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The differentiation and proliferation of follicle cells during oocyte growth in *Lacerta sicula*

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SUMMARY

The follicular epithelium of the lizard oocytes undergoes structural and morphological modifications throughout oocyte growth. During this process the number of follicle cells increases and the epithelium acquires a multilayered and polymorphic organization which is characterized by the appearance of large follicle cells (intermediate and pyriform cells). The number of large cells also increases during oocyte growth and this increase parallels that of small cells. However, only the small cells become labelled one hour after [^{3}H-]thymidine administration. Large cells have been found labelled after a longer period of time, i.e. 4–5 months after isotope injection.

All these results together indicate that large follicle cells arise from the differentiation of small cells.

INTRODUCTION

The follicular epithelium of the lizard *Lacerta s. sicula* Raf. undergoes morphological and structural modifications during oocyte growth. In oocytes 100 μm in diameter, the follicular epithelium is made up of a single layer of small cells. Subsequently, during oocyte growth, large round vesicular cells appear, so that the epithelium becomes polymorphic and multilayered. The follicular epithelium surrounding oocytes between 600 and 1500 μm in diameter maintains a polymorphic and multilayered organization. It contains small follicle cells, intermediate cells similar to the vesicular cells described above, and a new class of cells called, owing to their shape, pyriform cells (Trinci, 1905). At the beginning of yolk deposition, i.e. in oocytes exceeding 1500 μm in diameter, the intermediate and pyriform cells gradually disappear, so that the follicular epithelium reacquires a single-layered and homogeneous organization (Filosa, 1973).

With the light microscope (Trinci, 1905; Loyez, 1906; Regamey, 1935; Filosa, 1973) and with the electron microscope (E.M.) (Hubert, 1971*a*; Neaves, 1971; Taddei, 1972), the pyriform cells can be recognized in the follicular epithelium by their large nucleus, located in the enlarged body, and by an elongated apex pointing towards the oocyte surface. Furthermore, under E.M. the pyriform cells appear to be directly connected to the oocyte through intercellular bridges

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(Ghiara, Limatola & Filosa, 1968; Hubert, 1971*b*; Neaves, 1971; Taddei, 1972; Bou-Reslei, 1974). This is a remarkable feature of these cells, since in the vertebrates studied so far it is the only clear example of cytoplasm continuity between follicle cells and oocyte. The functional meaning of pyriform cells is not yet completely clear, but it has been inferred that they may have a nutritional role (Taddei, 1972; Andreuccetti, Taddei & Filosa, 1978).

According to most of the available data, these cells derive from the small follicle cells via intermediate cells (Trinci, 1905; Loyez, 1906; Hubert, 1971*a*). The only experimental evidence so far reported is based on histoautoradiographic data showing that only the small cells incorporate [^{3}H]thymidine, while this labelling is completely lacking in intermediate and pyriform cells (Hubert, 1973). However, these results are not sufficient to demonstrate the origin of the pyriform cells, since owing to the short-time labelling used, only the rapidly dividing cells were labelled. Furthermore, only large follicles were examined, and at this stage the follicular epithelium is already fully differentiated and organized.

In our opinion the recent finding (Hubert, 1977; Andreuccetti *et al.*, 1978) that small and intermediate cells are also connected to the oocyte through intercellular bridges is compatible with the hypothesis of a progressive differentiation of the small cells into pyriform cells, but does not demonstrate it, as claimed by Hubert (1977).

In order to investigate whether pyriform cells may originate from differentiation of small follicle cells we studied the increase in the number of follicle cells during oocyte growth and the incorporation of [^{3}H]thymidine in these cells. We also investigated the influence of gonadotrophin stimulation of the timing of the differentiation process.

MATERIALS AND METHODS

In *Lacerta sicula* Raf. the ovarian cycle lasts about one year (Filosa, 1973); adult animals were therefore collected in the neighbourhood of Naples at different periods of the year corresponding to the various periods of the ovarian cycle. Animals weighing about 14 g were used either in normal conditions or after stimulation by intraperitoneal injection of FSH (follicle stimulating hormone, porcine Calbiochem). The FSH was given every second day in doses of 200 μg/ml (specific activity 1 Armour unit/mg) (Angelini, D'Uva, Picariello & Ciarcia, 1978) and then killed after 3–6–10 injections. The ovaries were removed and fixed with ethanol/acetic acid (3:1 v/v), and processed for light microscopy.

