Sequential Ultrastructural and Histochemical Alterations
Associated with Atresia of the Preovulatory Follicle in the Rat

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SEQUENTIAL ULTRASTRUCTURAL AND HISTOCHEMICAL ALTERATIONS ASSOCIATED WITH ATRESIA OF THE PREOVULATORY FOLLICLE IN THE RAT

by

Mary Cristine England-Charlesworth

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Sciences

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VITA

The author, Mary Cristine England-Charlesworth, is the daughter of James D. England and Angeline (Corso) England, and is married to Jon E. Charlesworth. She was born on October 19, 1955 in Knoxville, Tennessee.

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INTRODUCTION

Shortly after birth in most mammals, stromal cells within the ovary surround the oocyte to form a primordial follicle. The follicle is destined to either grow and ovulate or degenerate at some stage in its development. In the rat ovary, the number of oocytes decreases from approximately 40,000 at birth to 10,000 by the onset of puberty (Arai, 1920). Although neonatal human ovaries contain close to two million ova, only 400 will be ovulated in the course of reproductive life (Baker, 1963). Thus, the majority of ova and follicles in the mammalian ovary will become atretic with only a small percentage ovulating.

The term "atresia" denotes the processes of deterioration that signal incipient follicular degeneration and death, and loss of viability of the oocyte. Aspects of follicular growth and function cannot be accurately defined without taking into account the factors which cause atresia and the possible consequences of atretic follicles on the ovarian environment.
REVIEW OF LITERATURE

FOLLICULAR GROWTH AND DEVELOPMENT

Follicular development is controlled by both intrinsic and hormonal factors. Investigations by Pedersen and Peters (1968) and Pedersen (1972) show that the follicle matures through progressive stages of growth from a non-proliferating primordial follicle to a large preovulatory follicle in nineteen days. The initial stimulus for growth is not known, but once started, the follicle continues to grow until it undergoes atresia or ovulates (Peters et al., 1975). Follicular growth appears to proceed in two phases. The first phase entails a slow proliferation of granulosa cells and growth of the oocyte lasting fourteen days; the second is characterized by antrum formation and accelerated granulosa cell growth rate lasting five days. Changes in gonadotropin receptor content of follicular components (Channing and Kammerman, 1974; Uilenbroek and Richards, 1979) and fluctuating endogenous hormone levels in the cycling animal (Butcher et al., 1974; Clark et al., 1979; Nequin et al., 1979), suggest that the progressive sequences of differentiation are under hormonal control.

Gonadotropins and/or estrogens do not seem to be involved in initiation of follicular growth (Eshkol and Lunenfeld, 1972; Goldenberg et al., 1973; Peters et al., 1973; Nakano et al., 1975), although serum levels of luteinizing
hormone (LH) and follicle stimulating hormone (FSH) are elevated during the early juvenile period up to day 20 (Meijs-Roelofs et al., 1973; Dullaart et al., 1975), when the rate of transition from non-proliferating to growing follicles is greatest (Pedersen, 1972). Studies of early follicular development in infant mice and rats demonstrate that FSH and LH can bind to small follicles in immature animals (Lamprecht et al., 1976; Peluso et al., 1976; Funkenstein et al., 1980), and are necessary for normal differentiation of the thecal and granulosa compartments (Lunenfeld et al., 1975). The presence of a well-developed thecal layer in the growing follicle is necessary for full response of the follicle to gonadotropins (Goldenberg et al., 1973).

Receptor sites for LH in the theca interna and FSH in the granulosa layer of prantral and antral follicles have been identified (LH: Midgley, 1973; Channing and Kammerman, 1974; FSH: Nimrod et al., 1976; Nakano et al., 1977; Zeleznik et al., 1977). LH and FSH acting on thecal cells and granulosa cells respectively, may synergistically increase the production of estrogen (Bjersing and Carstensen, 1967; Dorrington et al., 1975; Makris and Ryan, 1975; Armstrong and Papkoff, 1976; Ryan, 1979). Stimulation of estrogen synthesis by these hormones could induce further follicular growth since estrogen stimulates granulosa cell division (Bradbury, 1961; Merk et al., 1972; Harman et al., 1975a; Louvet and Vaitukaitis, 1976). Gonadotropins appear to increase steroidogenesis within the ovary by interacting
with specific receptor molecules in the membrane of target
cells, activating adenylate cyclase and thereby increasing
production of cyclic-AMP. Cyclic-AMP (cAMP) activates
protein kinase which mediates the effects of trophic hor-
mone by phosphorylation of specific enzymes necessary in
regulating target cell functions and protein synthesis,
leading to steroidogenesis (Catt and Dufau, 1976). LH and
FSH are both known to increase cAMP accumulation and are
believed to act by a cAMP dependent-protein kinase complex
(Tsafriri et al., 1976; Lindner et al., 1977; Zeleznik
et al., 1977; Channing et al., 1978; Richards et al., 1979).

