Morphological and Functional Development of the Ovary of the Mouse

I. Morphology and Histochemistry of the Developing Ovary in Normal Conditions and after FSH Treatment

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WITH THREE PLATES

Maturation and maintenance of ovarian function are under the control of the pituitary gonadotrophins. The capacity of the ovary to respond to FSH stimulation develops gradually with age, and is most pronounced when the animal approaches sexual maturity. In very young animals reactivity to gonadotrophins is found to be lacking, and up to a certain age, which is characteristic for each species, sexual maturity cannot be accelerated by exogenous gonadotrophic stimulation (for references see Price & Ortize, 1944). However, in transplantation experiments of young ovarian tissue into adult spayed hosts, the grafted ovary reaches maturity much earlier than in its normal surroundings (Goodman, 1934; Dunham, Watts & Adair, 1941).

In an attempt to clarify further the influence of the adult host on the development and maturation of the ovary, the normal growth of the mouse ovary and its reactivity to FSH from birth to maturity has been compared with the development of these young ovaries transplanted into adult hosts.

In this first part, dealing with normal growth the correlation between differentiation of ovarian tissue and its sensitivity to FSH is considered; morphological observations of the developing ovary are supplemented by histochemical data which could be instrumental in revealing tissue-differentiation.

In the second part of the investigation (in preparation) the development of the

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young transplanted ovaries is described. Special attention is focused on the relationship between age of donor and rate of acceleration of the maturation process.

MATERIAL AND METHODS

For each experiment 8–12 female albino mice of a local strain, and taken from 3–5 different litters, were used. Litter mates served as controls. The following age groups were experimented with: 1, 3, 6, 9, 12, 15 and 18 days old.

In an attempt to administer physiological doses of FSH, mice (18–22 days old), which previously had been found to be most sensitive to the hormone, were tested for their sensitivity to the gonadotrophin preparations so as to determine the optimal dose of the hormone and the time interval required for maximum effect. The gonadotrophins used were: Gonadotrophin FSH–Serum Gonadotrophin–Paybyrn, and Equinex–PMS Gonadotrophin–Ayerst. The first was given subcutaneously and the second intramuscularly in a single injection. The optimal dose for the 18-day ovary was 8 i.u., with the effect fully manifest after 72 hours. Considering the decline in sensitivity with the decrease in age (Smith & Engle, 1927), the dose of the hormone administered to the very young mice was relatively higher. (Details for each experimental group are given in the results.) Generally the animals were sacrificed 72 hours after treatment, except when the hormonal effect on the later development of the ovary was to be examined.

Ovarian response was determined as follows: 1. By following up the morphological development of the ovarian tissue and measuring the maximal length of the organ and diameter of the largest follicle present in histological preparations. The ovaries were fixed in Zenker-formal solution (9:1), sectioned in series at 8\(\mu\) for hematoxylin eosin staining, at 4\(\mu\) for Gomori's chrom–alum phloxin stain, and at 10\(\mu\) for the PAS reaction. 2. By observation of macro- and microscopical changes in the uterus. 3. By histochemical methods.

Histochemical methods

Lipids

The ovaries were fixed overnight in 10 per cent neutral formalin, sectioned at 15\(\mu\) on the freezing microtome, stained for total lipids in a 0.5 per cent solution of Sudan IV in 70 per cent alcohol, and for cholesterol by the Schultz method according to Lillie (1954).

Ascorbic acid

According to Dean & Morse (1948). Rapid fixation of the ovaries, 40–50 seconds after the animal's death in alcoholic silver nitrate for half an hour in a dark bottle, subsequently transferred to bisulphate–thiosulphate solution for 2 hours and washed overnight with tap water. The paraffin sections were cut at 5\(\mu\) and counterstained with azocarmine.
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Alkaline phosphatase

By the method of Gomori (1939). Ovaries were fixed in 80 per cent alcohol in the refrigerator at 5°C for 24 hours, and after rapid dehydration embedded in paraffin in vacuum for 10–15 minutes. Sections cut at 5 μm were incubated for half an hour in Na-glycerophosphate, hexosediphosphate and yeast nucleic acid, respectively. All solutions were made in barbital buffer at pH 9.6. Intensive enzyme activity appeared exclusively in the Na-glycerophosphate, so that only this substrate was used later.

