A HISTOCHEMICAL STUDY OF PRE-OVULATORY AND POST-OVULATORY FOLLICLES IN THE RABBIT OVARY

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Summary. Histochemical changes in the membrana granulosa and theca interna of oestrous, pre-ovulatory and post-ovulatory follicles in the rabbit ovary have been investigated.

Membrana granulosa in oestrous follicles shows two types of lipid bodies \( L_1 \) and \( L_2 \). The \( L_1 \) bodies consist of phospholipids and triglycerides. Their number is gradually increased in the pre-ovulatory and post-ovulatory follicles. The \( L_2 \) bodies consist of phospholipids. Their number is decreased in the pre-ovulatory and post-ovulatory follicles; simultaneously the \( L_2 \) bodies also develop triglycerides. Diffuse lipoproteins develop throughout the cytoplasm of granulosa lutein cells.

Theca interna cells in oestrous follicles contain lipid droplets (consisting of cholesterol and/or its esters, triglycerides and phospholipids) and diffuse lipoproteins; cholesterol or its esters are not seen during the pre-ovulatory period. In the post-ovulatory follicles, the theca interna cells are filled with lipid bodies consisting of triglycerides, cholesterol or its esters and some phospholipids.

The physiological significance of these histochemical changes has been discussed.

INTRODUCTION

Histological changes in the pre-ovulatory and post-ovulatory follicles in the ovaries of different mammals, including the rabbit, have been described in many studies (see reviews in Brambell, 1956; Young, 1961; Eckstein, 1962), but little work has been carried out using histochemical techniques on changes in the pre-ovulatory and post-ovulatory follicles (Young, 1961; Jacoby, 1962). In the present study, histochemical techniques for lipids, nucleic acids, carbohydrates and proteins were used to follow changes in the membrana granulosa and theca interna of oestrous, pre-ovulatory and post-ovulatory follicles of rabbits.

Recent electron microscope studies show that there is an increase in the agranular endoplasmic reticulum coincident with luteinization (Björkman, 1962; see review by Christensen, 1965). An attempt is made in the present study to correlate the histochemical changes in the granulosa cells during corpus luteum formation with the ultrastructural findings.
MATERIALS AND METHODS

Ovaries from sexually mature New Zealand giant white rabbits were used. They were divided into three groups: (1) from oestrous rabbits for the study of oestrous follicles; (2) from animals killed at different times (1 to 11 hr) after the intravenous administration of 100 i.u. human chorionic gonadotrophin (hCG) which causes ovulation 10 to 11 hr after injection; these ovaries were used to study histochemical changes during the pre-ovulatory swelling of follicles; (3) from animals killed 1 to 4 days after hCG-induced ovulation for the study of the histochemistry of luteal cells. The recovery of eggs from the oviduct as well as the presence of fresh corpora lutea were the criteria for ovulation. Follicles with their surrounding tissue were removed with a sharp razor blade and immediately transferred to a fixing fluid.

Fixatives used for the study of lipids included formaldehyde calcium with and without postchromation (Baker, 1944, 1946, 1956), formaldehyde saline with and without postchromation (Baker, 1949), and 10% neutral formalin (Lillie, 1954). After fixation and subsequent postchromation, material was embedded in gelatin (Baker, 1946, 1949). Frozen gelatin sections were cut at 10 µ, washed briefly in water and stained by the following methods: Sudan black B in 70% ethanol (Baker, 1944) and in propylene glycol (Chiffelle & Putt, 1951, cited in Pearse, 1960) for colouring lipids in general; Nile blue sulphate technique for neutral and acidic lipids (Cain, 1947, 1948); Sudan III and IV method for neutral fats (Kay & Whitehead, 1941, cited in Pearse, 1960); the gelatin Sudan III and Sudan IV method (Govan, 1944, cited in Pearse, 1960) for neutral lipids; the Fettrot method (Pearse, 1960) for neutral fats; acid haematein technique together with pyridine extraction control (Baker, 1946) for phospholipids; Schultz method (Gomori, 1952) for cholesterol and its esters. Small pieces of fresh material were also treated with cold acetone and ethanol for 24 to 48 hr with three changes of each of the solvents. After extractions with these solvents, the material in each case was treated with formaldehyde calcium to fix the lipids that resisted the action of acetone and ethanol. Frozen gelatin sections were treated with the various histochemical methods described above to determine the selective solubility of different lipids in cold acetone and ethanol. Sudanophilic lipids, which stained pink in Nile blue and red in red Sudan dyes, together with negative reactions in the material extracted with cold acetone, were interpreted as neutral fats (triglycerides). Acidic lipids (phospholipids), lipoproteins and proteins generally stain blue in Nile blue. Only those sudanophilic substances, which stained blue in Nile blue, and blue-black in acid haematein, together with negative reactions in the material treated with cold ethanol and pyridine, were interpreted to contain acidic lipids (phospholipids). Those substances, which stained blue-green with the Schultz test, followed by a negative reaction in the material extracted with cold acetone, were considered to be cholesterol and/or its esters.