Estrogen, in addition to its mitogenic effect on the
granulosa cells, increases the responsiveness of the cells
to FSH (Goldenberg et al., 1972b; Richards et al., 1976;
Richards and Midgley, 1976). In turn, FSH increases the
number of its own receptors and is able to stimulate the
final growth phase beginning with the development of the
antrum (Thibault, 1977; Zeleznik et al., 1979), and induc-
tion of LH receptors in the granulosa cells (Richards and
Midgley, 1976; Nimrod et al., 1977; Erickson et al., 1979;
Zeleznik et al., 1979). FSH not only increases the number
of LH receptors with time, it also enhances the cellular
response of ovarian adenylyl cyclase to LH (Lindner et al.,
1977). When the number of LH receptors is sufficient, the
follicle is able to ovulate in response to an LH surge
(Uilenbroek and Richards, 1979). The sequence of events
described for development of the preovulatory follicle
depends on a progressive differentiation of granulosa cell ability to respond to hormones. Subtle changes in the follicular environment or responsiveness of the follicle could initiate alterations that cause atresia.

MORPHOLOGY AND HISTOCHEMISTRY IN HEALTHY AND ATRETIC PREOVULATORY FOLLICLES

The ovarian follicle is composed of thecal and granulosa cells separated by a basement membrane, and an oocyte surrounded by granulosa cells. Each component is altered during atresia.

Granulosa Cells

The granulosa layer can be divided into three areas: the basal or mural granulosa near the basement membrane, the membrana granulosa and the antral granulosa. All the granulosa cells, including the cells of the cumulus oophorus which surround the oocyte, are either cuboidal or columnar in shape. The granulosa cell contains a large nucleus, oval lamellar mitochondria, Golgi apparati, multivesicular and lysosome-like bodies, rough endoplasmic reticulum, numerous ribosomes and a scattering of lipid droplets in the basal granulosa (Björkman, 1962; Bjersing, 1967a, 1967b; Byskov, 1969; Zamboni, 1974; Hay et al., 1976).

Lysosomes, in addition to their degradative function, may play a part in regulating cellular secretion (Motta,
1969) and contributing to steroidogenesis, possibly by reducing cholesterol esters to free cholesterol (Strauss et al., 1978). In large preovulatory follicles, the granulosa cells may show an increase in smooth endoplasmic reticulum and vesicular mitochondria resembling lutein cells of newly-formed corpora lutea (Björkman, 1962; Bjersing, 1967b). Granulosa cells have ultrastructural characteristics of actively growing, protein-synthesizing cells (Guraya, 1973), but they are steroidogenic also, producing estrogen, androgens and/or progesterone depending on species and stage of follicular development (Makris and Ryan, 1975; Moor et al., 1978; McNatty et al., 1979). Conversely, Channing and associates (1978) suggest that granulosa cells, at least in the pig and monkey, are steroidogenically inactive until luteinization.

The function of intercellular connections known as "gap", "tight" or "nexus" junctions and "zona occludens" are of particular interest. They are common between adjoining granulosa cells and are characterized by a particulate interface (Amsterdam et al., 1974) and a distinct pentalaminar appearance (Espey and Stutts, 1972; Merk and McNutt, 1972; Albertini and Anderson, 1974). Also, annular nexi, vacuoles delineated by the same 5-layered membrane, are often found in granulosa cells (Merk et al., 1973; Larsen and Tung, 1978). Espey and Stutts (1972) suggest the following possible functions of these connections: 1. to increase intercellular cohesion, 2. to transport nutrients
across the granulosa layer, 3. to facilitate movement of follicular fluid to the antrum and 4. to permit the layers of granulosa cells to respond as a unit. Sheridan (1971) has mentioned the possibility of intercellular passage of cAMP between cells joined by nexi in mouse brown fat. The junctions then may also serve to allow a more uniform response to gonadotropin stimulation, an idea that concurs with the suggestion that these junctions may be related to the appearance of gonadotropin receptors on the cell membrane (Albertini and Anderson, 1974; Fletcher et al., 1979). In atretic follicles, the zona occludens are reduced, probably accounting for the disruption of the granulosa cell layer and decreased cell adhesion (Hay et al., 1976).