Counting of follicle cells. The ovaries of animals were fixed and embedded in paraffin. Serial 6 μm-thick sections were stained with Feulgen. The oocyte diameter was measured on the largest oocyte section by an ocular micrometer. An estimate of the number of follicle cells present in the follicular epithelium was obtained by averaging the number of cells contained in each of the three sections close to the largest one. Since the largest cells do not exceed 20

μm in diameter, an interval of five sections between each of the three sections selected for counting was considered sufficient to avoid the mistake of counting the same cells twice. Since the thickness of the follicular epithelium around the oocyte is constant, this procedure gives a good estimate of the number of the follicle cells present in the follicle (see appendix).

The cells were grouped into two classes: one containing the small follicle cells, easily characterized by a small nucleus about 5 μm in diameter, and another including both intermediate and pyriform cells with a larger nucleus which can reach 15 μm in diameter. As a definite distinction between intermediate and pyriform cells was not possible, these were counted together.

Histoautoradiography. 100 μCi of thymidine methyl [^{3}H] (5Ci/mmole from Radiochemical Centre, Amersham) were injected intraperitoneally into adult animals. In some animals the treatment with labelled thymidine was followed 24 h later by an injection of 100 μl of 5 mM non-radioactive thymidine. The animals, in groups of three, were killed at various intervals (1, 3, 6 and 12 h and 1, 7, 30, 120 and 150 days) after the first injection. Experiments under the same conditions were also carried out by using either a lower (50 μCi) or a higher dose (150 μCi) of radioactive thymidine. Some animals were treated with a daily dose of 50 μCi of [^{3}H]thymidine for a week and then killed 6, 12 and 30 days later. The FSH-stimulated animals were given the [^{3}H]thymidine (100 μCi/injection) at the beginning or at the end of the hormone treatment and were always killed from 1 to 30 days after the beginning of the hormone treatment. The ovaries were removed, fixed, embedded in paraffin and 6 μm-thick sections were cut and mounted on gelatin-coated slides. The sections, after the removal of the paraffin, were treated with 5 % trichloroacetic acid at 5 °C for 5 min and coated with the photographic emulsion (NTB_2 Kodak). The slides were exposed for 5 days in light-proof boxes at 4 °C, developed in Dektol (Kodak), fixed in Unifix (Kodak), washed in running water and then stained with haemallumen and mounted with DPX.

As a control, some sections were treated for 4–6 h at 37 °C with deoxyribonuclease (0·2 mg/ml in buffer phosphate pH 7 with 3×10^{-3} M-$MgCl_2$) before being coated with the photographic emulsion.

RESULTS

(A) *Increase in the number of follicle cells during oocyte growth*

During oocyte growth the follicular epithelium gradually increases, reaching about 90–100 μm in thickness in the largest previtellogenetic follicles (1500 μm in diameter); then, at the beginning of yolk deposition, it rapidly decreases as the pyriform cells disappear.

Fig. 1 shows that the number of small follicle cells increases almost linearly until the oocyte reaches a diameter of 1300 μm. After this stage, the number of cells slightly decreases. The enlarged cells appear for the first time around oocytes

