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Histological Studies on Onset of Meiosis and on the Time of Appearance of Follicles in Different Stages in Immature Rabbit Ovaries

Masao SASABE, Atsushi FUKUNARI, Teruo MAEDA,
Takato TERADA and Yoshio TSUTSUMI

Animal Reproduction Laboratory, Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 724, Japan

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Abstract Progress of the prophase in meiosis I and time of the first appearance of follicles in each developmental stage in ovaries of young rabbits were investigated histologically. Oocytes in leptotene and zygotene were located in 5.6% and 1.4% of all ovaries of newborn rabbits, respectively, indicating that some transition of oogonia to oocytes had occurred at day 0. The percentage of oocytes in diplotene was very high (86%) on day 21, and this stage had been completed by day 28. Follicles of Type 2 (in the classification by PEDERSEN and PETERS, 1968) appeared first in ovaries of 7-day-old rabbits. Follicular formation was initiated on day 7 when oocytes within the follicles reached the stage of diplotene or the transitional phase between pachytene and diplotene. The first small antral follicles were recognized in ovaries on day 70, meaning that formation of such follicles started between day 57 and day 70, though atretic follicles were frequently found and secondary interstitial tissue appeared at this age. Fully developed follicles (Type 8) appeared in one of two does on day 107. Through all stages of follicular growth, more developed follicles were located generally in the deep ovarian cortex adjoining the medulla and containing well developed blood vessels.

INTRODUCTION

The oogonia in most mammals enter meiotic prophase during fetal life and the transition into oocytes is almost completed by the time of birth (FRANCHI *et al.*, 1962). In some species, including ferret and mink (BYSKOV, 1975) and golden hamster (SHALLONER, 1975), it has been confirmed that meiosis begins just after birth and oogonia accomplish the transition into oocytes during the early neonatal period. In rabbits, oocyte development takes place entirely in the post-natal period and completes up to diplotene by 21 days of age. Then soon after completion of the diplotene stage, the oocyte nucleus enters into the long resting phase (dictyotene) followed by follicular formation simultaneously. Some follicles develop fully to reach the stage of ovulation (TEPLITZ and OHNO, 1962; PETERS *et al.*, 1965). Although studies on the process of oocyte maturation and follicular growth have been carried out in morphology (SATO, 1973), there are very few detailed reports on the relationship between exact age and the initiation of each stage of the nucleus in the oocyte, or appearance of each type of follicular development.

The purpose of the present study was to pursue further investigation on the process of oocyte development and on estimation of days of age when each different type of follicles, especially antral follicles appear for the first time.

MATERIALS AND METHODS

Thirty-five female Japanese White rabbits from birth to adulthood were used. After being weighed, the animals were sacrificed by decapitation. Both right and left ovaries of each rabbit were fixed in Bouin's solution immediately after removal, and dehydrated. They were embedded in paraffin wax for serial sections at 6 μm and stained with hematoxylin-eosin (H. E.), Azan and periodic acid Schiff (PAS) reaction.

To study the progress of meiotic prophase I, ovaries from 25 animals were examined histologically at the following ages; day of birth and 7, 10, 14, 21, 25, 28 and 35 days after birth. All germ cells in eight microscopic fields (×400), randomly selected from ovarian tissue, were counted in every tenth serial section. The frequencies of oogonia (mitotic phase and premeiotic resting phase) and of oocytes in the four stages of meiotic prophase I (leptotene, zygotene, pachytene and diplotene) were estimated as the proportions of each stage to total germ cells counted in the ovarian tissue of infant rabbits.

Types of follicles in all serial sections of ovaries from both sides, collected from 33 rabbits (0 to 35 days of age and 57, 70, 107 and 161 days of age), were examined to determine the time of the first appearance of follicles in different developmental stages in rabbits. For classification of the follicles, the proposal by Pedersen and Peters (1968) for the mouse ovary was adopted in the present study. In addition, the diameter of oocytes within follicles at the stages after Type 5b was measured in 33 immature rabbits examined to estimate the oocyte development and two adult rabbits (more than 5 oocytes randomly selected for each follicular type) to detect the degree of oocyte growth.