For investigating substances other than lipids, some of the material was fixed in Carnoy’s, Zenker’s or Bouin’s fluid, embedded in paraffin wax, and sectioned at 10 µ. For the study of RNA in the material fixed in Zenker’s fluid, the methyl green–pyronine techniques were used (Jordan & Baker, 1955; Kurnick, 1955,
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cited in Pearse, 1960); control sections were pre-treated with ribonuclease and trichloracetic acid (Pearse, 1960). The periodic acid–Schiff (PAS) technique (Hotchkiss, 1948, cited in Pearse, 1960) was used for the localization of carbohydrates. The mercuric bromphenol blue method (Mazia, Brewer & Alfert, 1953) was used for the demonstration of proteins.

RESULTS

Histochemistry of oestrous follicles

The granulosa cells of oestrous follicles show lipid bodies (L₁ and L₂) and granular mitochondria (Guraya, 1959, 1964). The L₁ bodies, consisting of phospholipids and some triglycerides, are sparsely distributed in the basal portions of the membrana granulosa (Pl. 1, Fig. 1). The L₂ bodies consist of phospholipids. The mitochondria show phospholipids and proteins. The small Golgi zone stains for protein, lipoprotein and some RNA. The cytoplasm of granulosa cells is rich in RNA as judged by the strong positive reaction in the methyl green–pyronine technique and the negative reaction in the control sections.

The cytoplasm of the slightly hypertrophied theca interna cells is filled with sudanophilic lipid droplets (Pl. 1, Fig. 1), which in their histochemical reactions resemble the L₁ bodies of the membrana granulosa. They consist of phospholipids and triglycerides. The coarse lipid droplets of theca interna cells also stain moderately in the Schultz test. This indicates that they contain some cholesterol and/or its esters, in addition to phospholipids and triglycerides demonstrated in the previous study (Guraya, 1959). The cytoplasm and its organelles show the same histochemical reactions as those observed in the granulosa cells, except that the cytoplasm of theca interna cells contains diffuse lipoproteins and relatively less RNA.

Lying outside the theca interna is the broad theca externa (Pl. 1, Fig. 1), which shows a compact organization owing to its more fibrous nature. Its connective tissue elements, derived from the general stroma of the ovary, show some sparsely distributed lipid granules and rods. The lipids resemble histochemically the L₂ bodies of the membrana granulosa: they consist of phospholipids.

Histochemical changes in follicles after HCG administration

Pre-ovulatory changes. The growing follicles in the ovaries of rabbits killed 1 to 5 hr after injection of HCG do not show much histochemical change in the membrana granulosa and theca interna. However, cholesterol and/or its esters, present in the lipid droplets of theca interna cells in oestrous rabbits, are not seen, as evidenced by a negative reaction in the Schultz test. The L₁ bodies have increased slightly in number in the basal portion of membrana granulosa. Some L₂ bodies have coalesced with each other to form larger lipid bodies of irregular shape.

In the animals killed 6 to 10 hr after the administration of HCG, the cells of the membrana granulosa are separated by the follicular fluid, which accumulates in the intercellular spaces. The L₂ bodies stain pink in Nile blue followed by a
deep red coloration in red Sudan dyes. These reactions disappear in the material extracted with acetone, indicating that the $L_2$ bodies develop some triglycerides in addition to their phospholipids. The number of $L_1$ bodies is increased in comparison to the previous stages. They ($L_1$) are seen throughout the membrana granulosa. The theca interna cells are further hypertrophied. They continue to show sudanophilic lipids consisting of phospholipids and some triglycerides and also diffuse lipoproteins. The histochemical changes are well marked in the various components of newly ruptured follicles (Pl. 1, Figs. 2 and 3). The theca interna cells are loaded with the relatively coarser lipids consisting of triglycerides and phospholipids (Pl. 1, Figs. 2 and 3). Blood vessels appear among the granulosa cells, so that every cell is in intimate contact with blood capillaries. Mitoses are frequent. The $L_1$ bodies are still present in the membrana granulosa; diffuse, sudanophilic lipids, not seen in the granulosa cells of pre-ovulatory follicles, develop in the cytoplasm.