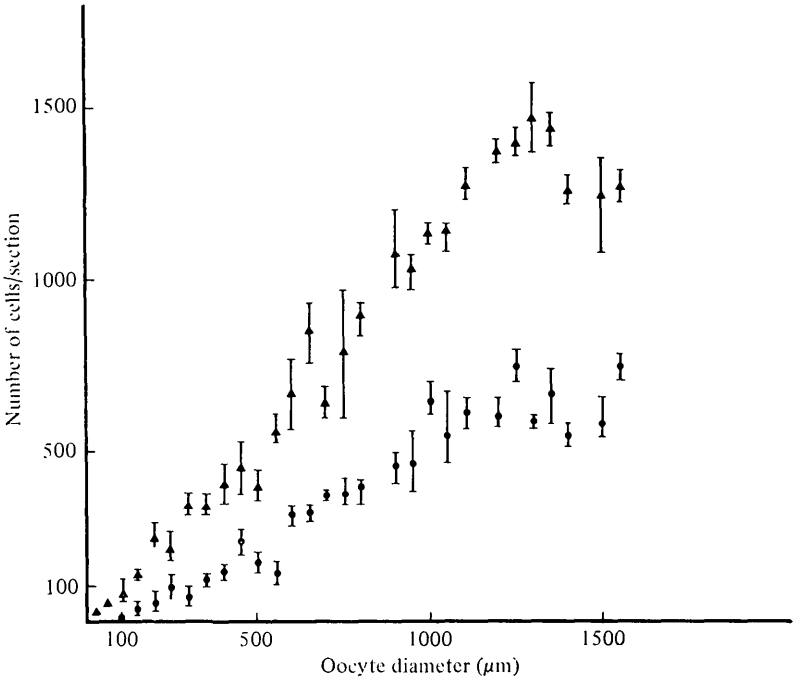


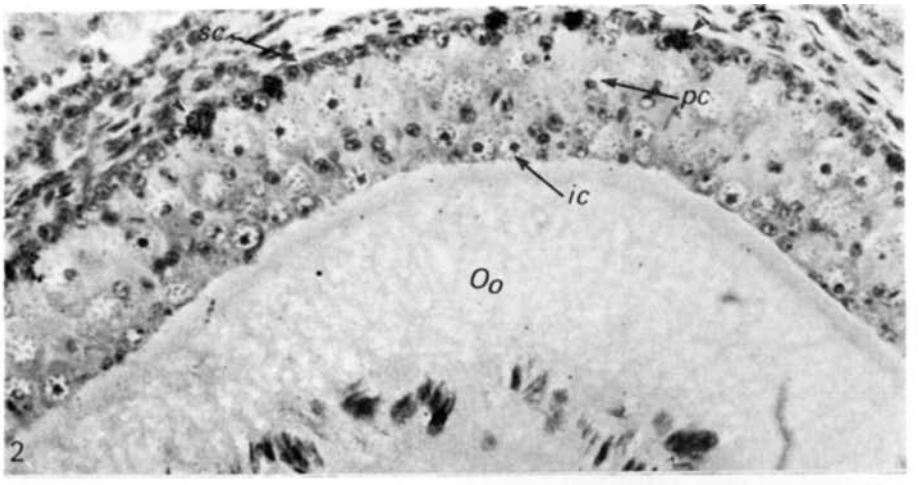
Fig. 1. *Relation between number of follicle cells and oocyte diameter.* Abscissa: oocyte diameter (μm); ordinate: number of cells/section. The cells were counted as described under Methods. The triangles refer to the number of small follicle cells, the circles to the number of pyriform and intermediate cells. Each point represents the mean value measured in 10 oocytes. The bars indicate the dispersion of the single measurements.

FIGURES 2–4

Fig. 2. *Histoautoradiography of the follicular epithelium of an oocyte* 1200 μm *in diameter.* The animal received a single dose of 100 μCi of [^{3}H]thymidine followed one day later by a dose of 100 μl of non-radioactive thymidine (5 mM) and was killed after 5 days. The follicular epithelium contains small follicle cells (*sc*), intermediate cells (*ic*) and pyriform cells (*pc*). The radioactivity is present only in the nuclei of the small follicle cells (arrow heads) of the outer layer. Note the absence of radioactivity on the intermediate and pyriform cells. Oocyte cytoplasm (*Oo*). × 350.

Fig. 3. *Histoautoradiography of the follicular epithelium of an oocyte 1200 μm in diameter.* The animal received a daily dose of 50 μCi of [^{3}H]thymidine for a week and was killed 6 days later. Note the higher number of labelled cells (arrows) of the outer layer as compared with that of Fig. 2. × 350.

