Ovaries of prepubertal white rats ranging in age from 0 days (time of birth) to 73 days were examined in detail for the distribution of osmiophilic lipid. The object of this examination was to throw light on the time when estrogen first appears and on the site of its formation and influence. There is no specific histochemical test for estrogen. The osmium reduction technique which was used in this study marks other unsaturated lipoids in addition to estrogen. The unsaturated compounds reduce the osmium tetroxide component of the Levi's fixative and adsorb the black reduction product. Osmophilic lipoids, other that estrogen, which are known to be present in the prepubertal ovary are phospholipids, free and combined cholesterol, and neutral fat.

Lipoid in the germinall epithelium is most abundant in the regions which are active in oocyte proliferation. There is no indication that estrogen is produced in the epithelium. Connective tissue of the stroma and tunica albuginea contains only scattered lipoid droplets the number of which decreases gradually with increasing age. The connective tissue plays no part in estrogen production.

Interstital tissue cells are not apparent in the prepubertal rat ovaries until the fifth day after birth. They first appear in small clumps in the inner cortex, increase steadily in number, and at 24 days form extensive continuous masses in the stroma of all the cortex except in the region of the ligament where oocytes are proliferating from the germinall epithelium.

In twelve-day and older rats interstitial tissue is quite obviously derived from the theca interna of atretic follicles. In younger animals the origin of the cells is less obvious. The cytoplasm of all interstitial cells is densely packed with droplets of lipid. The amount of lipid per cells undergoes no fluctuation during development of the ovary. There is nothing in the lipid picture to preclude the possibility that estrogen is produced or stored in the interstitial tissue.

There is no lipid in the ova of growing follicles. The epithelial cells of young, developing follicles contain no lipid, but scattered droplets may appear in those (granulosa cells) of some larger follicles. There is no indication that estrogen is produced in the granulosa, although the small amount of lipid sometimes found may represent estrogen which has entered from the theca and which may stimulate mitoses in the granulosa. A very thin theca is apparent for the first time around some of the follicles at three days. Lipoid which may be estrogen is noticeable in the theca interna as early as four or five days and increases gradually in amount concurrently with growth of the theca. The increase in width of the theca interna and in the lipid is more rapid from 36 days to maturity.

Lipoid is not found in the ova of atretic follicles. It is always found in the granulosa of the follicles during degeneration of the cells. The lipid is
believed to be the breakdown product of the cytoplasmic constituents and may be cholesterol esters. Atretic degeneration is well advanced in the granulosa before the ovum is visibly affected. The theca interna undergoes extensive proliferation during follicular atresia. Its cells assume an epithelioid form and become filled with droplets of osmiophilic lipoid; they are indistinguishable from interstitial tissue cells in late atresia. There is no invasion of the granulosa by the theca cells. The osmiophilic lipoid in the cells of the theca interna may be in part estrogen. The cause of atresia is discussed.
THE DISTRIBUTION
OF OSMIOPHILIC LIPOID
IN THE DEVELOPING RAT OVARY

by
BERTHA DANA CUTRESS

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OREGON STATE COLLEGE

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the requirements for the
degree of
MASTER OF SCIENCE
June 1948
APPROVED:

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Associate Professor of Zoology

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Chairman of Department of Zoology

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Chairman of School Graduate Committee

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Dean of Graduate School
ACKNOWLEDGEMENTS

Sincere expression of appreciation is due Dr. Ernst J. Dornfeld of the Zoology Department of Oregon State College for suggesting the problem for this thesis, for making available necessary materials and facilities, and for his patient criticism and guidance of the work throughout.

The writer is deeply obligated to Dr. Ivan Pratt of the Zoology Department of Oregon State College for the use of facilities in preparation of the photographs, and to Walter S. Vincent, Jr. for taking many of the photographs.

To her husband, Charles E. Cutress, Jr., she is especially grateful not only for his invaluable aid in preparation of the photographs but also for his constant encouragement and inspiration which made completion of this thesis a more pleasant task.
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THE DISTRIBUTION OF OSMIOPHILIC LIPOID IN THE DEVELOPING RAT OVARY

INTRODUCTION

In recent years interest has been increasing in the correlation of chemical and structural changes within tissues and organs during their various phases of growth and activity. The chemical analysis of substances present in certain of these has received vigorous attention. The morphological characteristics of cells and tissues have been described in detail by histologists. It remains necessary to localize the chemical substances and to correlate their changes in quantity and position with known changes in physiological and cellular activity. The newly active fields of histochemistry and histophysiology (18) are devoted to this objective.

The cyclic nature of the changes in the adult ovary has made study of this organ particularly fascinating, and it has been histochemically investigated by Dempsey and Bassett (17). In the immature ovary, however, the correlation of developmental changes with cellular activity has been neglected. In its stead there has been a tendency to extrapolate information from studies of the adult ovary in explanation of changes in the developing ovary. For
instance, evidence from histochemical studies of the adult organ (17) indicates early formation of estrogen in the thin theca interna of young, growing follicles. From this it has been suggested (23) that some estrogen may be secreted in the ovaries of very young animals. It is of importance to know the exact time and site of production and influence of estrogen, for there are several fundamental roles which the hormone may have in the immature ovary. However, the only significant analyses of the time of its production in the immature ovary have been assay studies (36) which rely upon the response of the uterus to stimulation. This gives an indication only of the time when estrogen is released into the blood in a concentration sufficient to stimulate the uterus. In all probability it is present in the ovary prior to the time of its measurable effect on other parts of the body.

It was with the hope of finding some indication of the time and site of formation of the estrogen that the work for this thesis was undertaken. There being no specific histochemical test for the hormone, it was not assumed that the problem could be solved by a simple, direct analysis. All available techniques mark other substances in addition to estrogen. It seemed worthwhile, however, to approximate a solution by using a method which marks the reactive substances, including estrogen, and at the same time gives good
morphological detail. In order to utilize paraffin-imbedded serial sections, best results were thought possible with the osmium reduction technique.

METHODS AND MATERIALS

Source and care of animals. Thirty-nine female and twenty-one male albino rats comprised the mature breeding stock used as source material for this thesis. All were animals from the inbred colony maintained in the Zoology Department at Oregon State College. This colony was derived from rats obtained from the Sprague-Dawley laboratories, Madison, Wisconsin.