Other structural changes associated with atresia appear to occur in stages similar to those described histologically. Byskov (1974) defined three stages of follicular atresia in the mouse using ³H-thymidine incorporation. Three similar stages were reported in atresia of ovine follicles (Hay et al., 1976). From these studies and observations in other animals (rat: Deane, 1952; Lobel et al., 1961; Peluso et al., 1977a; guinea pig: Harman and Kirgis, 1938; rabbit: Nicosia et al., 1975; hamster: Knigge and Leathem, 1956; sheep: Turnbull et al., 1977; Hay and Cran, 1978; Carson et al., 1979; cow: Lobel and Levy, 1968; human: Bomsel-Helmreich et al., 1979; six species: Guraya and Greenwald, 1964), atretic follicles can be classified histologically in one of three distinct stages. Follicles in Stage I exhibit pyknosis of
the granulosa cells (condensation and extrusion of the nucleus of dead cells into the antrum), a decrease in mitotic figures, some detachment and vacuolization of the antral granulosa cells and thinning of the granulosa layer. Stage II follicles show a marked increase of pyknosis, phagocytic cells, absence of dividing cells and disruption of the granulosa cells. In Stage III, the follicle is sometimes collapsed, obliterating the antral cavity; the granulosa layer is disintegrated, invaded by leukocytes, and the basal lamina is discontinuous.

Ultrastructural studies confirm these observations. Pyknotic nuclei are present in the antrum and vacuoles (possibly dilated endoplasmic reticulum) are numerous in antral granulosa cells. Increased lipid throughout the membrana granulosa, degenerating cells with autophagic vacuoles, phagocyte-like cells invading the granulosa layer through a disrupted basement membrane and disintegration of the cohesive granulosa cells are the characteristics of an atretic follicle (Vazquez-Nin and Sotelo, 1967; Hay et al., 1976; Peluso et al., 1979b). These features are exaggerated as atresia progresses.

Histochemical studies have also defined alterations in follicular function as atresia progresses in large follicles. Granulosa cells of large preovulatory follicles normally contain small amounts of lipid near the basement membrane. Lipid content in the membrana granulosa of atretic follicles is greatly increased (Lobel et al., 1961; Guraya and Green-
walld, 1964; Motta and Bourneva, 1970). One exception is in the guinea pig where the granulosa cells remain free of lipid during atresia (Adams et al., 1966). In addition, the content of lipid droplets in the granulosa is changed from primarily phospholipid to triglycerides, cholesterol and cholesterol esters. This conversion occurs before histological signs of atresia appear, indicating an early alteration in steroid metabolism (Guraya and Greenwald, 1964). However, 3-β-hydroxysteroid dehydrogenase (3-β-HSD) activity which is observed in mature follicles, remains even in late stages of atresia (Pupkin et al., 1966; Lobel and Levy, 1968; Motta and Bourneva, 1970; Motta, 1972; Bomsel-Helmreich et al., 1979; Hay et al., 1979; Peluso et al., 1979b). 3-β-HSD converts pregnenolone to progesterone; its presence indicates that atretic follicles are still steroidogenically active.

Activity of lactate dehydrogenase and glucose-6-phosphate dehydrogenase, important enzymes in carbohydrate metabolism, is decreased in atretic follicles (Pupkin et al., 1966; Peluso et al., 1979b). Concomitantly, the growth rate or mitotic index is decreased (Byskov, 1977; Peluso and Steger, 1978) and acid phosphatase activity is high (Lobel et al., 1961; Banon et al., 1964; Lobel and Levy, 1968; Peluso et al., 1977a). Using histochemical procedures, acid phosphatase, a hydrolytic enzyme of lysosomal origin, is not demonstrable in healthy follicles. However, electron microscopic cytochemical studies of at-
retic preovulatory follicles demonstrate an increase in acid phosphatase activity in autophagic vacuoles. Also, in both healthy and atretic follicles, acid phosphatase activity is found in the Golgi complex and smooth endoplasmic reticulum of granulosa cells, indicating a possible role for acid phosphatase in steroidogenesis (Dimino et al., 1977; Elfont et al., 1977). The presence of high acid phosphatase activity in degenerating follicles is predictable, but when it first appears and what triggers its activity in the atretic process is unknown.

