DETERMINATION OF FOLLICLE GROWTH RATE IN THE OVARY OF THE IMMATURE MOUSE

TORBEN PEDERSEN

Finsen Laboratory, Finsen Institute, Copenhagen 0, Denmark

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Summary. The cell population kinetics of the ovary in 28-day-old mice were investigated. A method to determine growth rates of follicles is described, and the results obtained are discussed.

The granulosa cells in each follicle were considered to represent a single cell population. The time taken to double such a cell population was calculated from the labelling index of the granulosa cells and the duration of the synthesis phase of the single cells. Both parameters have been determined in autoradiographs prepared at different time intervals after the injection of tritiated thymidine.

Different stages of follicle development were classified according to the number of their granulosa cells. The actual time taken to grow to different stages was calculated from the time taken to double the number of the granulosa cells in these stages. It was shown that medium follicles grow at a slower rate than large follicles. A small follicle with one layer of granulosa cells took 16 days to develop into a large one with several layers and antrum formation.

INTRODUCTION

Several methods have been used to investigate follicle growth in the mouse ovary. The number of follicles and the distribution of the different developmental stages can be determined by histological investigation (Brambell, 1928; Engle, 1931), but this method does not give any information on the dynamics of follicle growth. Bullough (1942) attempted to determine the growth rate of follicles by measuring the mitotic index in follicles of different sizes. He found a correlation between an increase in the mitotic index and an increase in the size of the follicles.

Recently, the technique of autoradiography after pulse labelling with tritiated thymidine has been applied to follow cell movements in the ovary (Peters & Levy, 1966). Using this method, it is possible to distinguish between growing, i.e. proliferating cell populations, and cell populations which are not growing, non-proliferating cell populations. It is also possible to determine the growth rate of different structures in the ovary under various physiological conditions.

It is the purpose of this paper to describe the cell population kinetics in the
mouse ovary at a fixed age, and to report on a method by which growth rates of follicles can be measured.

**Principles and definitions**

A distinction between the cell populations of the ovary can be made from autoradiographs prepared shortly after the injection of labelled thymidine, which is incorporated into all cells that are in the S-phase of the mitotic cycle, i.e. synthesizing DNA. Cell populations with no labelled nuclei are considered as non-proliferating.

The distribution of labelled cells within an organ or within a cell population represents the labelling pattern.

In order to be able to discuss their growth, the follicles were classified according to their stage of development. These stages are characterized by increasing numbers of granulosa cells around the oocyte. The follicles were therefore classified according to the number of cells surrounding the oocyte in a representative section of the follicle. The largest cross-section was chosen as such a section (Pedersen & Peters, 1968). Text-fig. 1 shows the different follicle types defined by the number of granulosa cells in the largest cross-sections.

The growth rate of follicles was defined as the time it took for a follicle to grow from one stage of development to the next, or from one type of follicle to another. The growth rate of a given follicle can be determined if (a) the total number of cells in the follicle, and (b) the 'doubling time', i.e. the time it takes for the cells to double their number, is known. The total number of
cells in follicles of different types can be calculated from the number of cells in the largest cross-sections and the diameters of the follicles. The largest cross-section is for practical purposes identical with the cross-section of a follicle in which the nucleolus of the oocyte is seen. The doubling time can be measured in different ways. In this investigation, it was calculated from two parameters: (1) the labelling index (LI) of granulosa cells after pulse labelling, i.e. the number of labelled granulosa cells expressed as a percentage of the total number of cells in the largest cross-section of the follicle determined in autoradiographs prepared 1 hr after injection of labelled thymidine, and (2) the length of the S-phase of the granulosa cells, determined in autoradiographs prepared at different time intervals after pulse labelling.

The doubling time is an expression of the growth rate of a cell population. The growth rate of a single cell can be expressed as the generation time, i.e. the time it takes a cell to go from one mitosis to the next, or from one point in the cell cycle to the same point in the next.

Under certain conditions, the doubling time of a cell population is identical with the generation time of its individual cells. This is true if the growth fraction is 1, i.e. all cells in the population are producing new cells by mitosis, and if the generation time of the granulosa cells is constant from cell to cell. Because the growth fraction in a follicle could not be determined with accuracy in these experiments, all calculations of growth rates of follicles are based on the determinations of doubling times of the granulosa cell population rather than of generation times of the individual granulosa cells.