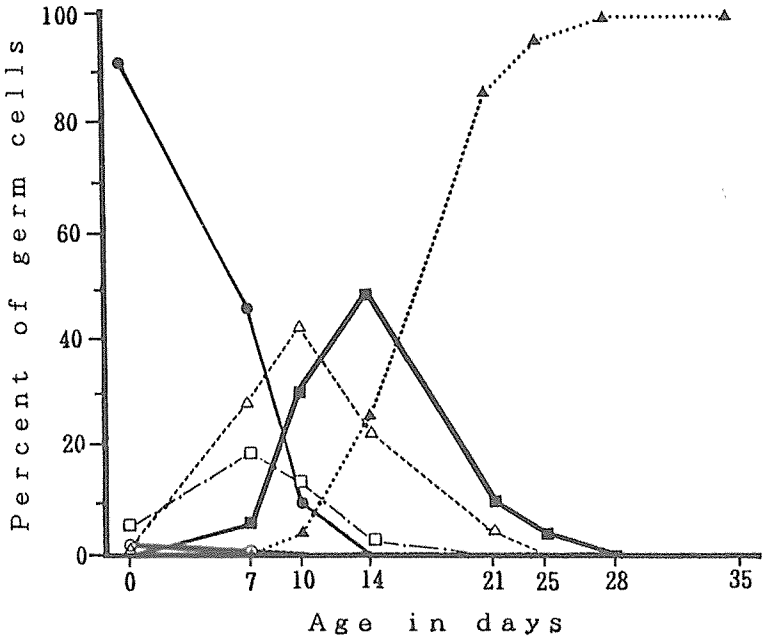
Statistical analysis

Data were examined by analysis of variance and differences among means of oocyte diameters according to follicular types and days of age were tested by Duncan's multiple range test.

RESULTS

1. *Development of germ cells after birth*

Table 1 shows the distribution of oogonia and oocytes in different stages of meiotic prophase I, which were found in ovaries with increasing ages from 0 to 35 days in early life. Text-fig. 1 is a summarized version of Table 1. On the day of birth, only a few oogonia were in metaphase, anaphase, telophase in mitotic division (Fig. 1), and most germ cells were oogonia in the premeiotic resting phase (Fig. 2). However, some oocytes in leptotene (Fig. 3) or zygotene (Fig. 4) stages were evident. With advancing age, the percentage of oogonia decreased rapidly owing to progressive transition to oocytes. The percentage of oocytes in leptotene increased from about 7% on the day of birth to about 19% on day 7, when the ratio of oocytes in leptotene reached a maximum. During the next week their number decreased gradually due to an increased number of oocytes in the later stages of meiotic prophase. In the ovaries of 14-day-old rabbits, only 2.5% of oocytes remained in



Text-fig. 1. Distribution of oogonia (mitotic phase, ○; premeiotic resting phase, ●) and oocytes in each stage of prophase (leptotene, □; zygotene, △; pachytene, ■; diplotene, ▲) with age in days.

leptotene, and in the following week oocytes of this stage could not be found any more.

The percentage of oocytes in zygotene increased gradually to nearly 30% of all germ cells after a week and peaked at about 42% on day 10, followed by a decrease to 0% on day 25.

Oocytes in pachytene (Fig. 5) were observed first on day 7 (5.9%). The percentage of oocytes in this stage increased rapidly to the maximum on day 14 (about 50%). Then, the oocytes in pachytene rapidly lessened in number during the following week and had almost disappeared by day 28, in contrast with a great increase in number of oocytes with nuclei in diplotene (Fig. 6) after day 14.

The maturation of oocytes from pachytene to diplotene occurred in a small number (0.02%) on day 7. The percentage of oocytes in diplotene increased rapidly to 26% on day 14 and 86% on day 21, and meiosis had almost completely progressed beyond all transitory stages of prophase by day 28.

As regards the progress of oocyte differentiation, there seemed to be a tendency that the more advanced stages of prophase were recognized in the ovaries of the does with heavier body weight at each age sampled.

2. *Time of first appearance of follicles in each developmental stage*

Type−1 follicles were consisted with small oocytes having no follicular cells, and existing in cell nests invaded to varying extent by stromal cells. The cell nests were composed of oocytes in various stages of development and located in the cortex of the neonatal rabbit ovaries.