Mucopolysaccharides and glycogen

The Periodic Acid Schiff technique (McManus, 1946; Hotchkiss, 1948) was used on some sections after Zenker-formol fixation and on others after 10 per cent neutral formalin. To distinguish between mucopolysaccharides and glycogen, sections were digested by saliva or 0.1 per cent solution of diastase (Lillie, 1954). The digestion was controlled by including liver sections in the jar.

RESULTS

The dimensions of the ovary at different stages of development in normal (control) conditions and after FSH treatment are summarized in Text-fig. 1. The long axis of the organ and the diameter of the largest follicle are presented. It may be seen that the life span from birth to maturity can be subdivided into three periods according to the degree of tissue reactivity to the trophic hormone. This is in agreement with the data reported by Rennels (1951), who subdivided this period in the rat into infantile, early juvenile and juvenile (or prepubertal) periods. The duration of each of these periods differs slightly from that reported for the rat.

The infantile period—from birth to the sixth day

Follicular organization in the ovary of the mouse begins soon after birth, and during the infantile period small primary follicles with two layers of typical granulosa cells are developed. The morphological characteristic at the end of this period is the establishment of the primary theca—a single layer of flattened, long nucleated cells.

The histochemical preparations from this period show that the amount of sudanophilic substance is very small; it is scattered in droplets in the granulosa of the primary follicles and takes the form of tiny islands in the interstitial cells at the end of the infantile period. The substance does not react when put to the Schultz cholesterol test. The ascorbic acid content of the ovaries is low, only a few black granules showing up in the egg cell. A slight alkaline phosphatase activity appears only towards the end of this period in some groups of the interstitial cells (Plate 2, Fig. 9). Tests with PAS technique reveal the fine fibrillar stroma, and at the end
of the sixth day the existence of a reactive membrana granulosa and zona pellucida. Only a few drops appearing in the egg cytoplasm proved to be glycogen.

Treatment with FSH 2 i.u. at birth or 4 i.u. on the third day, produced no deviation from the normal controls. As Text-fig. 1 shows, there are no changes in the dimensions of the organ nor in its morphology or histochemical reactions. The ovary is, as yet, refractory to hormonal stimulus.

The early juvenile period—from 7 to 15 days

The morphological development of the ovary during this period is a slow process. Follicles continue to grow into 3–4 granulosa layers and the second layer of the theca is established. An important morphological change at the end of this period is the beginning of antrum formation. The ovary is densely packed with primary follicles of different sizes separated by flattened cells of the stroma and by interstitial cells (Plate 1, Fig. a).

Histochemical preparations reveal the gradual process of differentiation of ovarian tissue during this period. There is an increasing amount of sudanophilic material in the granulosa of the larger follicles as well as in islets of interstitial cells. In time the sudanophilic material accumulates in the latter, forming a red background against which the reactive droplets in the granulosa become blurred. Positive reaction to cholesterol appears in some cords of the interstitial cells and as droplets in the granulosa at the end of this period only.

The content of ascorbic acid rises gradually, still scattered in the tissue as in the earlier stage, but tending to concentrate in the eggs of the larger follicles. It is mainly manifested in the ovaries of 12–13 days.

Signs of alkaline phosphatase activity appear in the theca cells in the course of their organization at about the ninth day and in the interstitial cells, mainly concentrated in the nuclei (Plate 2, Fig. h). Reactivity of the theca increases and by the twelfth–thirteenth day, all larger follicles are surrounded by a blackened theca (Plate 2, Fig. i).

Reactivity to PAS increases due to the gradual thickening of the fibrillar portions of the theca and the zona pellucida. The latter appears to be brought about by droplets of intensely reactive material, not affected by saliva, found in the egg cell and in the adjacent cells of the granulosa. New reactivity centres appear in the small follicular cavities and in the nearby intercellular substance.

In addition it was found that in some of the larger eggs the whole cell area reacted as intensely as its pellicle. Since such intense reaction to PAS is characteristic of the eggs of atretic follicles (Harter, 1948) found in the course of later development, it would appear that atretic features occur much earlier than hitherto assumed, and that their existence could be revealed through histochemical reactions though the morphological picture of the follicles at that age does not show atretic signs.

Treatment with 4 i.u. FSH at 6 days, or with 6 i.u. at 9 or 12 days, caused deviation from the normal developmental pattern. Changes first occur on the ninth
PLATE 1

FIG. A. Twelve days—control. Gomori’s chrom–alum phloxin stain.

FIG. B. Twelve days, 3 days after FSH. Note the lutein-like appearance of the interstitial cells. Gomori’s chrom–alum phloxin stain.