MATERIAL AND METHODS

Experiment A

This experiment was designed to distinguish between proliferating and non-proliferating populations in the ovary, and to determine the LI of different follicles:

Five mice of the Bagg strain, 28 days old, each received intraperitoneally 100 µc [³H]thymidine (Amersham Radiochemical Centre, specific activity ranging from 3.6 c/m-mole to 5.0 c/m-mole). All injections were given at some time between 09.00 and 13.00 hours. The animals were killed 1 hr after injection.

The age of 28 days was chosen, because, at this time, all follicle types except pre-ovulatory ones are already present in the ovary, but cyclic events which could influence the results have not yet started.

Experiment B

This experiment was designed to determine the length of different phases of the mitotic cycle:

Fifty mice, 28 days old, each received 50 µc [³H]thymidine intraperitoneally. They were killed at different time intervals after the injection, ranging from 1 to 48 hr.
**Autoradiographic procedure**

The ovaries were fixed in Bouin's solution for 20 to 30 min and embedded in paraffin. All ovaries were serially sectioned at 5 µ and, after coating with liquid photographic emulsion, Ilford K 2, the sections were exposed at 4° C for 1 to 4 weeks. After development, the sections were stained with haematoxylin and eosin.

**Counting procedure**

The LI of a follicle can be determined as the number of labelled cells expressed as a proportion of the total number of cells on that cross-section of a follicle in which the nucleolus of the oocyte is seen.

In Exp. A, every fifth section was examined. If such a nucleolus was seen in a follicle, the total number of cells as well as the number of labelled cells in the cross section of this follicle were counted and the LI calculated from these values. For every follicle type, a mean LI was calculated.

In Exp. B, the length of the S-phase of granulosa cells was determined by the method of labelled mitosis (Howard & Pelc, 1953; Quastler & Sherman, 1959). Labelled and unlabelled mitotic figures were counted in every specimen. As medium and large follicles only have few cells in mitosis, it was necessary to examine several follicles of the same type to find enough mitoses for the calculations. Twenty-five mitoses were counted in medium follicles, and 100 mitoses in large follicles. The percentage of labelled mitoses was calculated separately for every type of follicle at different time intervals after the injection.

In both experiments, cells were considered as labelled if they had 4 or more grains over the nucleus. The background labelling never exceeded 0.5 grains/100 µ². All counts were done under oil immersion.

**RESULTS**

**Labelling pattern**

The ovaries of 28-day-old mice injected with [³H]thymidine were found to contain structures in which labelled cells were seen, and others, that had none or very few labelled cells. Many labelled cells were seen in follicles above a certain size, and also in the surface epithelium. The stroma, however, was almost free of labelled cells and presumably constitutes a non-proliferating cell population. Only a few endothelial cells and histiocytes might have become labelled.

The proportion of labelled cells in the follicles varied according to their stage of development. Only a few cells in the small follicles were labelled. In the small follicle of Type 2, for example, labelled cells were rarely seen. About a third of the Type 3a follicles with one complete ring of granulosa cells, contained one or more labelled cells in the largest cross-sections.

Medium and large follicles, however, contained labelled cells in every cross-section of each follicle, but the labelling pattern and the proportion of labelled cells, the LI, differed. In the medium type follicles, only a small proportion of the cells scattered throughout the follicles was labelled and these
Fig. 1(a). Autoradiograph of an ovary from a 28-day-old mouse. In the centre, medium-sized follicles with few labelled cells are seen in cross-section. On the outside, large follicles are seen; they are not cut through their maximum diameter. ×75.

Fig. 1(b). Same section as in Fig. 1(a) (dark field illumination) in which only the grains over the labelled nuclei are seen.

Fig. 2(a). Autoradiograph of an ovary from a 28-day-old mouse, showing the labelling pattern of a Type 5b and a Type 6 follicle. Many labelled granulosa cells are seen in these follicles. A degenerating follicle with very few labelled cells is seen in the centre. ×75.

Fig. 2(b). Same section as in Fig. 2(a) (dark field illumination).

Fig. 3(a). Labelled granulosa cells are located around the oocyte and the cavity of a Type 7 follicle. ×75.