Post-ovulatory changes. The follicles are transformed into corpora lutea (Pl. 2, Fig. 4), which are fully differentiated and vascularized on Day 1 after ovulation. The granulosa cells have multiplied and hypertrophied to form the luteal cells which finally occupy the original antra cavity (Pl. 2, Fig. 5). Their cytoplasm shows more $L_1$ bodies; some $L_2$ bodies of irregular shape are also seen. The histochemical reactions of mitochondria are not changed. However, they have apparently increased in number with the hypertrophy of the luteal cells. The cytoplasm of luteal cells continues to stain positively for RNA. The Golgi zone, which was originally of compact organization, becomes loose. The luteal cells develop diffuse, sudanophilic, lipid substance throughout the cytoplasm (Pl. 2, Figs. 4 and 5), which is not seen in the granulosa cells, except for the Golgi zone (Pl. 1, Fig. 1). It reacts negatively to other techniques for lipids and appears to consist of diffuse lipoproteins. The theca interna cells can still be recognized, although much shrunken and degenerate, at the periphery of 1-day-old corpora lutea (Pl. 2, Fig. 4). They are filled with coarse lipid bodies which also show Schultz positive material (cholesterol and/or its esters), in addition to triglycerides and phospholipids. On Day 3, the luteal cells of granulosa origin contain increasing numbers of lipid droplets ($L_4$) in some of which cholesterol and/or its esters are present. In the 3- to 4-day-old corpora lutea, luteal cells develop vacuoles and coarse lipids (Pl. 2, Figs. 6 and 7). The sudanophilic lipids are

**EXPLANATION OF PLATE 1**

Figs. 1 to 3 are photomicrographs from frozen gelatin sections of follicles fixed in formaldehyde calcium, postchromed in dichromate calcium and coloured with Sudan black B.

* $\sigma$ = granulosa; $L_1$ = lipids of first type; $L_2$ = lipids of second type; $\sigma t$ = theca interna; $t e$ = theca externa.

Fig. 1. Portion of vesicular follicle from oestrous ovary, showing the distribution of lipids in the membrana granulosa and theca interna. $\times$ 373.

Fig. 2. Portion of newly ruptured follicle, showing the distribution of lipids in the membrana granulosa and theca interna. Follicle was removed 10 hr and 40 min after the injection of 100 i.u. HCG. $\times$ 233.

Fig. 3. High-power view of portion of follicle shown in Fig. 2. Note the accumulation of lipids in the cells of the theca interna. The number of $L_1$ bodies has increased in the membrana granulosa which also shows some heterogeneous $L_2$ bodies. Vascularization of the granulosa cells has started and they are developing diffuse lipids in their cytoplasm. $\times$ 373.
(Facing p. 384)
relatively more abundant in the outer portions of corpora lutea. The cell organelles of the 3-day-old luteal cells are obscured by the accumulation of cytoplasmic lipids. The cells derived from the theca interna cannot be distinguished from the granulosa lutein cells (Pl. 2, Fig. 6); they seem to have degenerated and disappeared. The 3- to 4-day-old corpora lutea are less vascular and more fibrous than the earlier stages. Further increase in lipids of 4-day-old luteal cells is due to the storage of more triglycerides and cholesterol and/or its esters.

**DISCUSSION**

The histochemical techniques used in the present investigation demonstrated orderly changes occurring in response to ovulatory gonadotrophin (hCG) in the membrana granulosa and theca interna of pre-ovulatory and post-ovulatory follicles in the ovaries of rabbits. The membrana granulosa in follicles of oestrous rabbits contains two types of lipid bodies (L₁ and L₂). The L₁ consist of phospholipids and some triglycerides. The presence of L₁ bodies in the basal portions of the membrana granulosa, lying near the blood vessels of the theca interna, is probably of physiological significance, as similar lipid droplets occur also in the theca interna cells. The amount of L₁ bodies is slightly increased in the membrana granulosa of pre-ovulatory and post-ovulatory follicles. The luteal cells (derived from the granulosa cells) of 3- to 4-day-old corpora lutea are filled with the L₁ bodies. Simultaneously, they (L₁) begin to show relatively more triglycerides and cholesterol and/or its esters. The sites of distribution of L₁ bodies suggest that they are intimately connected with the synthesis of steroid hormones. They are probably identical with the lipid inclusions of Björkman (1962) who studied them with an electron microscope in the granulosa cells of pre-ovulatory and post-ovulatory follicles of the rat ovary. The L₂ bodies consist of phospholipids. Such lipid bodies (L₂) also occur in the normal granulosa cells and oocytes of other mammals (Guraya, 1964, 1965a; Guraya & Greenwald, 1964a, b). They may supply energy-rich substances (phospholipids) for different metabolic activities (Guraya, 1965a, b). The L₂ bodies decrease in number.