The animals were housed in standard wire cages, not more than three or four being caged together. All were given ample quantities of commercial animal biscuits, along with a specially prepared meal containing the following:

- Yellow corn meal (whole corn) ------ 38.0%
- Whole wheat flour ----------------- 32.0%
- Powdered skim milk --------------- 20.0%
- Whole ground alfalfa meal -------- 6.0%
- Cod liver oil --------------------- 2.0%
- Fleischman's pure dry yeast ------ 1.0%
- Calcium carbonate ---------------- 0.5%
- Sodium chloride ------------------ 0.5%

This diet was supplemented once a week with lettuce or other greens. Pregnant females and lactating mothers were fed fresh liver once a week. All rats were usually fed daily between four and six o'clock in the afternoon.
Breeding methods. Vaginal smears were repeated daily for all non-pregnant females in order to follow the estrous cycle. The smears were made from four to six in the afternoon, when the animals were fed. Any female found to be in proestrus was immediately placed in a separate cage with a male and left until the following day. Another vaginal smear was made during the next feeding period to determine the presence of sperm in the vagina. In case of a positive test, the female was placed in a separate cage where she remained until it was determined that she was not pregnant or until birth of the litter. No care was taken to establish the exact time of fertilization, for, in all cases, the age of the young rats from which ovaries were taken was recorded as days post partum.

Three to five days before the expected birth of a litter, excelsior was placed in the cage with the pregnant female for use in nest-making. The litter remained with the mother until all the female young were taken for ovarian material or until the time of opening of the vaginal orifice when males and females were caged separately.

Preparation of material for analysis. Ovaries were taken from rats ranging in age from zero days (time of birth) to seventy-three days, with samples removed daily during the first twenty-four days and every other day thereafter. The first estrous cycle was established at 54 days in the rats of one litter, 61 in another and 73 days in a third.
Each young rat from which ovaries were taken was anesthetized with nembutal, injected intraperitoneally. Both ovaries were excised and immediately immersed in Levi's fixative (29) of the following composition:

\[
\begin{align*}
&2.5\% \text{ potassium dichromate} \quad \text{5 parts} \\
&5.0\% \text{ mercuric chloride} \quad \text{5 parts} \\
&40.0\% \text{ formalin (neutralized with magnesium carbonate)} \quad \text{1 part} \\
&2.0\% \text{ osmium tetroxide} \quad \text{1 part}
\end{align*}
\]

The ovaries were kept in a generous volume of the fixative from three to seven days, depending on their size. The fixative was renewed at frequent intervals, every second day in most cases, to ensure thorough blackening of all osmophilic substances.

At the end of the fixation period the ovaries were washed overnight in running water and then dehydrated rapidly in dioxan. The dioxan was renewed every half hour for not more than four hours; prolonged treatment tends to remove the blackened substances. Several drops of iodine were added to the first three dioxan changes to remove crystals of mercuric chloride likely to be left by the fixative.

After thorough dehydration of the ovaries, they were cleared for from ten to twenty minutes in xylol, infiltrated in at least five half-hourly changes of melted paraffin, and then imbedded separately in paraffin.

The ovaries were sectioned serially. The most
satisfactory thickness was found to be 6μ, and all but the first several ovaries, sectioned at 8μ, were so cut. Sectioning was facilitated by the use of dry ice, the vapors from which were directed across the microtome blade and paraffin block. The chilling of the paraffin aided in preventing compression of the sections.

All sections were affixed serially to microscope slides and were lightly stained with Heidenhain’s hematoxylin following the standard procedure. They were then mounted in neutral balsam and covered with Gold seal cover slips of No. 1 thickness.

**DATA**

Rats from the same litter were utilized whenever possible. The following sibling series were available: (1) 2, 3, 4, 7, 16, 17 and 19 days; (2) 5, 6 and 8 days; (3) 2, 3 and 4 days; (4) 7, 8, 9 and 10 days; (5) 11, 12, 14 and 15 days. The ovary sections were carefully studied both directly and by comparison of photographs. The day by day changes which have been observed are recorded in tabular form on pages 8, 9 and 10. This is followed by an account of the changes which occur with progressing age in the various parts of the ovary.

Osmium-blackened substances are commonly termed "lipoids". The word lipid in this case has a physical and
not a chemical connotation, embracing all substances, non-fats included, which are extractable with fat solvents. For convenience the term lipoid will be used synonymously with osmiophilic substances. It should be emphasized that we are describing not the distribution of all lipoids, but only the unsaturated lipoids, these alone being capable of reducing osmium.

Growing follicles have been classified according to the system proposed by Slater and Dornfeld (38) in which five stages have been designated:

00 - Ova characterized by the absence of follicle cells. This includes both the partially differentiated proliferations from the germinal epithelium and more fully differentiated oocytes.

01 - The early primary follicles. The granulosa cells are loosely arranged about the oocyte but soon become flattened and then closely surround the oocyte in a single layer.

02 - The later primary follicles, larger in size, in which the flattened cells have become cuboidal.

03 - The secondary follicles, with two or more follicle-cell layers but no antrum.

04 - Antra-containing follicles.

Key to symbols in table

- = Structure absent.
0 = Structure present but without lipoid.
1 = Lipoid droplets few, single and scattered.
2 = Droplets more numerous, in scattered clumps or single.
3 = Droplets in more closely spaced, small clumps.
4 = Droplets in closely spaced, large clumps.
5 = Droplets in continuous masses or in closely spaced large clumps.
## Relative Amounts of Lipoid in Ovary Structures

### 0 to 10 Days

<table>
<thead>
<tr>
<th>Ovary Structures</th>
<th>Age in Days Post Partum</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tunica albuginea</td>
<td></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
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<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td></td>
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<tr>
<td>Interstitial tissue</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
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</tr>
</tbody>
</table>

**Growing Follicles:**

0₀: Ovum
0.000000000

0₁: Ovum
0.000000000

Granulosa
-0.000000000

Theca interna
---1.11111111

0₂: Ovum
-0.000000000

Granulosa
-0.000000000

Theca interna
---1.11111111

Theca externa
---1.1111

0₃: Ovum
-0.000000000

Granulosa
-0.000000000

Theca interna
---1.11111111

Theca externa
---1.1111

0₄: Ovum
-0.000000000

Antrum
---1.1111

Granulosa
-0.000000000

Theca interna
---1.11111111

Theca externa
---1.1111

Atretic Follicles:

0₀: Ovum
0.000000000

0₁: Ovum
-0.000000000

Granulosa
-1.11111111

Theca interna
---1.11111111

0₂: Ovum
-0.000000000

Granulosa
-1.11111111

Theca interna
---1.11111111

0₃: Ovum
-0.000000000

Granulosa
-122222

Theca interna
---1.111111

Theca externa
---1.111111

0₄: Ovum
---1.111111

Antrum
---1.1111

Granulosa
---1.1111

Theca interna
---1.1111

Theca externa
---1.1111
### RELATIVE AMOUNTS OF LIPOID IN OVARY STRUCTURES
#### 11 TO 20 DAYS

<table>
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<th>OVARY STRUCTURES</th>
<th>AGE IN DAYS POST PARTUM</th>
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<tr>
<td>Stromal conn. tissue</td>
<td>1</td>
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<tr>
<td>Interstitial tissue</td>
<td>4</td>
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<tr>
<td>Growing follicles:</td>
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<td>0: Ovum</td>
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<td>Granulosa</td>
<td>0</td>
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<tr>
<td>Theca interna</td>
<td>1</td>
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<td>0: Ovum</td>
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<td>Granulosa</td>
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<td>Theca interna</td>
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<td>0: Ovum</td>
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<td>Antrum</td>
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<td>Theca interna</td>
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<td>0: Ovum</td>
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<td>Atretic follicles:</td>
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<td>Granulosa</td>
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## Relative Amounts of Lipoid in Ovary Structures

21 to 36 Days

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<td>Interstitial tissue</td>
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<td>Growing follicles:</td>
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**Germinal epithelium.** In the ovaries of newborn rats the cells of the germinal epithelium are cuboidal or nearly spheroidal. They are continually giving rise to new oocytes, especially in the region of the ligament. Lipoid droplets are sparsely scattered in the epithelium at 0 days (Fig. 1). At 1 day lipoid is more abundant but still is present in scattered droplets (Fig. 2). From 1 to 8 days there is no significant change in the lipoid. At 9 to 11 days the number of droplets decreases markedly. At 12 days they are again as numerous as at 8 days. From 12 days to maturity the lipoid gradually decreases in amount.

In 19- and 20-day ovaries the germinal epithelium begins to flatten on the convex side of the ovary. The area of flattening becomes progressively greater, encroaching upon the region of oocyte proliferation. At 36 days cuboidal cells are found only in the immediate vicinity of the ligaments and in the valleys formed by the irregular surface of the ovary, at which points ovogenesis still is apparent. The lipoid droplets become restricted for the most part to these areas of proliferation.

**Tunica albuginea.** This fibrous connective tissue layer lies to the inside of the germinal epithelium. It varies little during development of the ovary except for thickening slightly. At 0 days the droplets of lipoid are sparsely scattered (Fig. 1). At 1 day they are more numerous
(Fig. 2). In the 3-day ovaries the amount of lipoid in the tunica decreases markedly. During the rest of development it is found in gradually decreasing quantity.

**Stromal connective tissue.** The concave side of the ovary is attached to the ligaments. The connective tissue of the ligaments which continues into the ovary forms the medulla (Fig. 3). No follicles are present in the medullary connective tissue, but blood vessels and nerves enter at this point.

At 0 days there are numerous lipoid droplets scattered throughout the medulla. There is a decrease in the number at 3 days and during the rest of development the droplets are widely scattered.

In the outer portion of the ovary, the cortex, connective tissue trabeculae (Fig. 3) radiate outward from the medulla, carrying blood vessels and nerves. Connective tissue cells also surround the follicles. At 0 days lipoid droplets are numerous in this connective tissue, particularly in the inner cortex (Fig. 1). At 1 day additional lipoid appears in the outer cortex (Fig. 2). At 3 days there is a decided decrease in the lipoid in all of the cortex. During the rest of development the droplets are widely scattered in whatever connective tissue the constantly changing stroma contains.

As the ovary becomes larger an increasing portion of
the stromal connective tissue is permeated and replaced by functional ovarian tissues. A part of it goes to form the theca around growing follicles (Fig. 4) beginning about the third day. At five days small groups of lipid-laden interstitial tissue cells appear, embedded in the stroma of the inner cortex (Fig. 3). The amount of interstitial tissue increases until, at 12 days, only the stroma of the outer cortex resembles that in the ovary of the newborn (Fig. 6). Here it surrounds young follicles proliferating from the epithelium. The region of proliferation of the new follicles becomes restricted almost entirely to an ever-decreasing area in the region of the ligaments, and, as older follicles fill the cortex, lipid-filled interstitial tissue forms about them in continuous masses (Figs. 5 to 8).

From 24 days to maturity blood and lymph vessels which permeate the ovary increase in numbers and size, and the connective tissue accompanying them becomes more abundant and more loosely organized, making the ovary less compact (Fig. 16). The amount of lipid in any of this tissue is negligible, occurring only in occasional, widely scattered droplets.

**Interstitial tissue.** No interstitial tissue is found in the ovaries from 0 to 5 days. In the 5 and 6 day ovaries a few small clusters of cells, believed to be the beginning
of interstitial tissue, are embedded in the stromal connective tissue and are particularly apparent in the inner cortex (Fig. 3). The exact origin of the cells is not obvious in these young ovaries.

The interstitial tissue becomes increasingly abundant in the inner cortex, forming at 10 days large, though discrete, clusters of cells packed with lipoid (Fig. 5). At 12 days continuous masses of interstitial tissue are found, still largely restricted to the inner cortex (Fig. 6). All cells are solid with lipoid droplets. With further development of the ovary, young follicles in the outer cortex are replaced by older, large follicles, some atretic. Subsequently the outer cortex is invaded by interstitial tissue (Fig. 7), until, at 24 days, only a small area in the region of the ligament contains young follicles surrounded by connective tissue that is free of interstitial tissue cells. Blood and lymph vessels and nerves which enter the cortex from the medulla become larger and more numerous, and, with their accompanying connective tissue, surround and separate islands of the lipoid-filled interstitial tissue (Fig. 16).