FIG. C. Fifteen-day ovary, 6 days after FSH. Follicles disintegrate and contribute to increase in interstitial tissue. Hematoxylin-eosin.

FIG. D. Fifteen days, 3 days after FSH. Irregular follicular response persists. Hematoxylin-eosin.

FIG. E. Eighteen-day ovary—control. Hematoxylin-eosin.

FIG. F. Twenty-one days, 3 days after FSH. Morphological and functional maturity is reached. Hematoxylin-eosin.

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Plate 2

Alkaline phosphatase reactivity in the developing ovary

Fig. G. Six-day ovary. Fig. H. Nine-day ovary. Fig. I. Twelve-day ovary. Fig. J. Seventeen-day ovary. Fig. K. Thirty-day ovary.

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PLATE 3

PAS reaction in the early juvenile ovaries

Fig. L. Twelve-day ovary—control. Fig. M. Twelve days, 3 days after FSH. Glycogen is accumulated in the interstitial cells between almost all the larger follicles. Fig. N. Fifteen days, 3 days after FSH. Glycogen is accumulated in some nests of interstitial cells.

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day (3 days after FSH) the most prominent being the hypertrophy of the interstitial cells and the appearance of cavities among the granulosa cells, which cause a disturbance in their regular organization (Plate 1, Fig. b). The hypertrophied interstitial cells contribute to a slight increase in size of the ovary, while follicle dimensions remain unchanged (Text-fig. 1). The deviation becomes more pronounced with the passage of days and produces a disorganized follicular response involving almost all the follicles (Plate 1, Fig. c).

**TEXT-FIG. 1.** Dimensions of the ovary at different stages of development in normal conditions and after FSH treatment. Each point represents the average value of 8 ovaries. The vertical lines express S.D.
Histochemically there is an appearance of materials which do not occur in normal development and, conversely, an acceleration of already existing processes. The latter is evident in an increased accumulation of sudanophilic substances in the hypertrophic interstitial cells, the former in concentrations of PAS-positive material, apparently glycogen (Plate 3, Fig. m).

In the period between the twelfth and fifteenth day, the hypertrophy of the interstitial cells, though still marked, is on the decline when compared with the earlier days; disorganized follicular response persists (Plate 1, Fig. d). The accumulation of PAS-positive material is smaller than previously (Plate 3, Fig. n) and sudanophilic material from interstitial cells is on the decrease. In correlation with the latter fact, the uterus shows a slight response to hormonal activity of the ovaries; it becomes distended and hyperaemic, and the endometrial epithelium is folded.

The juvenile (or prepubertal) period

From the fifteenth day to onset of ovulation

The earliest ovulation occurs at 30 days. The following 15–18 days mark the later phases in the development of the primary follicles. They are characterized by the high mitotic activity of the granulosa and a further thickening of the theca owing to an increase in its cellular and fibrillar constituents and the multitude of capillaries present in the theca interna. During the process of follicle growth mitotic figures are found side by side with cell death shown by pycnotic nuclei (Plate 1, Fig. e).

In the last stages towards maturity, when the Graafian follicles develop, atresia becomes evident for the first time. The cells of the disintegrating atretic follicles contribute by adsorption to the increase in interstitial tissue.

The lipid background, in which the 18-day follicles are embedded, spreads so as to include the theca with the interstitial cells. Part of the lipids proved to be cholesterol. It is characteristic of the 15–18 day ovaries that the granulosa loses the hitherto present reactivity. Sudanophilic substances abound in the maturing ovary: in the interstitial cells, in the theca and in the granulosa of some Graafian follicles. Part of the sudanophilic substances respond to Schultz reagent. The cholesterol content of atretic follicles is very pronounced.

Ascorbic acid concentrates in the eggs of small and medium follicles rather than in those of the larger ones. Toward maturity, local concentrations of the vitamin are found in the theca interna and in the interstitial cells. Alkaline phosphatase activity, in the 15–18-day ovaries is high in the walls of the arteries and in the theca of some of the larger follicles and absent in those that have shown enzyme activity at an earlier stage (Plate 2, Fig. j). Later on, towards the end of the juvenile period, high enzyme activity is evident in the theca of almost all maturing follicles (Plate 2, Fig. k). By the PAS technique, in addition to the reactive sites already mentioned, the reactivity of the follicular fluid is prominent. Upon
treatment with diastase the pink cytoplasm of the eggs shows a brighter colour, indicating uniform distribution of glycogen.