Fig. 4. *Histoautoradiography of an oocyte from an animal treated with [^{3}H]thymidine and FSH.* The animal received three injections of FSH, one dose of 100 μCi of [^{3}H] thymidine after the third injection of FSH and was killed after 3 days. A large number of small follicle cells (arrow) of the outer layer is labelled. × 140.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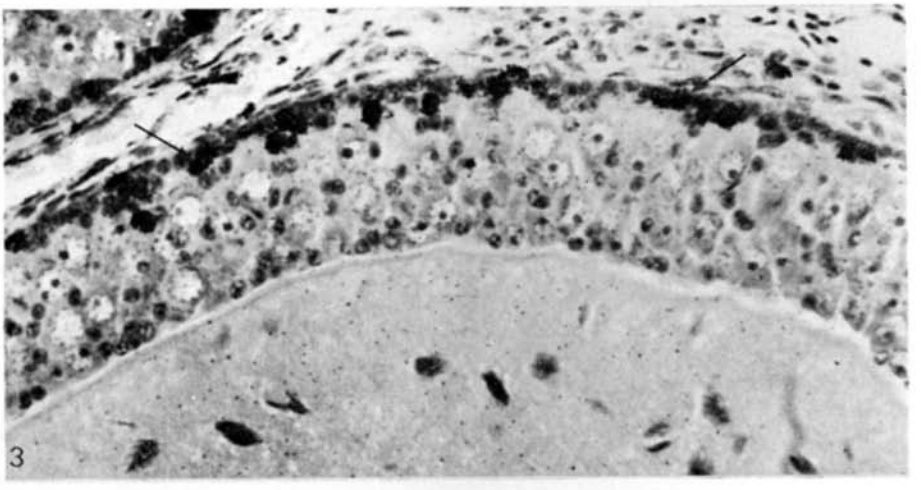
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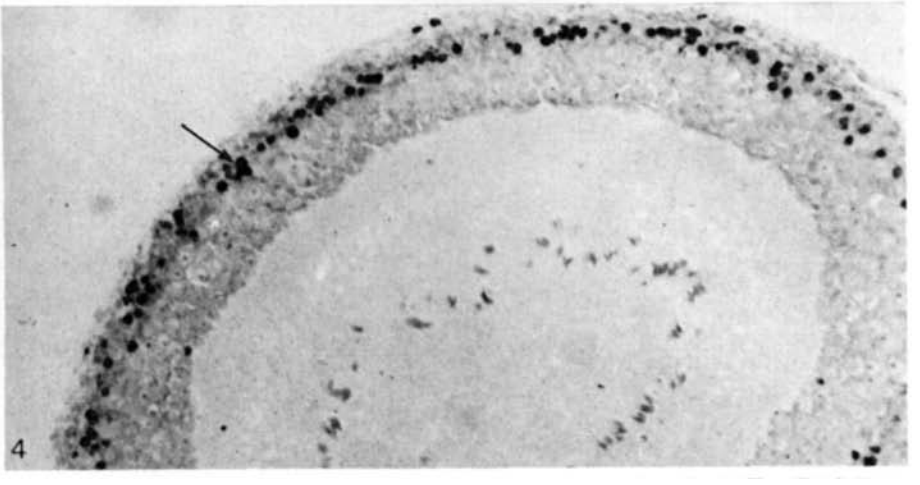
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100 μm in diameter; their number also increases throughout oocyte growth, reaching a plateau when the oocytes are about 1000 μm in diameter.

(B) *Labelling of follicle cells with [^{3}H]thymidine*

In all the experiments the [^{3}H]thymidine-labelled nuclei were clearly distinguishable from unlabelled ones by the conspicuous deposition of the grains (more than 50/nucleus). Furthermore the specificity of the labelling was checked by its sensitivity to DNAse treatment as described under Methods.