Thecal Cells

The thecal layer is divided into the theca externa consisting of fibroblasts, connective tissue and contractile elements, and the theca interna, which is composed of fibroblasts, transitional cells (thought to be undifferentiated gland cells; Hiura and Fujita, 1977) and gland or steroidogenic-like cells, similar to steroid-secreting cells in other tissues (Byskov, 1969; Christensen and Gillim, 1969; Motta, 1969; Hiura and Fujita, 1977). Features of steroid-secreting cells include large quantities of lipid, abundant tubular smooth endoplasmic reticulum, dispersed Golgi elements and vesicular mitochondria (Christensen and Gillim, 1969). Thecal cells are connected by gap junctions, although the junctions appear less frequently than in the granulosa layer, and annular nexi are rarely seen (Amsterdamm and Lindner, 1977; Fletcher, 1977). The thecal layer also is wellvascularized (O'Shea et al., 1978).
During atresia in most mammals studied, the thecal cells do not show degenerative changes at all or not until late atresia. Guraya (1974) proposed that thecal cells from atretic follicles are the source of interstitial gland tissue on the basis of ultrastructural characteristics such as luteinization and hypertrophy of the thecal cells. O'Shea and associates (1978) and Lobel and Levy (1968) find no evidence for this theory in the sheep and cow where degenerative modifications including nuclear and cytoplasmic condensation and fragmentation in the thecal layer occurred by Stage III atresia. A decreased blood supply in the theca interna has also been proposed as contributing to atresia (Hay et al., 1976).

Lipid content in thecal cells of healthy follicles is mainly phospholipid and cholesterol, and atresia does not seem to change the lipid composition (Guraya and Greenwald, 1964). 3β-Hydroxysteroid dehydrogenase activity is at a level comparable to that of the thecal layer in healthy follicles (Lobel and Levy, 1968; Motta and Bourneva, 1970; Bomsel-Helmreich et al., 1979). Histochemical observations of the thecal compartment are sparse due to the much greater attention focused on the changing function of the granulosa cells and their relationship with the oocyte.

The Oocyte

Two to four layers of granulosa cells called the cumulus oophorus, surround the oocyte and connect it to the
membrana granulosa. The cumulus-oocyte integrity remains stable during atretic alterations in the granulosa cells. The corona radiata, the layer of cells closest to the oocyte, send numerous cytoplasmic projections through the zona pellucida to contact microvilli on the oocyte. Gap junctions are often found at these points of contact (Amsterdam and Lindner, 1977). This interaction between cumulus cells and the oocyte is thought necessary to provide nutrients and other materials to the oocyte (Guraya, 1973; Zamboni et al., 1974). It may also be of importance in maintaining the oocyte in meiotic arrest at the diplotene state (Tsafriri and Channing, 1975; Channing et al., 1978). During atresia, the close spatial relationship is lost when the coronal cell projections retract, the microvilli decrease in size and number and the zona pellucida thickens. Concurrently, germinal vesical breakdown and an apparent resumption of meiosis within the oocyte occurs as a consequence of isolation of the oocyte (Bjorkman, 1962; Vazquez-Nin and Sotelo, 1967; Peluso et al., 1977a). These degenerative changes are not seen, however, until late stages of atresia.

For a healthy follicle in the mouse to reach the final stages of atresia takes four days according to labeling index studies on granulosa cell incorporation of $^{3}$H-thymidine (Byskov, 1974). Pyknotic nuclei are the initial criterion for atresia used in most investigations. Clearly, the presence of pyknotic nuclei from dead cells indicates that
Degenerative changes are occurring in the follicle before atresia is detectable by histochemical and/or histological methods.

STERIDOGENESIS IN HEALTHY AND ATRETIC PREOVULATORY FOLLICLES

During the antral phase of follicular growth, follicles are very active steroidogenically. Follicular development up to this stage has been discussed previously. This section reviews aspects of steroidogenesis in the pre-ovulatory follicle.

The rat comes into estrus every four to five days. Gonadotropin levels surge to about ten times the basal level on the afternoon of proestrus (Butcher et al., 1975; Nequin et al., 1979). LH initiates the process of ovulation, but the significance of high levels of FSH is not clear. It is certain, however, that both LH and FSH act on the preovulatory follicle to stimulate steroidogenesis.