Follicular development was initiated on day 7. A follicle with an incomplete ring of

Table 1. Distribution of germ cells in the different stages of meiotic prophase I

Rabbit No.	Days of age	Body weight (g)	Number of germ cells[1]	Oogonia (%)		Oocytes (%)			
				mitotic	resting[2]	leptotene	zygotene	pachytene	diplotene
1	0	41	1782	1.6	97.6	0.8	0	0	0
2		63	4701	1.4	88.7	8.9	1.0	0	0
3		72	6140	1.7	89.5	6.1	2.7	0	0
4	7	125	2234	0	39.7	19.1	22.4	2.8	0.05
5		94	7402	0.05	55.6	19.1	32.5	8.7	0
6		140	5037	0.2	41.5	18.8	33.3	6.1	0.1
7	10	120	885	0	8.8	11.0	44.9	32.6	2.7
8		160	1886	0	10.4	13.7	41.6	29.4	4.9
9	14	105	918	0	1.5	3.9	27.7	51.9	15.0
10		200	1279	0	0	0.7	29.1	50.2	20.0
11		320	1823	0	0	1.1	10.3	42.6	46.0
12	21	230	656	0	0	0	5.5	14.0	80.5
13		280	482	0	0	0	3.7	16.2	80.1
14		300	608	0	0	0	1.6	1.6	96.8
15		290	1169	0	0	0	2.5	10.8	86.7
16		370	601	0	0	0	0	0.2	99.8
17		300	637	0	0	0	0.2	7.4	92.4
18	25	250	502	0	0	0	0	0.6	99.4
19		220	1096	0	0	0	0	6.5	93.5
20	28	280	1437	0	0	0	0	0	100
21		260	900	0	0	0	0	0.1	99.9
22		340	661	0	0	0	0	0	100
23	35	490	808	0	0	0	0	0.2	99.8
24		575	814	0	0	0	0	0	100
25		580	982	0	0	0	0	0	100

[1]: Total number of germ cells counted in eight microscopic fields (×400) in every tenth serial section.
[2]: Premeiotic resting phase.

follicular cells on a small oocyte was designated as Type 2 (Fig. 7). Nuclear stages of the oocytes of Type−2 follicles were diplotene or transitional phase between pachytene and diplotene.

Follicles containing oocytes covered with one complete ring of flat follicular cells (Type 3a; Fig. 7) were first found on day 10. And follicles with oocytes surrounded by one cell layer of cuboidal follicular cells (Type 3b; Fig. 7) appeared on day 14.

Type−4 follicles, which had two layers of follicular cells (Fig. 8), were observed in all ovaries of rabbits older than 21 days.

Follicles with three layers of follicular cells (Type 5a; Fig. 9) were noted for the first time in the ovaries of one of eight 21 day-old rabbits. On day 25, follicles of Type 5a were found in all rabbits.

Oocytes in Type−5b follicles (Fig. 10) were surrounded by more than four layers of follicular cells and covered with definitive PAS-positive substances situated between the oocyte and the layers of follicular cells. This follicular type appeared in ovaries of all rabbits on day 57, though such follicles were few.

Follicles in which there were many layers of follicular cells on the oocytes and some

Table 2. Mean diameters of oocytes within follicles of Types 5b to 8 at various days of age

Days of age	Oocyte diameter (μm)			
	Type 5b	Type 6	Type 7	Type 8
57	74.2 ± 7.0^{a} (15)	—	—	—
70	$86.1\pm 5.8^{b,A}$ (10)	$101.5\pm 6.7^{a b,B}$ (10)	—	—
107	$104.4\pm 13.7^{c,A}$ (13)	$94.4\pm 7.8^{a,B}$ (20)	$94.7\pm 7.0^{a,B}$ (5)	$97.4\pm 4.2^{a,AB}$ (12)
161	96.6 ± 8.8^{d} (15)	$97.2\pm 5.7^{a b}$ (16)	$100.1\pm 3.2^{a b}$ (5)	$102.1\pm 4.4^{a b}$ (10)
adult	103.0 ± 8.7^{c} (22)	102.0 ± 6.3^{b} (25)	105.0 ± 4.4^{b} (11)	106.4 ± 4.2^{b} (13)

Values indicate mean±S. D.

Significant differences are seen between different letters (a, b, c, d) in the same column ($p<0.05$).

Significant differences are seen between different letters (A, B) in the same line ($p<0.01$).

Figures in parentheses show the number of oocytes measured.

small cavities in the granulosa layer, containing follicular fluid (Type 6; Fig. 11), were recognized in ovaries of 70-day-old rabbits.

In the process of follicular growth, such cavities became integrated into a single large cavity and an ambiguous cumulus oophorus was formed. Such Type-7 follicles (Fig. 12) were found on day 107.