Fig. 3(b). Same section as in Fig. 3(a) (dark field illumination).
were found just as frequently in the inner as in the outer layers (Pl. 1, Figs. 1a, b).

In Type 6 and Type 5b follicles (many layers of granulosa cells, with and without follicle fluid respectively), a high proportion of labelled cells was found randomly distributed throughout the follicles (Pl. 1, Figs. 2a, b).

There were still many labelled cells in every cross-section of Type 7 follicles, but these were mainly located close to the antrum and around the oocyte. In the periphery of the follicle, only a few cells were labelled (Pl. 1, Figs. 3a, b).

The labelling pattern of the degenerating follicles was quite different. A degenerating follicle may be defined as showing one or both of the following criteria: (1) a shrunken oocyte, (2) more than 5% pyknotic degenerating granulosa cells in a cross-section.

Degenerating medium-sized follicles were rarely or never seen in the ovary of 28-day-old mice, but many large follicles were seen in more or less advanced stages of degeneration. Large follicles in early degeneration still had labelled cells, but fewer than the 'healthy' follicles of the same type. At a late stage of degeneration the cells no longer incorporate \(^{3}\text{H}\)thymidine.

The labelling pattern of theca cells varied from that of granulosa cells. In Type 3b and Type 4 follicles, the theca layer was poorly developed, consisting of only one layer of cells, and few of these were labelled. The theca around Type 5a and Type 5b follicles consisted of several layers of cells, many of which were labelled, while only a few labelled theca cells were seen around Type 6 and Type 7 follicles. In the haematoxylin–eosin stained sections used in this study, the delimitation between the theca layer and follicle cells was well defined, but there was no clear separation between the theca layer and the stroma. It was therefore not possible to count labelled and unlabelled theca cells to determine the LI, or to calculate their growth rates. Although an exact measurement could not be obtained, there was no doubt that more theca cells were labelled in follicles of Types 5a and 5b than in other types, indicating that the theca layer grew faster in these follicles than in other types.

**Labelling index**

The relation between follicle size, represented by the number of cells on the largest cross-section of each follicle, and LI is shown in Text-fig. 2. In the medium-sized follicles about 8 to 10% of the cells were labelled, while 35 to 40% of the cells were labelled in the large follicles.

The number of follicles examined, and the distribution of their different types is recorded in Table 1. As every fifth section of five ovaries was examined, these numbers represent the mean number of follicles present in one ovary at the age of 28 days. In addition, the mean LI of the different follicle types is recorded.

Type 7 follicles were not present in all ovaries of 28-day-old mice, but where present, the LI was found to vary considerably within the same follicle; the cumulus contained many labelled cells resulting in a high LI, while the periphery showed only few of the granulosa cells to be labelled. It seems that Type 7 follicles consist of two different cell populations, a central one which
is growing, and a peripheral one, which has ceased growing, or proliferates only at a slow rate.

**Determination of S-phase-lengths of granulosa cells**

The length of the S-phase, which is needed for the calculation of doubling time, was measured here by the method of labelled mitosis. The percentage of

![Graph](image)

**Text-fig. 2.** Labelling index of follicles in 28-day-old mice.

**Table 1**

<table>
<thead>
<tr>
<th>Type of follicle</th>
<th>No. of follicles ± S.D.</th>
<th>Total cells counted</th>
<th>No. of labelled cells</th>
<th>Mean of LI ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium-sized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>follicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>142 ± 20</td>
<td>5452</td>
<td>474</td>
<td>9.5 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>94 ± 13</td>
<td>7130</td>
<td>534</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>5a</td>
<td>60 ± 7</td>
<td>7897</td>
<td>1417</td>
<td>17.2 ± 0.9</td>
</tr>
<tr>
<td>Large-sized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>follicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5b</td>
<td>22 ± 4</td>
<td>6106</td>
<td>2431</td>
<td>38.7 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>19 ± 8</td>
<td>11780</td>
<td>3942</td>
<td>34.4 ± 1.4</td>
</tr>
</tbody>
</table>

labelled mitoses was determined at various time intervals after the injection of the label. By plotting these values against time after injection, a cyclic curve has been obtained. On this curve the duration of the different phases of the mitotic cycle can be measured (Quastler & Sherman, 1959; Takahashi, 1966). Text-fig. 3 shows two examples of such curves. The curve for Type 6 follicles
shows two waves of labelled mitosis. The second wave is not as well defined as the first one because of the variations in the rate at which cells progress through the cell cycle (Cleaver, 1967). The time interval represented by the distance between the peaks of the two waves, corresponds to the generation time. As the peak of the second wave is ill-defined, the generation time can only be approximately estimated. It averages 16 hr for cells in Type 6 follicles.