In vivo and in vitro studies have shown that estrogen is produced by the aromatization of androgens, specifically testosterone and androstenedione. The enzymatic reaction occurs primarily in the granulosa cells and is controlled by FSH (Dorrington et al., 1975; Armstrong and Papkoff, 1976; Armstrong et al., 1977; McNatty and Baird, 1978). Although Channing and Coudert (1976) maintain that the theca interna is the main source of estrogen in the human and primates, McNatty and Baird (1978) demonstrated that the granu-
losa layer is a main site of estrogen synthesis in the human. LH enhances conversion of cholesterol (the precursor of all steroid hormones) to pregnenolone and increases 3-β-hydroxysteroid dehydrogenase activity (Armstrong, 1968; Marsh, 1976; Edwards et al., 1977). A primary site of LH action is on the cells of the theca interna to increase androgen synthesis (Fortune and Armstrong, 1977; Ahren et al., 1979). This is the main source of androgen that is converted to estrogen in the granulosa compartment.

LH and FSH also stimulate progesterone secretion in the thecal and granulosa cells. Estrogen inhibits LH-stimulated progesterone production in granulosa cells of small porcine and rat follicles in vivo and in vitro (Thanki and Channing, 1977; Channing et al., 1978; Leung et al., 1979), presumably to prevent premature luteinization. Androgens, however, act with FSH to enhance progesterone secretion in granulosa cells (Nimrod and Lindner, 1976; Derrington and Armstrong, 1977; Lucky et al., 1977; Leung et al., 1979). That this is a true androgenic effect is supported by the fact that dihydrotestosterone (DHT), a non-aromatizable androgen, has the greatest synergistic action (Derrington and Armstrong, 1977; Leung et al., 1979). The ability of androgen to increase progesterone secretion is diminished as the follicle matures (Zeleznik et al., 1979). This may be due to an increased estrogen production during the last phase of growth and subsequent overriding inhibition of the stimulatory effect of androgen, although estrogen enhances
progesterone secretion in preovulatory follicles (Goldenberg et al., 1972a). A large dose of LH injected into estrogen- and FSH-primed immature rats initially increased the production of androgen and estrogen (Hillensjö et al., 1976). Dorrington and Armstrong (1977) have speculated that increased steroid and gonadotropin levels on proestrus induce luteinization of the granulosa cells and the increase in progesterone synthesis seen on late proestrus.

Thus, androgen, estrogen and progesterone production in the follicle is controlled by gonadotropins and modulated by steroids. A comparison of follicular fluid steroid content in healthy and atretic follicles gives an indication of steroidogenic activity in the follicles. In large atretic ovine follicles, total steroid secretion into follicular fluid is less than half the amount found in healthy follicles. Estrogen is the major steroid produced in normal follicles, whereas androgen is the prominent steroid found in fluid from atretic follicles. However, the total synthesis of androgen is decreased by 30-50% in atretic follicles (Moor et al., 1978). Progesterone concentrations remain essentially the same (Nicosia et al., 1975; Moor et al., 1978). Similar results were reported from studies on steroid production in granulosa cell cultures from healthy and atretic follicles (McNatty et al., 1979). It can be concluded from these data that one characteristic of atresia is a loss of aromatization ability in the granulosa cells.
where FSH is present (McNatty et al., 1979). Receptors for FSH and LH are greatly decreased in the granulosa cells of atretic follicles (Richards et al., 1976; Peluso et al., 1977a; Peluso and Steger, 1978; Carson et al., 1979), which may explain the lack of estrogen and build up of testosterone in the follicular fluid. Whether the loss of FSH receptors occurs prior to a decline in estrogen levels has not been determined, but Moor and associates (1978) suggest estrogen synthesis decreases very early in the atretic process.

HORMONAL INDUCTION OF FOLLICULAR ATRESIA

As ovarian follicles mature, they become increasingly dependent on gonadotropins and more sensitive to changes in hormone concentrations (Richards, 1975). Decreasing levels of FSH near the end of the estrous cycle has been suggested as a cause of antral follicle atresia which is high during this time (Thibault, 1977). However, the surge of FSH on proestrus may "save" a selected cohort of these follicles from atresia, thus enabling them to mature and ovulate in response to the LH surge of the next cycle (McClintock and Schwartz, 1968; Greenwald, 1974; Richards and Midgley, 1976; Hirshfield and Midgley, 1978a, 1978b). Injection of pregnant mare serum gonadotropin (PMSG) or FSH into immature animals increases the number of large healthy follicles by delaying or preventing atresia (Greenwald, 1974; Peters et al., 1975; Richards, 1975; Peluso et al., 1977a; Turnbull
et al., 1977). PMSG also decreases acid phosphatase and acid ribonuclease release, maintaining a stabilizing effect on lysosomal activity (Dimino and Reece, 1973). Deprivation of gonadotropins by hypophysectomy leads to atresia in medium and large follicles (McCormack and Meyer, 1962; Peluso and Steger, 1978). Antiserum to FSH given on proestrus impairs follicular maturation and ovulation in the succeeding cycle (Schwartz et al., 1973). FSH may exert its anti-atretic effect by stimulating estrogen production. Estrogen has an anti-atretic action independent of its positive effect on cell proliferation (Ingram, 1959; Harman et al., 1975b).