From 24 days to maturity there is no doubt that a large part, if not all, of the interstitial tissue is derived from the theca interna of atretic O₃ and O₄ follicles. In atresia of these follicles the granulosa
degenerates and disappears, followed in late stages by degeneration of the ovum. The theca interna alone escapes destruction. Concurrently with degeneration of the granulosa and ovum, the theca interna undergoes proliferation, in most cases to at least twice its original width. The cells become rounded and the cytoplasm becomes completely filled with droplets of lipoid. In late stages of atresia when the granulosa and ovum have almost completely disappeared, the theca interna is continuous with, and indistinguishable from, the interstitial tissue (Fig. 16).

Growing follicles: O₀ oocytes alone characterize the ovaries at 0 days (Fig. 1). Although new oocytes are constantly proliferating from the epithelium, the total number of O₀ stages decreases steadily as development proceeds, both through atresia and through development into O₁ stages. With very few exceptions no visible lipoid is found within the ova at any age.

Early O₁ follicles develop by the first day. These have a very thin theca by the third day (Fig. 3). The number of follicles decreases rapidly, for those which develop to later stages and those lost through atresia are replaced only in part by new follicles proliferated from the germinal epithelium. Their ova contain no lipoid and there is none in the granulosa as long as the follicle remains non-atretic. The theca often contains a few
scattered droplets of lipoid.

O₂ follicles are found by the third day in the ovaries used in the present study (Figs. 3 and 5). They are always few in number. As in the younger follicles, the ova and granulosa lack lipoid. The theca interna is thin and with few scattered droplets.

O₃ follicles in early stages are found by the fourth day. Up to the fourteenth day some of these follicles may develop until the granulosa is five to eight cell layers thick, but all such undergo atresia before an antrum is formed. At 16 days O₃ follicles of larger size are more numerous and an increasing number continue development into the antrum stage before becoming atretic (Fig. 7). The ova do not contain lipoid. In some of the O₃ follicles fine droplets of lipoid are apparent in the granulosa cells which lie next to the basement membrane (Fig. 6). This deposition of lipoid may be the first sign of atresia. There is as yet no degenerative nuclear pyknosis and cellular disintegration which characterize the more typical atretic granulosa, but shortly after the appearance of a number of these follicles with a small amount of lipoid, there are found follicles which have slightly more granulosa lipoid and in which cellular degeneration is apparent.

The theca interna around early O₃ follicles is thin with widely scattered droplets (Figs. 5 and 7). As the
layer increases in width with growth of the follicle, there is a greater amount of lipoid (Fig. 14). By 24 days the droplets are found in small clumps. In the 7-day ovaries a thin theca externa can be distinguished around some of the follicles. Even in the older ovaries this layer remains thin and contains very little lipoid (Fig. 13).

The total number of growing O3 follicles decreases steadily although the follicles develop to a larger size with increasing age of the ovary.

Non-atretic O4 follicles are first seen at 16 days (Fig. 7). They become more numerous and larger with increasing age (Fig. 13). However, in the immature ovaries, all undergo atresia before they are capable of ovulation. The ova and follicular fluid do not contain lipoid. Fine droplets may be found in the granulosa cells of a few (Fig. 11). Here again such deposition of lipoid may be heralding the approach of atresia. Lipoid droplets are found in closely spaced, large clumps in the theca interna (Figs. 11 and 13). The theca externa contains scattered droplets.

Atretic follicles: The largest number of O0 oocytes in atresia is found at 0 days (Fig. 1). Atresia of the ovum is not accompanied by the presence of lipoid. The nucleus forms granules which stain deeply with iron hematoxylin but which do not reduce osmium.
Atretic $O_1$ follicles are first found in 1-day ovaries. The ova of all are without lipoid. Granulosa cells contain a few widely spaced lipoid droplets. The theca interna does not widen during atresia and contains only sparsely scattered droplets.

$O_2$ follicles in atresia are never numerous. Most which develop beyond the $O_1$ stage continue development to $O_3$ stages. As before, the ovum has no lipoid. The granulosa and theca contain scattered droplets.

Atretic $O_3$ follicles are most numerous at 10 days when there is a peak in atresia, and again at 24 days with the onset of widespread degeneration of follicles which continues till maturity. There is a striking difference between atresia at these ages. Before 24 days degeneration of the follicles is apparently a gradual process; there are early and intermediate stages in slow succession but few late stages. After 24 days atresia is more rapid and more widespread.

Particularly outstanding in the atretic follicles after 24 days is the deposition of rapidly increasing amounts of lipoid in the cells of the granulosa. This is accompanied by the rapid degeneration of the cells (Figs. 17 to 20). Although lipoid first appears at the periphery of the granulosa, cellular and nuclear changes are first apparent near the ovum (Fig. 17). As atresia progresses
many of the lipoid droplets coalesce and are densely packed in the rapidly thinning granulosa. In some follicles a few small areas in the granulosa stand out as islands of black, containing particularly dense concentrations of lipoid droplets. These islands are larger than the limits of a single cell (Figs. 10, 19 and 20).

In late atresia the granulosa has almost completely degenerated, remaining only as a thin band surrounding the ovum (Figs. 10 and 20). It is often identified only by its former boundaries, the zona pellucida of the ovum and the basement membrane of the granulosa (Fig. 20). Until final stages there usually are some large lipoid droplets or groups of small droplets in and among the degenerating cells. The invasion of the granulosa by the theca, observed in some animals, is not a factor in atresia in the rat ovary.

There is often little change in the ovum until the granulosa has almost entirely disappeared. The theca interna, however, becomes wider, largely through increase in the size of the cells and perhaps also by their proliferation. The cytoplasm of the cells rapidly fills with droplets of lipoid. In late atresia, the ova are the last to disappear and they fill the interior of the ovaries after 24 days, lost in masses of interstitial tissue (Fig. 16).

O4 follicles in atresia are first found at 14 days.
They become increasingly numerous, especially after 24 days (Figs. 9, 15, 18 and 19). The changes in atresia follow much the same pattern as in the O3 follicles. The antrum decreases in size until it finally disappears. Lipoid-laden cells may break away from the granulosa and float in the follicular fluid (Figs. 9 and 18), but other than this, lipoid is not found in the antrum. In late stages atretic O4 follicles can not be distinguished from the O3.