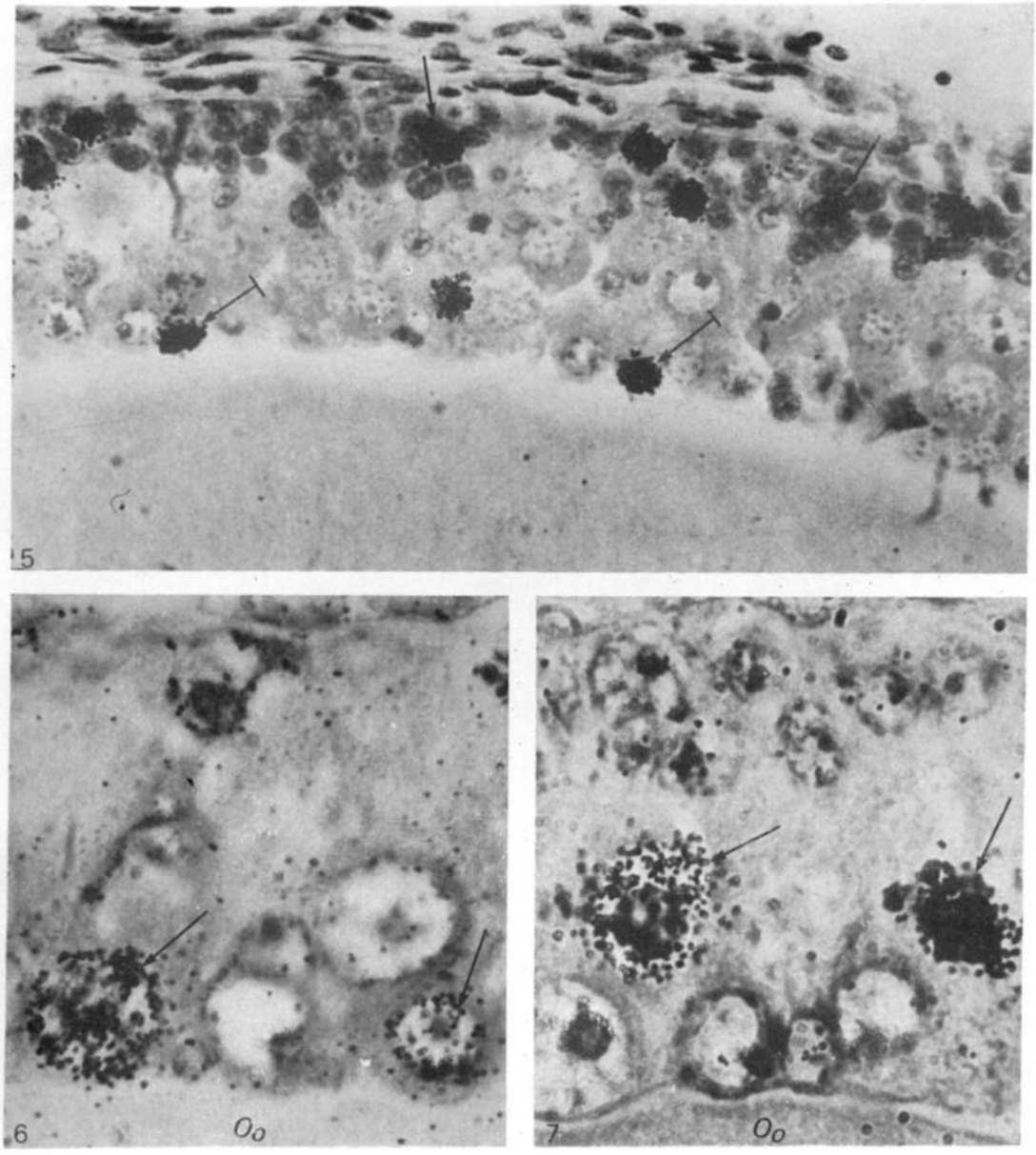
Autoradiographs of the sections through the follicular epithelium of animals treated with 100 μCi of [^{3}H]thymidine showed that small follicle cells located just beneath the connectival theca became labelled 1 h after isotope injection, while small follicle cells of the inner layer, intermediate cells and pyriform cells showed no trace of radioactivity (Fig. 2). Labelling exclusively on the small peripheral follicle cells was observed in all the follicles, regardless of the stage of oocyte growth and the ovarian cycle. The same result was obtained using the lower (50 μCi) or the higher (150 μCi) dose of [^{3}H]thymidine.