Follicles of Type 8 (Fig. 13) were identified as more developed follicles with a single cavity and definitive cumulus oophorus, and were found in the ovaries of one of two rabbits at 107 days of age.

Ovaries of 161-day-old rabbits were almost comparable to ovaries of mature rabbits in histology and size. Some large follicles were located scattered within the fully developed ovarian cortex, and follicles of Type 3a were non-contiguously distributed at the periphery.

In general, more developed and advanced follicles tended to be located at the deeper sites of the ovarian cortex in contact with the ovarian medulla containing well developed blood vessels. In ovaries after day 70, large follicles which had an oocyte with condensed chromosomes and/or enlarged follicular cells like lutein cells were frequently identified as atretic follicles. Antral follicles of Type 6 and the secondary interstitial tissues appeared first in ovaries on day 70, and full development of interstitial tissues was evident on and after day 107 (Fig. 14).

3. *Relationship between follicular types and oocyte diameters at various ages*

Mean diameters of oocytes within the follicles of Type 5b to 8 are shown in Table 2. Diameters of oocytes in Type-5b follicles at 57 and 70 days of age were much smaller than those at 107 or 161 days of age or in adult does. Although the diameters of oocytes in Type-6 follicles were larger than those in Type-5b follicles at day 70, at each day of age sampled after day 107 the diameters of oocytes in Type 8 (full ripeness) were not always larger than those in Type 5b. There was a significant ($p<0.05$) but small difference in oocyte diameters between 161 days of age and adult in Type-5b follicles. In addition, oocyte diameters in this type of follicle at both 57 and 70 days of age were markedly small in comparison with oocyte diameters at the other ages.

DISCUSSION

The present result on oocyte development, from oogonia to oocytes in the diplotene

stage, in ovaries of immature Japanese White rabbits are rather different from those reported by PETERS *et al.* (1965). According to their observations on the ovaries of Copenhagen White rabbits, meiosis I was initiated only after day 1; but our study showed that oocytes with nuclei in leptotene were found in all ovaries of 3 does at day 0. They reported also that oocyte development up to diplotene was completed by 21 days of age. In the present study, oocytes in zygotene were still found in the ovaries of five of six young does at 21 days of age, and this is at least four days later than that reported by PETERS *et al.* (1965). These differences may be caused mainly by the difference in breed, gestation length and degree of fetal growth. Therefore, it may be that the transition of oogonia to oocytes of meiotic prophase I is initiated in fetal life and that oocyte maturation up to diplotene requires a longer period in the Japanese White rabbit than in the Copenhagen White rabbit.

It has been shown that follicular growth from Type 1 to Type 5b does not require the action of pituitary gonadotropin (NAKANO *et al.*, 1975), and that sudden changes in the endocrine system, especially in the plasma FSH/LH ratio, occur around 70 days of age in rabbits (DE TURCKHEIM *et al.*, 1983). Since the first antral follicles appeared between 57 days and 70 days of age in the present study, synchronous changes in the endocrine milieu as described above might occur during this period.

According to BYSKOV (1975) and DEANESLY (1975), the first follicular formation in the cat, mink, ferret, guinea-pig and rabbit occurs at the innermost part of the ovarian cortex, where rete tubules from the ovarian medulla are mediating. They stated also that there was no evidence that the tubules from the medulla were contributing to formation of growing follicles, though those tubules were sometimes in contact with primordial or growing follicles. In the present study, more developing follicles were always found in deeper sites of the ovarian cortex in contact with the medulla, which may supply some requirements for follicular growth. Therefore, it is supposed that there are some interactions between follicular growth and development of cortical connective tissue.

The secondary interstitial tissue appeared first in ovaries at 70 days of age and the tissue developed fully on and after day 107. This fact is roughly in agreement with observations by GURAYA and GREENWALD (1964), who reported that the interstitial tissue consisted of lutein-cell-like elements originating from granulosa cells in small atretic follicles and from cells of the tunica interna folliculi in large follicles. MORI and MATSUMOTO (1973) also reported that large-follicle atresia occurred at and after 3 months of life, transforming into secondary interstitial tissue.