DISCUSSION

Osmium tetroxide reduction method. This investigation was intended as a preliminary step in the search for the time when estrogen first appears in the immature ovary, the regions of the ovary in which it is found, and, if possible, the relative amounts present at various ages.

Unfortunately there is no specific histochemical test for estrogen. There are certain staining procedures which have been used for steroid hormones (18). These methods, however, are dependent upon the reaction of certain side groups and bonds in the hormone molecule, and the positive reactants to any one of the methods are not limited to the steroid hormones. The evidence derived, therefore, is of a negative sort. A negative test is definite evidence for the absence of hormones in a given area. But a positive test
may or may not indicate the presence of hormones. It is only by the comparison of the results of several methods that more conclusive evidence will be had.

For the preliminary study it was thought best to select a method of preparation allowing for permanent mounts of serial sections of the ovaries. Also it was desirable to use a method of marking the estrogen which would give maximum morphological detail. These requirements were best met by the osmium reduction technique.

The osmium tetroxide component of Levi's fixative is a strong oxidizing substance. It oxidizes, and in turn is reduced by, compounds having an unsaturated bond in their molecules. The exact nature of the reduced osmium compound is unknown. Black (8) believes it to be a lower oxide of osmium, or metallic osmium. According to Lee (26) Mann believes the osmium-tetroxide to be reduced to osmium tetra-hydroxide, and Partington and Huntingford find the product to be a hydrated form of osmium dioxide. The reduced osmium, whatever its nature, is a black compound. It precipitates on the reducing substance and marks it with good morphological detail. This reduction and precipitation process is evidently distinct from the process of lipoid fixation which is also a characteristic of osmium tetroxide (24).

Osmium-reducing compounds in the ovary. There are a
variety of substances which will reduce osmium tetroxide, including both aromatic and aliphatic reducing substances and unsaturated compounds (7). Those which are found in the ovary are limited almost entirely to certain lipid compounds and to closely related ovarian hormones. All are extractable with fat solvents and are often collectively termed "lipoids". This term has been used in the foregoing analyses and discussion.

The lipids known to be present in the ovary are phospholipids, free and combined cholesterol and neutral fat (9, 25, 30). Most of the neutral fat is stored in tissues as depot fat. As such it varies in concentration directly with the nutrition of the animals (41). Such non-essential fat is found, in most rat organs tested, to decrease during growth of the animal (42).

The phospholipids and cholesterol are the so-called "essential lipids". They are normal protoplasmic constituents and are not affected by nutritional factors, except in extreme inanition (41). However, they do seem to vary in concentration with the activity of the organ. This has not been demonstrated in the immature ovary, but it has been shown to be true in the mature ovary and in other organs (9, 10, 12). With increase in the activity of a tissue there is found to be a significant rise in phospholipid and a lesser increase in free cholesterol. With a
decrease in activity the concentration of these fractions drops and the cholesterol ester concentration rises. The latter is particularly high in degenerating tissues.

No analyses have been made in the ovary of the changes in essential lipids with increasing growth and age. In all the many other organs studied, however, there has been an increase in essential lipid, particularly the phospholipid components, with increasing size and age. It is likely that the same is true in the ovary.

Two types of formed elements which contain lipid are found in cells of the ovary, as well as in the cells of other tissues. These are mitochondria and Golgi bodies. These may blacken with osmium just like other lipid materials or estrogen. They can be readily distinguished from secretions droplets, however, by their small and irregular form.

The presence of these and other lipid must be taken into account in evaluating the results of osmium reduction.

Lipoid in the germinal epithelium. The lipoid droplets in the germinal epithelium are never present in great numbers at any time during development of the ovary. They are found chiefly in association with cells in the region of the ligaments. That this area is especially active in oocyte proliferation seems apparent in the ovaries used and has been noted by others (31).
That estrogen is secreted or stored in the germinal epithelium is in no way indicated. On the basis of an observed correlation of increased ovogenetic and mitotic activity of the germinal epithelium with the presence of concentrated estrogen in the ovary (2, 14, 21), it might be supposed that the lipoid we have observed in the region of proliferation is estrogen. However, it is doubtful that estrogen would be available to the germinal epithelium in effective amounts in the very young ovary. During the first five days after birth, particularly, no structures in the ovary show indications of hormone secretion. Some estrogen reaches the ovary through the blood (37), having been absorbed from the mother's milk by the suckling rats. However, experimentally-injected estrogen fails to affect the mitotic activity of the germinal epithelium unless it is administered in such a way that it is in direct contact with the cells of the epithelium (13). Furthermore, observations by Swezy and Fencharz (40) and Swezy and Evans (39) that stimulation of ovogenesis is correlated with the luteinizing process in the ovary do not substantiate a theory of estrogen stimulation.

The droplets could conceivably be neutral fat or essential lipoid. Since the greatest abundance of droplets is found in the most active regions of the epithelium, it is not unlikely that the droplets contain concentrations of
phospholipid.

**Lipoid in the connective tissue of stroma and tunica albuginea.** Little osmium-reduced material is found in ovarian connective tissue at any time during development. The total amount of connective tissue increases slowly during development (19); the concentration of lipoid, if it varies at all, decreases slightly in amount.

The connective tissue functions presumably only as a supportive framework. As such it plays no part in estrogen production. Lipoid droplets found within its cells may be neutral fat, essential lipids, or possibly droplets of estrogen which are diffusing from one part of the ovary to another.

**Lipoid in the interstitial tissue.** No agreement has been found in the literature as to the exact time in the development of the rat ovary when interstitial tissue first appears. One of the distinguishing features of interstitial tissue cells, besides their epithelioid appearance, is known to be the constant presence of clumped, osmiophilic lipoid granules within the cytoplasm. For that reason the method of preparation of material in this study is of value in the identification of the cells, for all osmiophilic lipoid is distinctly marked. In the ovaries studied a few small clusters of lipoid-filled cells which are believed to be the beginning of the interstitial tissue appear at five days.