FSH treatment during the juvenile period accelerates the natural development of the ovary. Six i.u. administered to 15-day old animals induced morphological and histochemical changes which follow the pattern of normal development, disorganized follicular response recedes and growth becomes regulated. At that time only 1–2 follicles reach the Graafian stage. Follicular growth being confined to only a small number of follicles does not contribute much to the increase in size of the organ (Text-fig. 1).

FSH causes depletion of sudanophilic substances located in the theca and interstitial cells. Accordingly, there is an enhanced uterine response; new endometrial glands being formed. PAS positive material, which accumulated in the earlier period under the influence of gonadotrophin, is now almost completely lacking.

In the next 18–21 days, in which the optimal dose of FSH (8 i.u.) is administered, the response reaches a peak (Plate 1, Fig. F). The ovaries, being most reactive, were examined 24, 48 and 72 hours after treatment, so as to follow the developing reactivity. In this reactivity process two main features could be observed: 24 hours after stimulation there was a pronounced reactivity of the interstitial cells and the theca, which underwent hypertrophy with the cytoplasm becoming strongly chromaffinic. Histochemically, there was a depletion of sudanophilia in the wake of stronger hormonal secretion. Forty-eight hours after treatment the follicles grew rapidly, while interstitial reactivity declined to a point of being almost non-existent after 72 hours, when Graafian follicles reached full maturity. At that time (72 hours) the ovary is 2–2.5 times larger than that of the control. Atretic follicles are found among the ripening ones. Ovulation and luteinization follow soon after. Regarding histochemical changes, after the depletion of sudanophilic substances, there is a continuous production and re-accumulation, with the highest content of cholesterol appearing 48 hours after treatment.

**DISCUSSION**

Division of the immature period into the ‘infantile’ (when the ovary is refractory to gonadotrophins) and the ‘juvenile’ (the interval between the appearance of responsiveness and the first oestrus) was already suggested by Clauberg (cited by Hisaw, 1947) in 1932. The results of observations on normal development and the parallelisms and deviations occurring under the influence of FSH treatment, as presented in this paper, justify the further subdivision of the juvenile period into the ‘early juvenile’ and ‘juvenile or prepubertal’, as suggested by Dawson & McCabe (1951) and by Rennels (1951) for the rat.

FSH accelerates growth and maturation of the ovary only when given in the prepubertal (juvenile) period. Administration of the hormone at earlier stages, in the infantile period (0–6 days) finds the ovary refractory to stimulation, while
during the early juvenile period (7–15 days) it causes deviation from normal development.

Histochemical tests for lipids and cholesterol, the latter assumed to be a precursor of the active oestrogenic hormone (Cleasson & Hillarp, 1947a and b), point to the interstitial tissue of the immature mouse ovary as being the primary source of oestrogen production. This is in agreement with the findings of Dawson & McCabe (1951) and Rennels (1951) for the rat.

The correlation between the decrease in total ovarian lipids and the biological effect on the uterus suggests that most of the lipids are pro-oestrogenic and perhaps even active hormonal substances (Emmens, 1940–41). The initial accumulation of this sudanophilic material occurs already from the sixth day and it is not clear whether gonadotrophins are needed for its initiation, as the early juvenile ovary does not depend on FSH (Smith, 1930). It appears that the gonadotrophins accelerate the already existing process in the interstitial cells while initiating its appearance in the theca interna and enhancing the secretory activity.

The nature of the lipid droplets which appear in the granulosa during the early juvenile period, and are clearly absent at later stages of development of the primary follicles, is unknown and requires study with more specific methods.

High ascorbic acid concentrations are known to be found in steroid producing tissues—such as in the adrenal cortex and the ovary. The amount is affected by the action of the trophic hormones ACTH (Sayers, Sayers & Woodbury, 1948), PMS gonadotrophin (Cleasson, Hillary, Högborg & Hökfelt, 1949) and LH (Miller & Everett, 1948). However, the ascorbic acid content in young immature mouse ovaries was found to be very low (though steroid synthesis was shown to exist), and FSH treatment did not produce any change in these ovaries. Concentrated amounts of the vitamin were found only in the cytoplasm of the growing egg cells. The appearance of specific localization of ascorbic acid in the theca and the interstitial cells was detected at the end of the prepubertal period only.