**EXPLANATION OF PLATE 2**

Figs. 4 to 7 are photomicrographs from frozen gelatin sections of post-ovulatory follicles (corpora lutea) prepared as in Figs. 1 to 3.

**Fig. 4.** Portion of 1-day-old corpus luteum, showing the distribution of lipids in the luteal cells (derived from the granulosa cells) and theca interna cells. The latter, which are filled with coarse lipids, are lying at the periphery of the corpus luteum. Vascularization of the luteal cells has reached its maximum development. × 229.

**Fig. 5.** Higher power view of luteal cells shown in Fig. 4. Note the appearance of diffuse lipids in the cytoplasm of luteal cells which are of spherical shape. Such lipids do not show any conspicuous development in the granulosa cells shown in Pl. 1, Fig. 1. The luteal cells are arranged into groups of various sizes by the stromal tissue. × 367.

**Fig. 6.** Peripheral portion of 3-day-old corpus luteum, showing the accumulations of coarse lipids, and sudanophobe vacuoles in the luteal cells. × 367.

**Fig. 7.** Central portion of 3-day-old corpus luteum, showing the accumulations of coarse lipids and sudanophobe vacuoles in the luteal cells. Note that the central portions of such corpora lutea contain relatively less lipid accumulations in comparison to the outer portions shown in Fig. 6. × 367.
during the pre-ovulatory and post-ovulatoty periods when they also develop triglycerides in addition to their phospholipids. In the luteal cells of 3- to 4-day-old corpora lutea the distinction between the L₁ and L₂ bodies disappears as the sudanophilic lipids (L₁) (consisting of mainly triglycerides, some phospholipids, and cholesterol and/or its esters) accumulate in them. Similar lipids are stored in the 3- to 4-day-old corpora lutea of non-pregnant hamsters (Guraya & Greenwald, 1965). It is generally believed that when the steroidal lipids are abundantly present in the steroid-secreting cells, storage is taking place, and when the amount is less, release of hormone is occurring (Guraya, 1966).

The most significant change in the post-ovulatoty follicles is the sudden demonstration of diffuse lipoproteins throughout the cytoplasm of granulosa lutein cells. Similar lipoproteins also develop during the transformation of granulosa cells into luteal cells in the human ovary (Guraya, 1968). They seem to derive from the abundant agranular endoplasmic reticulum which develops during the so-called luteinization of granulosa cells (Björkman, 1962; Green & Maqueo, 1965; see also review by Christensen, 1965). The increase in diffuse lipoproteins may be related to enzyme systems involved in steroid synthesis (see Christensen, 1965; Christensen & Fawcett, 1966). It is also possible that the diffuse lipoproteins might bind sufficient amounts of cholesterol for use as a substrate for steroid synthesis. This ‘bound’ cholesterol could not be demonstrated with the histochemical techniques. The scarcity of diffuse lipoproteins or agranular endoplasmic reticulum (Björkman, 1962) in the granulosa cells during follicular growth suggests that they are probably not synthesizing much steroid. This is also supported by histochemical studies on the oxidative enzymes in the granulosa cells (Deane, Lobel & Romney, 1962; Fienberg & Cohen, 1965). However, all the histochemical observations including the present one, as well as the ultrastructural studies, disagree with in-vitro experimental studies which have implicated the granulosa cells of follicles in steroidogenesis (Ryan & Petro, 1966; Ryan & Short, 1965).

The hypertrophied theca interna cells of pre-ovulatory follicles have been called the theca gland cells (Mossman, Koering & Ferry, 1964). In the present material, cholesterol and/or its esters are stored in the theca interna cells of oestrous follicles but not in the theca gland cells. This indicates that the thecal gland cells are metabolically more active, presumably in the production of oestrogens, during the pre-ovulatory growth of the follicle. Immediately after ovulation, the thecal gland cells (theca lutein cells) begin to store coarse lipids consisting of triglycerides and some phospholipids, suggesting some change in their lipid metabolism. The thecal gland cells filled with coarse lipids are still visible at the periphery of 1-day-old corpora lutea. In the 2- to 3-day-old corpora lutea, they degenerate and disappear from view by storing cholesterol and its esters. The thecal gland cells in the human ovary also store coarse lipids just after ovulation (Guraya, 1968).

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