The first wave shown in both curves is asymmetrical. This is due to a variation in the duration of the S-phase in different cells in addition to the variation in the G2-phase (the post synthetic phase between S-phase and mitotic phase).

The labelled mitosis curve for Type 5b follicles is similar to the curve for Type 6 follicles, but that for Type 4 follicles has no well-marked second wave within the time interval investigated. The generation time could not, therefore, be estimated from the curve. Curves obtained for Type 3b and Type 5a follicles are similar to the curve for Type 4 follicles.

The duration of the S-phase \( (t_s) \) corresponds to the interval on the abscissa between the first and second 50% intercept (see Text-fig. 3). The minimum duration of the G2-phase corresponds to the time between injection and the first appearance of labelled mitosis.

The duration of the G2-phase + half of the duration of the mitotic phase \( (G_2 + \frac{1}{2}M) \) (see following section) giving a value \( (t_2) \) corresponding to the time interval between injection and the first 50% intercept, are needed for calculation of the doubling time.

The mean values obtained for \( t_s \) and \( t_2 \) are listed in Table 2.
The S-phase length of granulosa cells is considerably longer in medium follicles than in large follicles. The longest S-phase occurs in cells of the Type 4 follicle (average 11·2 hr) but it is only a little shorter in Type 3b and Type 5a follicles (9·8 and 10·3 hr respectively). When the follicles reach the stage of development corresponding to Type 5b, the duration of the S-phase is reduced to 6·8 hr and it remains at this level in Type 6 follicles.

The duration of $t_2$ is similar in medium follicles and large follicles.

The standard deviation (S.D.) of phase duration can also be measured on the curves. It can be shown that the slope at 50% points on the ascending and descending part of the first wave of labelled mitosis is inversely proportional to the S.D. of $t_2$ and S.D. of $t_2 + t_s$ respectively (Takahashi, 1966). For practical purposes, the interval on the abscissa between the 32 and 68% intercepts of the ascending part of the first wave represents the S.D. $t_2$. The interval on the abscissa between the 68 and 32% intercepts of the descending part of the first wave indicates S.D. $t_2 + t_s$. The S.D. $t_s$ can be calculated from:

$$\text{S.D. } t_s = [(\text{S.D. } t_2 + t_s)^2 - (\text{S.D. } t_2)^2]^\frac{1}{2}$$

The S.D. of the $t_s$ values shows that the $t_s$ of granulosa cells varies considerably in medium follicles in contrast to granulosa cells in large follicles.

Calculation of doubling times of granulosa cells

Every follicle is assumed to consist of a single, exponentially growing, cell population. To meet the requirements of exponential growth, the following conditions should be fulfilled (Cleaver, 1967): there should be no cell loss either from cell death or from cells leaving the population, and all cells produced by mitosis should remain within the population. Cells in ‘healthy’ follicles fulfil these requirements: they do not wander through the theca layer, there is no obvious loss of cells by degeneration, and all cells produced by mitosis remain in the follicle.

The doubling time of an exponentially growing cell population can be calculated from the following formula (Cleaver, 1965):

$$LI = (\exp. \frac{t_s}{T_D} \ln 2 - 1) \exp. \frac{t_2}{T_D} \ln 2$$

where $T_D$ = the doubling time, $\ln = \log_e$ and $\ln 2 = 0·693$.

The doubling time of granulosa cells belonging to different types of follicles varies considerably (Table 3).