Determination of alkaline phosphatase in the young developing ovary is of particular interest because of its apparent activity in processes of differentiation and morphogenesis (Moog, 1944; Karczmar & Berg, 1951; Hinsch, 1960). The results here presented show a high concentration of the enzyme in two different stages of ovarian development. The first significant concentration appears in the theca of the follicles of ovaries 12–13 days old, and the timing of its increased activity coincides with the process of differentiation. The fact that the second appearance of a high content of alkaline phosphatase was found to coincide with the secretory activity of the theca might corroborate the suggestion that the function of the enzyme is connected with active transport (Moog, 1946).

While Moog & Wenger (1952) found that only the fibrillar theca and the fibres of the stroma showed alkaline phosphatase activity in the adult mouse ovary, the results of the present work indicate it to be mainly confined to the nuclei of the cells; the connective tissue fibres (in the theca and the medullary stroma) were found to be negative. The discrepancy in these findings may perhaps be due to
artefacts caused by diffusion of the enzyme or of calcium phosphate from sites of high activity, the adjacent fibres, and its adsorption upon the nuclei. The possibility of artefacts is reviewed by Novikoff (1955) and further discussed by Hinsch (1960).

Reactivity of the ovary to PAS technique, as well as the nature and function of its various mucopolysaccharide components, have been described and discussed by Harter (1948), Leblond (1950), Moog & Wenger (1952) and others. The present discussion is confined to glycogen accumulation in the early juvenile ovary induced by FSH treatment.

Deposition of glycogen is a phenomenon characteristic of the initial stages of lipogenesis (Wertheimer & Shafrir, 1960), and was described as occurring in the cumulus oophorus of atretic follicles in adult rats (Dean, 1952).

Production of glycogen in young ovarian tissue may be caused by the increased blood supply which is the first manifestation of FSH treatment. Its abnormal accumulation during the early juvenile period under the influence of FSH, may indicate the lower capacity of the ovaries to utilize it at this stage.

When the ovary becomes competent to react to FSH in the juvenile period, its metabolic rate is much increased, as shown, for instance, by succinic dehydrogenase activity (Eckstein, 1960). It would be interesting to follow the activity of this enzyme in the younger stages.

During the development of competence to react to FSH, the interstitial and theca cells are the first to respond. In the competent juvenile ovary, the hypertrophy of the interstitial cells disappears 24 hours after stimulation while oestrogen has already been secreted. Reactivity is then transferred to the follicular cells, which divide intensely and develop into the Graafian stage. The abnormal reactivity of the early juvenile ovary is manifested in the persisting hypertrophy of the interstitial tissue. This phenomenon is in contrast to the findings of Pfeiffer & Hooker (1942) in the mouse, but in agreement with investigations carried out on the rat (for references see Price & Ortize, 1944). Another irregularity lies in the development of the follicles which have not yet attained the full competence to react to FSH.

During the infantile and early juvenile period organogenesis of ovarian tissue is established. During this period inductive processes similar to those common in embryological systems take place (Hisaw, 1947). Grobstein (1956) has stated that 'Inductive processes have a measurable duration during which stability of new properties resulting from the induction is gradually rising. . . . Premature interruption of the process can give incomplete or atypical responses.' It would appear that FSH administration in the course of the early juvenile period is a further example of such an interruption. It is well known that ovarian follicular development, until the beginning of antrum formation, does not depend on FSH (Smith, 1930). The trophic hormone enhances normal development only from the time the ovary becomes dependent on it, i.e., by the end of the early juvenile period. Any earlier stimulation interferes with normal development and brings
about retrogression which is mainly expressed by the atypical response of the follicles.

**SUMMARY**

1. The post-natal development of the ovary during normal growth and under FSH treatment was analysed by morphological and histochemical methods.
2. The period from birth to maturity was found to be divided into three stages according to the degree of ovarian reactivity to FSH treatment.
3. Attention is mainly concentrated on the early juvenile period (7–15 days) in which the gonadotrophic stimulation interferes with the normal developmental process and brings about retrogression.

**RESUME**

Développement morphologique et fonctionnel de l'ovaire chez la Souris. I.—Morphologie et histochemie de l'ovaire en croissance dans des conditions normales ou après traitement à l'aide de FSH

1. Le développement post natal de l'ovaire en croissance normale ou soumis à l'influence de FSH a été analysé à l'aide de méthodes morphologiques et histo-chimiques.
2. Il a été observé que la période s'étendant de la naissance à la maturité peut être divisée en trois étales en se basant sur le degré de réactivité au traitement FSH.
3. L'attention s'est surtout concentrée sur la période juvenile précoce (7 ème au 15 ème j.) dans laquelle la stimulation gonadotrophe interfère avec le processus de développement normal et produit la rétroggression.

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