It is generally allowed that very few oocytes can survive to the stage of ovulation, and that most oocytes are eliminated during oocyte maturation. The degenerative process in folliculogenesis is known as atresia. Recently, many attempts have been made to recover and utilize follicular oocytes (which are essentially destined to be wasted) prior to their degeneration for *in vitro* fertilization, by mincing or enzymatic digestion of the ovary (e. g., mice—SZYBEK, 1972 and SORENSEN and WASSARMAN, 1976; rabbits—BAE and FOOTE, 1975 and FUKUNARI *et al.*, 1989; rhesus monkeys—GOULD and GRAHAM, 1976). IWAMATSU and YANAGIMACHI (1975) showed that fully grown oocytes in follicles had the capacity to undergo meiotic maturation *in vitro*. In the present study, the mean diameter of fully grown rabbit

oocytes was roughly about 100 μm, and this size is comparable with that in Type-5b follicles after 107 days of age and that in all Types 6-8 follicles after 70 days of age. Thus the results of the present study suggest that it would be feasible to use ovaries of about 2-month-old rabbits for research on oocyte recovery, since all oocytes in diplotene, located in the antral follicles, would be able to resume meiotic maturation.

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幼若家兎卵巣における減数分裂の開始と各発育段階の卵胞の出現時期についての組織学的観察

笹部　雅男・福成　篤・前田　照夫
寺田　隆登・堤　義雄

広島大学生物生産学部，東広島市 724

幼若（生後0，7，10，14，21，25，28，35，57，70，107，161日齢）および成熟家兎卵巣を用いて，減数分裂の進行と各発育段階にある卵胞の出現時期を組織学的に観察した。

生後0日齢の卵巣内で減数分裂は既に開始されており (leptotene, 5.6%; zygotene, 1.4%), 10日齢では zygotene (42.6%), 14日齢では pachytene (51.0%) が多く，21日齢では多くの卵母細胞が diplotene (88.0%) に達していたが，殆ど全ての卵母細胞が diplotene に達したのは28日齢であった。

卵胞形成が初めて認められたのは7日齢であり，28日齢では多くの卵胞が一層の卵胞細胞層を持つ卵胞 (Type 3a) に発育していた。Type 5b の卵胞（未熟な胞状卵胞）は57日齢以降の卵巣で観察された。107日齢では Type 7 の卵胞（単一な卵胞腔を持つ卵胞）が出現した。161日齢では間質が卵巣表層部から髄質にかけて広く分布し，Type 8 の卵胞（成熟卵胞）が出現した。57日齢以降の全ての胞状卵胞内の生殖細胞の直径を type 別に測定したところ，70日齢の Type 6 及び107日齢の Type 5b の卵胞内の生殖細胞の直径と成熟家兎卵巣の Type 8 のものとの間に有意差は認められなかった。

以上の結果より家兎における第一減数分裂は0日齢では既に開始しており，28日頃に完全に休止するものと考えられた。また，各発育段階の卵胞を組織学的観点から見た場合，70日齢頃より成熟卵子回収の可能性を示唆する卵胞が発育していることが明らかとなった。

EXPLANATION OF FIGURES

PLATE 1

Fig. 1. Oogonia in mitotic metaphase (arrows) were frequently found on the day of birth (day 0). S, stromal cells; R, oogonium in resting phase. H. E. ×805.

Fig. 2. Oogonia in premeiotic resting phase in an ovary at day 0 (arrows). H. E. ×805.