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**Table 2**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Type 3b (hr ± S.D.)</th>
<th>Type 4 (hr ± S.D.)</th>
<th>Type 5a (hr ± S.D.)</th>
<th>Type 5b (hr ± S.D.)</th>
<th>Type 6 (hr ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_2$</td>
<td>2·0±0·4</td>
<td>2·0±0·4</td>
<td>2·0±0·4</td>
<td>1·8±0·4</td>
<td>1·8±0·4</td>
</tr>
<tr>
<td>$t_s$</td>
<td>9·8±1·5</td>
<td>11·2±2·9</td>
<td>10·3±2·3</td>
<td>6·8±0·8</td>
<td>6·8±0·8</td>
</tr>
</tbody>
</table>

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In contrast to medium follicles, all granulosa cells in large follicles remain 'healthy'. However, the number of 'healthy' cells may vary considerably in the same follicle. In 'healthy' and 'latent' follicles the number of 'healthy' cells is only about 10 times larger than the number of 'healthy' cells in 'latent' follicles. The number of 'healthy' cells in large follicles is remarkably the same as in medium follicles. This is also true of the number of 'healthy' cells in large follicles.
Determination of total number of cells in follicles

To determine the time it takes a follicle of a given type to grow to another type, it is necessary to know the total numbers of cells in different follicle types. These can be calculated from the number of cells in the largest cross-section and the diameters of the oocyte and the whole follicle. The calculations are described in detail in the appendix.

The number of cells counted in the largest cross-sections and the calculated total number of cells of the corresponding whole follicles, are recorded in Table 4.

### Table 3

**Doubling times of granulosa cells in different types of follicles, and growth rate of follicles expressed as their transit time**

<table>
<thead>
<tr>
<th></th>
<th>Type 3b (hr)</th>
<th>Type 4 (hr)</th>
<th>Type 5a (hr)</th>
<th>Type 5b (hr)</th>
<th>Type 6 (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doubling time of granulosa cells</td>
<td>76</td>
<td>110</td>
<td>46</td>
<td>15.4</td>
<td>17.0</td>
</tr>
<tr>
<td>Transit time of follicles</td>
<td>167</td>
<td>126</td>
<td>56</td>
<td>23</td>
<td>14</td>
</tr>
</tbody>
</table>

### Table 4

**The relation between number of cells on the largest cross-section and total number of cells in follicles**

<table>
<thead>
<tr>
<th></th>
<th>No. of cells on largest cross-section</th>
<th>Total no. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 3b</td>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>410</td>
</tr>
<tr>
<td>Type 4</td>
<td>80</td>
<td>635</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>905</td>
</tr>
<tr>
<td>Type 5a</td>
<td>150</td>
<td>1420</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>2100</td>
</tr>
<tr>
<td>Type 5b</td>
<td>300</td>
<td>3000</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>5950</td>
</tr>
<tr>
<td>Type 6</td>
<td>500</td>
<td>8050</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>10400</td>
</tr>
<tr>
<td>Type 7</td>
<td>800</td>
<td>15600</td>
</tr>
</tbody>
</table>

**Calculation of follicle growth rate**

During its development, every follicle undergoes a transition from a type with a certain number of cells to another one with a larger number of cells. The time a follicle takes to move from one type to another is defined as the transit time. In reality, the doubling time of granulosa cells varies gradually as the follicles grow, but in the present calculations the doubling time was assumed to be constant for a given type of follicle. The mean doubling time of granulosa cells in Type 4 follicles is 110 hr (Table 3). The time it takes for 410 cells to become 905 cells, i.e. the transit time, t, can be calculated from the formula:

\[ N_t = N_0 \exp \left( \frac{t}{T_D} \right) \]
$N_o =$ minimum number of cells corresponding to a follicle type
$N_e =$ maximum number of cells corresponding to the same follicle type

The calculated transit times for follicles of different types are recorded in Table 3. The variation in the transit times is considerable. An early Type 3b follicle with 90 cells needs 7 days to grow to a follicle with 410 cells, while the growth of a Type 6 follicle with 5950 cells to one with 10400 cells takes only about 14 hr. It can also be seen that the time a follicle remains as a medium-sized follicle is about 14 days, but that the growth through the large types only takes about $1\frac{1}{2}$ days.

The doubling time of granulosa cells in follicles of the same type does not vary between the ages of 21 and 35 days (Pedersen, unpublished data). This means that the transit time of a certain type of follicle is constant within the same period. It is therefore possible to calculate the time it takes for an early medium-sized follicle to grow to a large follicle by adding the calculated transit times. Table 3 shows that during the juvenile period, it takes an early Type 3b follicle (with 20 cells in its largest cross-section) about 16 days to develop to a Type 6 follicle (with 600 cells in its largest cross-section).