In another set of experiments one group of animals which received a single dose (100 μCi) of [^{3}H]thymidine was killed after a longer period (from 3 h up to 1 month). A second group received a daily dose of [^{3}H]thymidine for a total of 7 days and was killed at the same intervals as the former group. The results of these experiments showed that also in the animals killed within 7 days after the isotope injection, only the small follicle cells of the outer layer were labelled. Similar results were obtained when the animals were treated with daily doses of [^{3}H]thymidine, the only difference being that a larger number of follicle cells was labelled (Fig. 3). In animals which were killed between 7 and 30 days after the isotope injection, the radioactivity was present not only in the small follicle cells of the outer layer, but also in the small cells located inside the follicular epithelium and in those in contact with the oocyte surface (Fig. 5). The intermediate and pyriform cells were never labelled in any of these experiments.

To find the conditions under which pyriform cells could be labelled, we extended the period of exposure to [^{3}H]thymidine up to 4–5 months. In these experiments, the animals received the isotope injection in two doses given at an interval of one day. The treatment began in August, as in this month the ovary contains only small oocytes (less than 1000 μm in diameter) and the number and size of previtellogenic oocytes increase during the following months (Filosa, 1973).

Intermediate cells were labelled for the first time in the ovaries of animals killed 4 months after the isotope injection (Fig. 6). Pyriform cells were labelled 5 months after the isotope administration (Fig. 7). However, both intermediate and pyriform cells were labelled in follicles less than 1000 μm in diameter, while in large follicles only small follicle cells appeared labelled.

The same results were obtained with animals in which the specific activity of the isotope was reduced by a successive injection of unlabelled thymidine as described under Methods.



FIGURES 5–7

Fig. 5. *Histoautoradiography of the follicular epithelium of an oocyte of 900 μm in diameter.* The animal received a single dose of 100 μCi of [^{3}H]thymidine and was killed 21 days later. The labelling is located on the small cells (arrows) of the outer layer and in some cases on the small cells of the inner layers (arrows with bar). The intermediate and the pyriform cells are unlabelled. × 600.

Fig. 6. *Histoautoradiography of the follicular epithelium of an oocyte 450 μm in diameter.* The animal was treated with 100 μCi of [^{3}H]thymidine and killed 4 months later. Grains are present on some intermediate cells (arrows) in contact with the oocyte (*Oo*). × 1400.

Fig. 7. *Histoautoradiography of the follicular epithelium of an oocyte 700 μm in diameter.* The animal was treated as that of Fig. 6 and killed 5 months later. The grains are detectable on the nucleus of the large pyriform cells (arrows). × 1450.

(C) *Labelling of follicle cells in FSH-stimulated animals*

Animals were injected with gonadotrophin FSH and [^{3}H]thymidine as described under Methods. The hormone treatment resulted in a marked increase in the percentage of labelled small follicle cells (Fig. 4). In addition, some intermediate cells were also labelled in the follicular epithelium of oocytes 400 μm in diameter of animals which had received the isotope at the beginning of the hormone treatment and had been killed one month later. Large follicle cells were never found labelled in animals which had received the isotope at the end of the hormone treatment.

DISCUSSION

The results reported in this paper lend further support to the hypothesis that the pyriform cells in the follicular epithelium of lizard differentiate from small follicle cells (Trinci, 1905; Loyez, 1906; Hubert, 1971*a*).

The autoradiographic experiments show that intermediate and pyriform cells are labelled only after prolonged exposure to the labelled precursors. Indeed, large cells incorporate tritiated thymidine in their DNA 4–5 months after isotope injection, while small cells are labelled within the first few hours. These results cannot be explained as being due to a different growth rate of the two cell populations. In fact, the increase in the number of the large cells in the follicular epithelium parallels that of the small follicle cells. Therefore the only possible explanation of these results is that large cells differentiate slowly from a subpopulation of small cells.