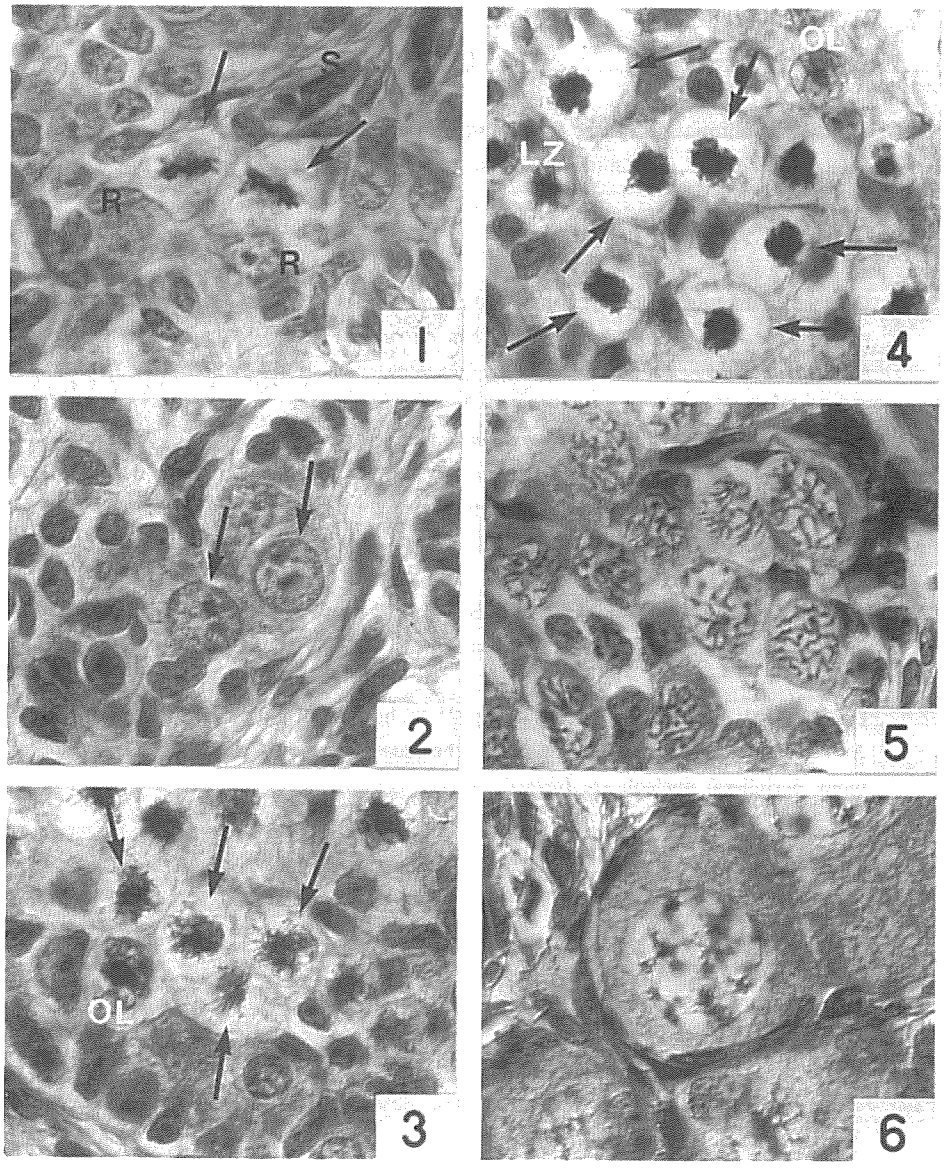
Fig. 3. Oocytes in leptotene at day 0 (arrows). The chromosomes become visible as long, thin, single strands. On this day, meiosis has already begun. OL, oocyte in the transitional stage between oogonium and leptotene (preleptotene). H. E. ×805.

Fig. 4. Oocytes with nuclei in zygotene at day 7 (arrows). The homologous chromosomes come together in close apposition and are located laterally in their nuclei as a mass. OL, transitional stage between oogonium and leptotene; LZ, transitional stage between leptotene and zygotene. H. E. ×805.

Fig. 5. Oocytes with nuclei in pachytene at day 14. The paired chromosomes begin to contact, becoming shorter and thicker. S, stromal cells. H. E. ×805.

Fig. 6. Oocytes with nuclei in diplotene at day 21. The paired chromosomes separated partially. At certain points, the homologous half chromosomes make contact with one another and many peripheral nucleoli are formed. H. E. ×805.

PLATE 1



EXPLANATION OF FIGURES

PLATE 2

Fig. 7. A longitudinal section showing the ovarian cortex with follicles of Type 2, 3a, 3b (arrows) at day 28. Progressively higher-numbered types are found in sites progressively further from the ovarian surface. H. E. ×175.

Fig. 8. Two follicles of Type 4 at day 57. H. E. ×175.

Fig. 9. A follicle of Type 5a with three layers of follicular cells at day 57. H. E. ×175.

Fig. 10. A follicle of Type 5b with more than four layers of follicular cells at day 57. H. E. ×175.

Fig. 11. A follicle of Type 6 with some cavities containing follicular fluid at day 107. H. E. ×95.

Fig. 12. A follicle of Type 7 with a single cavity containing follicular fluid at day 107. H. E. ×95.

Fig. 13. A follicle of Type 8 with a fully developed large cavity at day 161. At the periphery of the ovary, follicles of Type 3a are evident. H. E. ×40.

Fig. 14. Ovary at day 107 showing the secondary interstitial tissue. A degenerating follicle (arrow) is found, also. H. E. ×95.

PLATE 2