located in the parietal layer of the Graafian follicle survive after ovulation and become lutein cells. The ones surrounding the oocyte degenerate (Björkman, 1962). Jacoby (1962) cites evidence that the liquor folliculi is secreted by the granulosa cells. It has not yet been established whether the zona pellucida is secreted by the granulosa cells or by the oocyte (Odor, 1960).

Meiotic activity in the eight-day-old hamster ovary

The finding of three-filament chromosome cores typical of meiotic prophase in the 8-d-old hamster was quite unexpected. Oogenesis in the chick ceases at hatching (Hughes, 1963), and in both mouse and rat meiotic activity ceases by 4 d *post partum* (Parsons, 1962; Franchi & Mandl, 1962). It has been recently accepted as established that oogenesis does not occur in the mammalian adult (Franchi, 1962).

No evidence of meiotic activity was seen in the ovaries of the older animals used in this study. However, routine light microscopy of adult hamster ovaries reveals a small number of polyovular follicles to be present. These have also been previously reported by Kent (1962), who dimisses them as abnormal phenomena.

Nucleocytoplasmic relationships

Nucleocytoplasmic relationships in oocytes have received limited attention at the ultrastructural level, and it is difficult to ascertain whether certain phenomena differ with species or simply have not yet been seen in material studied. Reports on budding of vesicles and annulate lamellae from the outer nuclear membrane, and the extrusion of nuclear contents, have been reported in oocytes and in a variety of embryonic tissues (Swift, 1956; Ackerman, 1962; Hadek & Swift, 1962; Kessel 1963; Clark, 1960; Weakley, Patt & Shepro, 1964).

In the hamster oocyte, long lobes of the nucleus extending into the cytoplasm were seen in oocytes with a single row of flattened follicle cells. One is tempted to speculate that in the primordial polyovular follicle, where the oocyte is much smaller, interplay between follicle cell pseudopodium and egg nucleus can take place almost by direct contact, as in Fig. 3. Nuclear lobes in the enlarging oocyte may provide a transitional mechanism for communication, while in the still larger oocyte the complex system of cytofilaments or tubules might take over this function. At any rate, it seems likely that the cytofilaments, and possibly the two categories

of smooth endoplasmic reticulum, play some role in transport or communication in the rapidly expanding cytoplasm. This communication would presumably be two-way, since oocyte and granulosa cells are mutually dependent for full development (Björkman, 1962).

Cytoplasmic bridges between developing oocytes in the primordial polyovular follicle are probably comparable to those reported in developing spermatocytes (Fawcett, Ito & Slautterback, 1959), and may also serve as communication channels for developmental information.

Other cytoplasmic organelles

Round electron-dense profiles measuring 250–300 Å in diameter appear in the developing oocyte at the same stage as the cytofilaments, and continue to be present during the subsequent developmental stages. These particles do not appear to be artifacts since they are seen only in the cytoplasm of the oocyte and not in the zone pellucida, liquor folliculi or granulosa cells. In sections stained with uranyl acetate alone, the particles take up the stain while other cytoplasmic elements do not. This introduces the possibility that the particles contain nucleic acid. Perhaps what we have here is a dual population of ribosomes, the smaller clusters contributing to the so-called 'maintenance metabolism' of the oocyte and the larger single ones carrying on another function.

Changes in mitochondrial morphology and distribution in the developing hamster oocyte are not significantly different in kind from those described in rat and guinea-pig. Some difference in timing is noted, however. Mitochondria containing vacuoles are characteristic of meiotic pachytene in the late foetal and newborn rat (Franchi, 1962), whereas in adult guinea-pig they are seen in oocytes with one row of flat follicle cells (Adams & Hertig, 1964). In the hamster they occur in both the prepubertal and adult animal, always in oocytes with one row of flattened follicle cells. The embedding of clusters of mitochondria in an electron-dense matrix has also been reported in the hamster by Odor (1965) but not, so far, in other mammalian species. The matrix could represent a product elaborated by the mitochondria, or alternatively it could serve as a means of 'cementing' mitochondria into a close functional relationship. Such structural rigidity also appears to be characteristic of the stacks of cytotubules, which are interconnected by a system of fine fibrillar elements.

The round electron-dense bodies aligned beneath the plasma membrane of the oocyte from the multilaminar follicle stage onwards are apparently similar to or identical with the so-called 'cortical granules' in unfertilized tubal ova of hamsters (Austin, 1956, 1961) and rabbits (Hadek, 1963). These authors suggested a role for these granules in the fertilization reaction of the zona pellucida. Adams & Hertig (1964) reported similar granules in multilaminar follicles of the guinea-pig and described their evolution by coalescence of tiny vesicles. They suggested that the granules act as a storage depot for membrane-forming materials. In view of the probable transition of multivesicular bodies into lysosomes (Novikoff, Essner & Quintana, 1964), it would be pertinent to carry out electron histochemical tests for the presence of acid phosphatatase in these cortical granules of the hamster oocyte. There may well be a 'reservoir of membrane-forming materials' in the rapidly growing oocyte, as Adams & Hertig suggest. A reasonable location for this function would be the concentric

membrane-containing bodies which are largest in the late single and early multilaminar follicle stages of oocyte development. By the time the oocyte has reached maximum size, these bodies are no longer in evidence.

SUMMARY

Five developmental stages of the ovarian follicle in the golden hamster have been studied by electron microscopy. Fixation with glutaraldehyde followed by osmium tetroxide was employed.

1. Primordial polyovular clusters (predominant in ovaries from 8-d-old animals) contain oocytes in prophase of meiosis, identified by the presence of tripartite chromosome cores. Nucleoli in the oocyte tend to be linearly dispersed beneath the nuclear membrane. Two distinct categories of smooth endoplasmic reticulum are present in addition to the Golgi apparatus. Free ribosomes averaging 170 Å in diameter occur singly or in clusters. Cytoplasmic bridges between developing oocytes are present. The polyovular clusters are surrounded by flattened granulosa cells with extremely dark background nucleoplasm and background cytoplasm.

2. Unilaminar follicles with flattened granulosa cells are predominant in ovaries from 26-d-old animals. The granulosa cells may all be of the 'dark' type, or a variable number may lack this electron density. Oocytes are no longer in meiosis and the nucleoli have changed in morphology. Clusters of mitochondria are embedded in an electron-dense matrix and contain pale areas bounded by unit membranes. Systems of concentric membranes (lamellar bodies) and short rods with bulbous ends are numerous within the cytoplasm.

3. Unilaminar follicles with cuboidal granulosa cells. 'Light' and 'dark' granulosa cells differ somewhat in morphology. Cytoplasmic organelles have become peripherally distributed in the oocyte, the inner cytoplasm being filled with long filaments or tubules averaging roughly 150 Å in width. Electron-dense particles 250–300 Å in diameter are associated with the filaments and are thought to be large ribosomes. Electron-dense bodies measuring up to 1·8 μm in diameter, some of which contain lamellar membrane systems, are abundant in the peripheral cytoplasm.

4. Multilaminar follicles exhibit a varying ratio of 'light' to 'dark' granulosa cells. The filaments in the cytoplasm of the oocytes have more than doubled in width and exhibit a compartmented nature. Lamellar bodies, multivesicular bodies, and entities resembling lysosomes are present.

5. Follicles with antra: at least two types of granulosa cell are present, with intergrading between them. Lamellar bodies are no longer present in the cytoplasm of the oocyte. Lysosome-like bodies are aligned beneath the plasma membrane of the oocyte. Cytoplasmic filaments and associated electron-dense particles are still present.

The relation of certain methods employed to the results obtained is discussed.

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