Despite their early identification, the exact origin
of the cells is not clear in the material studied. In older ovaries the interstitial tissue is very obviously derived from the theca interna of atretic follicles. However, in the five-day ovaries all the follicles are small, none farther advanced than early $O_2$ stages; the theca interna around even the largest of these is very thin with little lipoid, even when the follicles are in atresia. There is no indication whatever that the theca contributes to interstitial cell formation at this age. This has been noted by others (reviewed by Kingsbury, 26), but there has been no agreement as to the real origin of the cells in the very young animals. Perhaps the most authoritative of the theories have been advanced by Corner (15) who attributes interstitial tissue formation to "modifications of the cells of the stroma and of the various epithelial proliferations", and by Kingsbury (26) who suggests that the cells are derived from stromal connective tissue through increase in cytoplasm followed by deposition of lipoid in the cytoplasm.

As to the stimulus which might cause these changes in the connective tissue cells or the possible origin of the lipoid, there have been no suggestions. In older ovaries the cells of the theca interna of atretic follicles acquire additional lipoid before becoming interstitial tissue. At least a part of this lipoid may enter from the degenerating granulosa cells. These contain abundant lipoid in beginning
stages of atresia, but it disappears gradually as the cells are destroyed. The products of cellular degeneration may also provide the stimulus for the increase in size of the thecal cells. There may be similar factors responsible for interstitial tissue formation in the very young ovaries. Here interstitial tissue first appears in the inner cortex where atretic follicles, all of small size, are most abundant. Total destruction of the granulosa of the degenerating follicles such as is observed later in large follicles, is not so obvious here but is assumed to occur. It is suggested that products of degeneration in the granulosa stimulate certain of the fibroblastic stromal cells to increase in size, and that the lipoid in the enlarged cells is derived at least in part from that in the atretic granulosa.

Although there is a very obvious increase in the total interstitial tissue lipoid by virtue of the increase in the total numbers of cells, there is no apparent fluctuation in the amount of lipoid per cell at any time during development of the ovary. This, along with the fact, observed by others, that there is little or no interstitial tissue in the ovaries of many mammals, makes it doubtful whether the cells are involved in hormone production. That estrogen is stored in the interstitial tissue has been suggested but not demonstrated. According to Cowdry (16) Mulon has observed anisotropism of the granules in the
interstitial tissue cells, a fact which, he says, suggests
the presence of cholesterol.

**Lipoid in the growing follicles.** With very few
exceptions none of the ova of the growing follicles contain
osmiophilic lipoid. This agrees with the observation (16)
that "the eggs of rats and mice are entirely free from
visible cytoplasmic inclusions so that in the fresh ovum
the nucleus is the only formed structure in a field of
homogeneous cytoplasm". This is not to say that there is a
complete lack of lipoid in the ovum but that any therein
contained must be suspended in ultramicroscopic particles.
The zona pellucida in large follicles is very well preserved,
in the material studied, and apparently takes a little of
the reduced osmium, appearing quite gray.

In the adult ovary experimental evidence has largely
eliminated the granulosa of the follicles as a source of
estrogen (15). That the granulosa of the immature ovary is
likewise not involved in secretion of the hormone is indi-
cated by the lack of significant lipoid in its cells. How-
ever, the possibility that the small amount of lipoid found
is estrogen can not be denied. There is some evidence (13)
that the mitoses responsible for the growth of the granulosa,
at least to a certain point, are stimulated by estrogen,
presumably from the theca. It would be expected, then, that
at least a small amount of estrogen would be found in the
granulosa of the follicles which have a well-developed theca.

The possibility that the lipoid may be yolk-forming material diffusing toward the ovum must be considered. Any yolk in the ovum is present in such small amounts and in such a finely divided state as to be invisible. Thus it would not be expected that large amounts of yolk-forming lipoid would be passing through the granulosa. The amount of lipoid in the granulosa cells is, in fact, never great.

There is a third possible explanation for the presence of the lipoid in the granulosa. In the ovaries herein analyzed the presence of such lipoid seems to herald the approach of atresia. In 0₁ follicles cellular degeneration is usually apparent around the antrum at the time lipoid appears. However, in many 0₂ follicles such degenerative changes are not obvious until after the appearance of considerable lipoid. In the latter case the deposition of lipoid may actually be the first signs of atresia in these follicles and may be products of degeneration.

Lipoid is not found in the antrum of 0₁ follicles in either mature or immature ovaries. Why this should be so is not altogether obvious, for in the adult ovary estrogen can be isolated from the follicular fluid.

The theca remains as the only part of the growing follicle in which estrogen may be produced. In the mature ovary there is abundant evidence (15, 32) that the theca interna is the chief source of the hormone. That the theca interna is likewise involved in the immature organ has been
assumed but the actual presence of estrogen has never been demonstrated.

In the ovaries analyzed in the present study, it is not until the third day that a very thin theca interna is observed around some of the follicle. A very few scattered lipid droplets may be present in the theca at this age. That this lipid represents estrogen secreted by the theca is not likely. The theca cells are derived from connective tissue cells of the stroma which, it has been noted, often contain lipid. It is not unlikely that the lipid was already present in the cells before their differentiation into thecal tissue. As the theca interna increases in width, however, additional lipid is deposited in the cells which may be estrogen which they have secreted.

Price (36) has found that estrogen in amounts large enough to affect the weight of the uterus is produced by 14 days post partum; it was not determined how far in advance this estrogen first appears in the ovary, however. In the ovaries analyzed, lipid which may be estrogen is apparent in the cells of the theca interna as early as four and five days. It increases gradually in amount as the size of the follicle increases. However, the thecal lipid in the largest of the follicles remains in discrete, scattered droplets until about the twelfth day when scattered clumps are found. This clumped lipid, if it is
estrogen, is evidently present in sufficient concentration by 14 days to evoke the response noted in the uterus by Price.

Price has found that there is an increase in estrogen concentration after 36 days. In our material there is a corresponding increase in thecal lipoid after 36 days. The theca interna around the largest follicles not only undergoes additional proliferation with increase in lipoid, but more important, the number of these large follicles increases.

It is of interest to examine the changing lipoid picture in the theca interna for possible correlation with the two fundamental roles which have been suggested for the estrogen produced in the ovary.