**DISCUSSION**

Most of the small follicles in the 28-day-old ovary are of Type 2 and a smaller proportion of Type 3a. Follicles of Type 1, ‘naked oocytes’, are no longer seen at this age. Only a few Type 2 follicles incorporate $[^{3}\text{H}]$ thymidine into their granulosa cells; the LI of the granulosa cells is low. This could suggest that all granulosa cells in Type 2 follicles are growing, but at an extremely slow rate. In this condition, the cells would have to have a very long generation time, and specifically a long G\textsubscript{1}-phase compared to the S-phase. Alternatively, it could suggest that granulosa cells of most Type 2 follicles are non-proliferating. The latter possibility seems more likely as, even in old mice, Type 2 follicles are still found in the ovaries, suggesting that these follicles have not grown, but have remained unaltered in the ovary since the immature period. It therefore seems that the group of Type 2 follicles in the 28-day-old ovary of the mouse is not homogeneous, but can be divided into two groups: one group, a resting stage, in which neither the oocyte nor the accompanying granulosa cells grow, and another group, in which both the oocyte and the granulosa cells have started to grow. Labelled granulosa cells in Type 2 follicles are found in the latter group.

One third of the small follicles of Type 3a contained labelled cells in their largest cross-sections. This does not necessarily imply that follicles with no labelled cells in their largest cross-sections are in a ‘resting’ stage, as labelled cells are often found in other sections of these follicles. The total number of cells in a Type 3a follicle never exceeds ninety and only a small fraction of these are scanned for labelling, when the largest cross-section is examined. Moreover, as the proportion of labelled cells in this follicle type is low, not every large cross-section examined will contain labelled cells. It seems probable that all Type 3a follicles are growing. None is in a ‘resting’ stage, and all have a low LI, indicating a slow growth rate.
In medium-sized follicles of the same size, the labelling indices are the same. This indicates that all medium follicles are growing, and that follicles of the same size grow with the same speed. Medium follicles are seldom seen degenerating at this age, which suggests that all follicles which have reached the Type 3b stage of development will continue to grow further until they have reached Type 5b or later developmental stages.

It has been suggested that follicles of Type 3a and 3b in the mature mouse ovary could remain unaltered in size for a long time, suggesting an inactive stage (Peters & Levy, 1966). In the immature mouse ovary, no resting follicles of these types are seen. It seems that all Type 3a follicles grow, even if only slowly, while all Type 3b follicles are in a continuous state of development.

That large follicles are also in a continuous state of development is inferred from the fact that they contain many labelled cells.

Ovulation has not yet started in 28-day-old mice, and large follicles, which have reached the stage of Type 6 or Type 7 cannot develop further. Such follicles do not remain in the ovary as healthy, resting follicles (large unlabelled follicles were never seen), but degenerate. Medium follicles contain a growing oocyte, while the oocytes in large follicles have reached their final size, but it is not known if it is the presence of a growing oocyte that protects the medium follicle against degeneration.

The LI is much higher in large follicles than in medium ones. For example, the LI of a Type 4 follicle is only one fourth of that of a Type 5b follicle (cf. Table 1). The length of the S-phase of the granulosa cells of Type 5b follicles is shorter than in cells of Type 4 follicles. Both changes lead to a reduction in the doubling time, with the result that cells in Type 4 follicles take 110 hr to double their number while cells in Type 5b follicles only need 15.4 hr.

The change in the growth characteristics of the granulosa cells is accompanied by a simultaneous increase in the growth rate of the theca layer. This increase in the growth rate of the theca layer is only of short duration, as indicated by a decrease in the number of labelled theca cells around Type 6 follicles, while the granulosa cells continue to grow rapidly in Type 5b and Type 6 follicles.

The marked differences in the doubling time of granulosa cells in medium- and large-sized follicles could be due to variations in the generation time alone, or could be due to a combined effect of variations in the generation time and in the growth fraction. That the growth fraction in the large follicles is 1, i.e. all cells are proliferating, is suggested by the fact that the generation time of single granulosa cells measured on the labelled mitosis curve is about the same as the calculated doubling time for the granulosa cells (16 to 17 hr).