The differentiation of large cells from the small ones and in particular the inability of the former to divide is also supported by the histophotometric data on the DNA content of the nuclei of follicle cells, which show that both intermediate and pyriform cells bear a diploid genome, while a portion of the small cells contains a higher amount of DNA (Olmo & Taddei, 1974). This result, together with the observation that the small cells can be rapidly labelled by [^{3}H]thymidine, leads to the conclusion that these cells only are able to divide within the follicular epithelium.

The observation that the first cells which incorporate [^{3}H]thymidine are located at the periphery of the follicular epithelium suggests that the proliferation of the small cells occurs in proximity to the connectival theca. This is also supported by the observation (Filosa, unpublished results) that colchicine-blocked mitotic cells are present only in this region.

The appearance of labelled follicle cells inside the follicular epithelium and close to the oocyte surface, long after the isotope injection, suggests that these cells migrate from the outer layer towards the oocyte surface. This migration takes approximately one month because it is only after this period that we have found labelled small follicle cells in contact with the oocyte. After cell contact with the oocyte has been established, the long time (3 months) required for the

small cells to differentiate into intermediate cells could be due to the morphological modification of the cells and to the formation of intercellular bridges recently observed between these cells and the oocyte (Andreuccetti *et al.*, 1978). These bridges are similar to those found between pyriform cells and oocytes (Ghiara *et al.*, 1968; Hubert, 1971*b*; Neaves, 1971; Taddei, 1972; Bou-Reslei, 1974).

The further differentiation of intermediate cells into fully differentiated pyriform cells proceeds more rapidly, taking about one month.

Moreover, our results show that the labelled intermediate and pyriform cells are detectable only in follicles less than 1000 μm in diameter, while labelled small cells are detectable also in larger follicles. These data suggest that the differentiation of small cells into large ones can occur only in follicles less than 1000 μm in diameter. In fact, the number of large cells does not increase after the follicles have reached 1000 μm in diameter, while the small follicle cells, which appear labelled also in larger follicles, increase in number until the follicles reach 1300 μm in diameter. The thickening of the follicular epithelium in this phase could be related to the increased number of small cells and to a further enlargement of the differentiated cells.

The time required for the differentiation of the follicle cells appears reduced in animals treated with FSH. Our results demonstrate that the hormone, beside inducing oocyte growth as previously reported by Licht (1970), stimulates the small cells' proliferation and accelerates their differentiation.

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APPENDIX

Let us consider a spherical follicle of radius r. We may divide the follicular epithelium surrounding the oocyte into small cubes of edge l with $l < < r$ (Fig. 8). If N is the number of cubes on the sphere the whole surface S is

$$S = Nl^2$$

and in first approximation

$$Nl^2 = 4\pi r^2. \tag{1}$$

Let us now consider the equatorial section of the sphere (Fig. 9). The length of the circumference is related to the number n of cubes in the section by the relation

$$nl = 2\pi r. \tag{2}$$

By substituting l from (2) into (1) we have a relation between N and n,

$$N = \frac{1}{\pi} n^2. \tag{3}$$

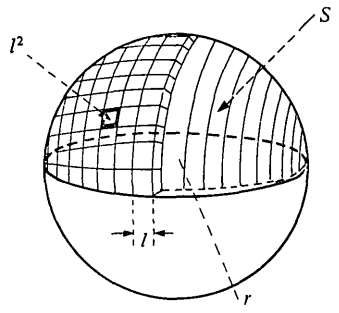

Fig. 8

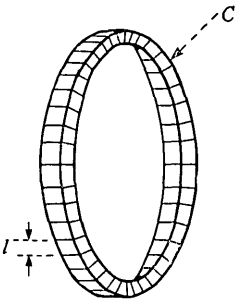

Fig. 9

FIG. 8 Schematic representation of a spherical follicle of radius r with the epithelium subdivided into N cubes of edge l.

FIG. 9 Equatorial section of the sphere of Fig. 8.

Let x be the number of cells in the unit volume. Therefore the total number of cells C_t in the follicular epithelium is

$$C_t = Nl^3x.$$

The number of cells C_s in the section is

$$C_s = nl^2x$$

hence

$$C_t = \frac{N}{n} l C_s = \frac{nl}{\pi} C_s.$$

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