The possibility that estrogen, produced in the theca interna, acts as a stimulatory agent for follicle growth has already been indicated in the foregoing discussion. It has been found (13, 23) that the period of greatest growth of the granulosa, as measured by the mitotic activity, is preceded by the formation of the theca interna. Furthermore, when the rate of mitosis in the theca interna reaches a peak and begins to decline, there is a corresponding decrease in the granulosa.

In the present study it is found that the lipoid deposition in the theca interna increases simultaneously with growth of the granulosa. If we assume that this lipoid is
estrogen, these observations are in agreement with the hypothesis that mitotic activity in the granulosa is stimulated by estrogen.

There is a possible correlation between the increase in thecal lipoid and stimulation of the ovary by FSH and LH. These hormones are known to be present in the anterior pituitary of the immature animal (1, 5, 33). However, the ovary is not sensitive even to injected gonadotrophin until 21 days (5). All evidence points to the fact that a well-developed granulosa is necessary for a response by the follicle. The thecal estrogen may control the development of the granulosa to a point where it is sensitive.

The FSH is primarily responsible for the later development of the follicle. The LH apparently is not effective in the ovary until just before puberty, being responsible then for the sudden increase in size of the Graafian follicle, and the subsequent maturation and ovulation of the ovum. The release of LH from the pituitary is believed to be stimulated by estrogen. Apparently estrogen is not present in high enough concentration until just before puberty to bring about release of LH in sufficient amounts to precipitate ovulation. Mossman (32) calls attention to the fact that in the many mammalian species investigated the epithelioid cells of the theca interna reach their maximum functional state, as judged by their cytology and by vascularity of the theca, at or near the time of ovulation.
Assuming that the lipoid we have noted in the theca interna is estrogen, our observations are in agreement with these facts.

**Lipoid in the atretic follicle.** One of the characteristics of the atretic follicle is the deposition of lipoid in the granulosa accompanied by degeneration of the cells. Just what the nature and origin of the lipoid may be is not known. Mitochondria have been observed to form fat droplets in degenerating tissue (11) and some of the granulosa lipoid may have such an origin. Degeneration lipoid has also been attributed to breakdown of protoplasmic constituents or to fat of extracellular origin which diffused into the cell (11). Cholesterol esters are found to be particularly evident in degenerating tissues (9) and may constitute at least some of the osmiophilic lipoid.

In the granulosa of many of the larger follicles are found small areas which contain particularly dense concentrations of lipoid droplets. There apparently has been no description of such areas in the rat by other workers. According to Cowdry (16) peculiar bodies have been observed in the granulosa of the rabbit, some monkeys and possibly of the human. They are commonly called the bodies of Call and Exner for want of agreement as to their exact nature. They are described as spherical masses of homogeneous material about which are grouped a small number of granulosa
cells. Some believe them to be the first stages of follicular fluid, others suggest that they are degeneration products or misplaced zona pellucida secretion. It may be that the areas which we have observed in the rat are similar in nature to these Call and Exner bodies. However, those which we have found consist mainly of lipid-laden cells and do not include a definite homogeneous mass in the center.

No suggestions were found in the literature as to the nature of the lipid in the theca interna of atretic follicles. There is abundant evidence that estrogen is secreted in the theca interna of growing follicles. It may be that the great increase in thecal lipid during atresia may be due to increased secretion of the hormone. Some of the lipid may also be derived from that which disappears from the granulosa during atresia.

The cause of atresia is uncertain. Allen et al (3) suggest that the nutriment material available to the follicle and ovum is limited and will allow for the maturation of a limited number of ova. As the follicles increase in size their nutritional requirements increase, and, in the resultant struggle for survival, many undergo degeneration. A correlation is found by others (32, 35) between degeneration of the follicle and an insufficiently developed blood supply.

The lack of hormones has also been suggested as a cause of atresia. This has stemmed largely from
observations that in the mature animal waves of atresia occur during certain stages in the estrous cycle (Engle (20) in the mouse and Allen et al (3) in the pig). Engle found the number of atretic follicles to be highest during diestrus, when estrogen levels are low. There are conflicting observations, however; Asami (4) and Pincus and Engleman (34) in the rabbit and Harman and Kirgis (22) in the guinea pig have noted no cyclic variation in the number of atretic follicles in adult ovaries.

In considering whether hormone lack may be a factor in atresia in the prepubertal ovary, it must be remembered that the situation is not entirely comparable to that in the mature organ. In the immature animal there is as yet no estrous cycle with its periodic fluctuations in production and release of hormones. The processes of formation and secretion of ovarian and pituitary hormones and the parts of the animal involved are still undergoing development.

Estrogen, released from the theca interna, is believed to control development of the follicle up to a certain size. When the follicle reaches this minimum size, all evidence indicates that continued development is influenced by FSH from the anterior pituitary. And, at the time of puberty, the changes which bring about ovulation are believed to be under control of LH, also from the anterior pituitary.
That estrogen induces release of LH has been indicated.

In view of these facts, if the theca interna has lacked sufficient stimulus for differentiation, if the substances which are precursors of estrogen do not reach the theca in adequate concentration, in all probability the estrogen supplies will be insufficient and the small follicles will degenerate. Likewise, if the factors which stimulate the production and release of gonadotrophins or if the precursor substances are not provided in sufficient amount, follicular development will be impeded. Even in the mature animal, where the hormone concentration undergoes cyclic variation, the level of a hormone at any one period is directly influenced by the nutrition and well being of the animal. Indeed, during periods of the estrous cycle when estrogen levels are low, the concentration will be high enough to prevent atresia in many of the follicles in the well-developed, well-nourished animal, but in a less fortunate animal the amount of atresia probably would be greater.

Although there is no estrous cycle in the prepubertal animal, cyclic variation has been observed in the young rat (38). Engle (20) did not observe waves of atresia in the prepubertal mouse ovary. In the present study it is found that there are peaks of atresia but that these do not occur at regular cyclic periods. Any cyclic variation which
has been observed may be due to the influence of maternal hormones reaching the animal through the milk. This has been suggested by Slater and Dornfeld (38) who noted 5-day cycles in atresia which would correspond with the mother's estrous cycle, normally repeated every five days. Maternal hormones are known to reach the circulation of the suckling rat (37). The widespread atresia seen in our material at 0-days may have been precipitated by the sudden termination of placental supplies of maternal estrogen. The 10- and 24-day peaks of atresia may also have been influenced, at least in part, by low levels of maternal estrogen.