The doubling time would be identical with the generation time if all cells were proliferating. If, however, only a part of the cells were proliferating (a growth fraction below 1), the generation time of individual cells would be shorter than the doubling time of the whole population. This seems to be the case in medium-sized follicles. In the labelled mitosis curve, the descending part of the first wave flattens out and remains on a level of 30 to 40% labelled mitosis for several hours. There is even an increase in the percentage of labelled mitosis to about 50% at 20 to 25 hr after the injection (this part of the curve
is not seen in Text-fig. 3), although a peak is never seen. This high level of labelled mitosis in the interval from 14 hr to about 25 hr after the injection may represent a second wave of labelled mitosis. The wave is flattened out, probably due to large variations in the generation time of the individual cells. The mean generation time therefore seems to be of the order of magnitude of 20 to 25 hr, which is much shorter than the doubling time. This suggests that in medium-sized follicles only a small part of the granulosa cells are proliferating and that the growth fraction is below 1.

The variations in the doubling time are responsible for the variations in transit time for different types of follicles. It can be seen that follicles spend about 14 days in the ovary as medium-sized follicles, developing from early Type 3b to Type 5a, but that they grow through the stages of Types 5b and 6 in less than 2 days, and can even start on the degenerative process within this time interval. Paesi (1949) found almost the same relationship between the growth rates of follicles by determining the frequency curve of follicles of different sizes.

The mitotic index of granulosa cells in follicles of different sizes has been investigated in mature mice by Bullough (1942), who found that it increases with increasing size of the follicles. He concluded that large follicles grow much faster than medium ones.

The growth of follicles can be summarized as follows: Type 2 follicles can be divided into two groups, the smaller of which consists of follicles which already have started on their development, while the larger group contains follicles in a ‘resting’ stage. The follicles remain in this group for an undefined period of time and then start to grow. The group represents a pool from which follicles develop throughout the lifetime of the mouse, and which is not exhausted even in old age.

All Type 3a follicles are growing at a slow rate.

Follicles of Types 3b to 6 inclusive are all growing, but at different rates. When medium follicles pass through the transitory stage of Type 5a, their growth rates are speeded up. In large follicles more granulosa cells divide, and divide more often compared with granulosa cells in medium-sized follicles. The doubling time of the granulosa cells decreases when the follicles grow from medium to large follicles. This decrease is due to a shorter generation time of single cells plus an increase in the growth fraction.

**APPENDIX**

*Determination of total cell numbers in follicles*

The total number of cells in the follicles of different types must be known in order to determine the growth rates of follicles. By making certain assumptions, it is possible, to calculate the total cell number in a follicle from the number in the largest cross-section and from the diameters of the follicle.

The assumptions are that the follicle itself, the oocyte and the nucleus of the single granulosa cells are spherical. If the diameters of the follicles are determined as the mean of two diameters at right angles to each other, the error introduced by this assumption is minimal.
Follicle growth rate in the immature mouse

The space between the oocyte and the theca layer forms a spherical shell with an inner radius (r) and an outer radius (R). These radii are measured for the largest-cross section (the median cross-section of the shell).

The volume of the shell \( V_1 \) is calculated from

\[
V_1 = \frac{4}{3} \pi (R^3 - r^3)
\]

The section thickness (f) is 5 \( \mu \). The volume of the cross-section of the shell \( V_2 \) is calculated from

\[
V_2 = \pi f(R^2 - r^2)
\]

The number of cells (n) counted in the largest cross-section should be multiplied by a correction factor \( (k) \), which depends on the radius of the nuclei of the cells and on the section thickness (Pedersen, B. personal communication; Haug, 1967). The factor \( k = 2r_c + f + 2p \), where \( r_c \) represents the mean radius of the nucleus of the granulosa cells. When nuclei have been tangentially sectioned, it is only possible to recognize them when the section is of a certain size. The \( p \) value represents the height of the smallest segment of a nucleus which is recognizable, and \( p \) has been found to average 0.6 \( \mu \). The mean radius of granulosa-cell-nuclei has been estimated by measuring 200 cells, and was found to average 3.2 \( \mu \). The value of the correction factor can then be calculated to 10.2.

The number of cells in the entire follicle \( (N) \) can then be calculated from

\[
N = \frac{n}{kV_2} fV_1
\]

From determinations of the total number of cells in 100 different follicles, it has been possible to evaluate the relation between the number of cells in the largest cross-section and the total cell numbers in a follicle (Table 4).

REFERENCES