Formation of polar bodies and even cleavage stages resembling those in the normal ovulating ovum have been observed in ova of atretic follicles (reviewed by Pincus, 33). That such abortive maturation in the atretic follicle may be stimulated by products of the degenerating tissue is of course possible. Atretic changes occur in the granulosa and theca before the ovum is affected in many animals.

There can be no adequate comparison of maturation in the normal and atretic ovum for the nature of the activating substances for the normal ovum is not known. The increase in follicular fluid and subsequent expulsion of the ovum are under direct influence of the pituitary, but whether the maturation changes which occur concurrently in the ovum are under the same control is not known. It may be that some
specific substance present in the follicular fluid is responsible for the maturation changes. This substance might be produced by the granulosa or it could originate extraneously and diffuse to the antrum at a critical period. Bassett (6) has found that in the normal follicle the basement membrane is more pervious at the time of ovulation to materials which it does not ordinarily pass, and Cowdry (16) reports a similar condition in the basement membrane of atretic follicles. Thus the maturation and cleavage in atretic follicles may be physiologically comparable to the normal.

SUMMARY

1. Ovaries of prepubertal white rats ranging in age from 0 days (time of birth) to 73 days were examined in detail for the distribution of osmiophilic lipoid. The object of this examination was to throw light on the time when estrogen first appears and the site of its formation and influence.

2. There is no indication that estrogen is produced in the germinal epithelium or in the connective tissue of the stroma and tunica albuginea.

3. Interstitial tissue cells are not apparent in the prepubertal ovaries herein analyzed until the fifth day after birth. The cells increase steadily in number, forming at
24 days continuous masses in the stroma of the entire cortex, except in the ligament region where oocytes are proliferating from the germinal epithelium. The cytoplasm of all interstitial tissue cells is densely packed with droplets of lipoid. The amount of lipoid per cell undergoes no fluctuation during development of the ovary. There is nothing in the lipoid picture to preclude the possibility that estrogen is produced or stored in the interstitial tissue.

4. There is no lipoid in the ova of growing follicles. The follicular epithelium of growing follicles contains no lipoid, but scattered droplets may appear in the granulosa cells of some larger follicles. There is no indication that estrogen is produced in the granulosa, although the small amount of lipoid sometimes found there may represent estrogen which has entered from the theca. A very thin theca is first apparent around some of the follicles at three days. Lipoid which may be estrogen is apparent in the theca interna as early as four or five days and increases in amount concurrently with growth of the theca. The increase in width of the theca interna and in the lipoid is more rapid from 36 days to maturity. These observations are discussed in the light of supposed effects of estrogen in the prepubertal rat.

5. Lipoid is not found in the ova of atretic follicles. It is always found in the granulosa of the follicles during
degeneration of the cells. The lipoid is believed to be the product of the breakdown of cytoplasmic constituents and may be cholesterol esters. Atretic degeneration is well advanced in the granulosa before the ovum is visibly affected. The theca interna undergoes extensive proliferation during follicular atresia. Its cells assume an epithelioid form and become filled with droplets of osmiophilic lipoid; they are indistinguishable from interstitial tissue cells in late atresia. There is no invasion of the granulosa by the theca cells. At least a part of the osmiophilic lipoid in the cells of the theca interna may be estrogen. The cause of atresia is discussed.
BIBLIOGRAPHY


EXPLANATION OF PLATES

The abbreviations used to label the photomicrographs are listed below. The lipoid has not been labelled, for its distinct black color identifies it in most cases. Structures which may be mistaken for lipoid have been labelled.

a. - antrum
a.p. - area of proliferation
at.f. - atretic follicle
b.m. - basement membrane
b.v. - blood vessel
c.t. - connective tissue
g.b. - granulosa body (Call and Exner body ?)
g.c. - granulosa cell
g.ep. - germinal epithelium
g.l. - granulosa with lipid
gr. - granulosa
i.t. - interstitial tissue
med. - medulla
00. - 00 oocYTE
01. - 01 follicle
02. - 02 follicle
03. - 03 follicle
04. - 04 follicle
0v. - ovum
s.l. - suspensory ligament
t.a. - tunica albuginea
th.e. - theca externa
th.i. - theca interna
trab. - trabecula
z.p. - zona pellucida

Figure 1. Ovary at 0 days (time of birth); #0-2a/r3-s7; x 200.

Figure 2. Ovary at 1 day; #1-2a/r4-s11; x 234.

Figure 3. Ovary at 5 days; #5-la/r4-s4; x 200.

Figure 4. Ovary at 7 days; #7-1b/r3-s17; x 225.

Figure 5. Ovary at 10 days; #10-2b-2/r3-s8; x 225.

Figure 6. Ovary at 12 days; #12-2a-1/r4-s4; x 234.

Figure 7. Ovary at 16 days; #16-1b-2/r3-s4; x 180.

Figure 8. Ovary at 19 days; #19-1b-3/r1-s3; x 180.
Explanation of Plates Continued

Figure 9. Ovary at 24 days, outer cortex; #24-la-2/r5-s6; x 200.

Figure 10. Ovary at 24 days, inner cortex; # as above; x 225.

Figure 11. Ovary at 30 days, outer cortex with 04 follicles; #30-1b-2/r5-s2; x 180.

Figure 12. Ovary at 30 days, outer cortex near area of proliferation; # as above; x 180.

Figure 13. Ovary at 36 days, outer cortex; #36-2a-4/r3-s2; x 200.

Figure 14. Ovary at 36 days, inner cortex; # as above; x 240.

Figure 15. Ovary at 36 days, outer cortex; # as above; x 198.

Figure 16. Ovary at 36 days, inner cortex with late stages of atretic follicles; # as above; x 200.

Figure 17. Ovary at 17 days, early atresia in 03 follicle; #17-la-1/r3-s7; x 666.

Figure 18. Ovary at 36 days, early atresia in 04 follicle; # as in figure 13; x 250.

Figure 19. Ovary at 36 days, moderately advanced atresia in 04 follicle; # as in figure 13; x 300.

Figure 20. Ovary at 36 days, advanced atresia in 03 follicle; # as in figure 13; x 250.