In contrast to atresia caused by hormone deficiency, androgens have been implicated in atresia of preantral follicles. Louvet and associates (1975) have shown that androgen produced in response to gonadotropins (specifically LH) in hypophysectomized immature rats acts to inhibit the stimulatory effects of estrogen on preantral follicle growth. This androgen-mediated response may also account for reports of atresia in small to medium follicles caused by LH (Harman et al., 1975b; Richards, 1975) and the large number of follicles which become atretic on proestrus (Hillier and Ross, 1979). The atretic effects of androgen on these follicles may be mediated through an altered granulosa cell estrogen receptor caused by translocation of the testosterone receptor complex (Hillier and Ross, 1979), thus withdrawing estrogenic support of growth. Alternatively, those follicles with insufficient numbers of FSH receptor may re-
spond to increased LH levels by degenerating, while those destined to ovulate will be "rescued" by the surge of FSH (Richards, 1975).

In larger preovulatory follicles, however, androgens have a stimulatory effect on follicular growth and ovulation. Preovulatory follicles gain an increased ability to produce androgen (Uilenbroek and Richards, 1979). Mori and co-workers (1977) demonstrated that testosterone administered one hour after an ovulating dose of human chorionic gonadotropin (hCG) and injection of antiprogestosterone restored ovulation that would have been blocked by the antiprogestosterone. Peluso and associates (1979d) have shown that blocking androgen action with antiandrogens, cyproterone acetate and flutamide, changes the preovulatory follicle so that it cannot respond to an LH surge by ovulating. In addition, in ovaries of rats treated with cyproterone acetate, the percent of atretic preovulatory follicles is greatly increased (Peluso et al., 1979c). Antiandrogen treatment to intact cycling rats decreased estrogen levels and follicular growth (Kumari et al., 1978). These observations suggest that a decrease in androgen at a critical stage of growth inhibits normal follicular maturation by a direct effect on the granulosa cells. Cyproterone acetate acts to inhibit dihydrotestosterone binding at its nuclear receptor (Liao et al., 1974), thus ruling out an effect in these studies mediated by loss of estrogen substrate.

From the above observations, it is clear that normal
growth of the follicle is dependent on a delicate ratio of androgens and estrogen in the follicle, and that much of gonadotropic action is mediated by steroids.

THE PMSG-PRIMED RAT MODEL

One problem encountered by researchers examining the atretic process is the lack of a uniform population of follicles in an ovary composed of numerous tissue types and steroidogenic potentials. Several models have been developed to study follicular atresia of a selected population of follicle types. Many of the investigations cited in this review have utilized immature hypophysectomized or intact female rats as a model system. Prepubertal rat ovaries, as described previously, contain a large population of small to medium follicles which undergo atresia in the absence of adequate gonadotropin stimulation. Administration of estrogen to hypophysectomized immature rats causes increased granulosa cell proliferation and the development of large preantral follicles. Subsequent treatment with FSH stimulates antrum formation and a uniform population of large normal-looking antral follicles. 48 to 60 h after a priming dose of FSH or PMSG, LH (hCG) can induce ovulation of the gonadotropin-induced preovulatory follicles (Peluso et al., 1977a). Induced follicular growth in this immature rat model system has been used extensively by different investigators in conjunction with cell culture techniques and studies of normal cycling adults to determine the pro-
gressive changes in hormonal secretion and response necessary for normal development of the preovulatory follicle.

Peluso and co-workers have refined the immature rat model for the purpose of studying the process of atresia in preovulatory follicles. 5 IU of PMSG given to 24-day old immature female rats induces maximal development of preovulatory follicles by 48 h after treatment (Steger et al., 1975; Peluso et al., 1977a; Peluso and Steger, 1978). This dose of PMSG will not elicit ovulation of the follicles. Instead, if an exogenous dose of LH or hCG is not given between 48 and 60 h after PMSG treatment to induce ovulation, the follicles will begin to degenerate (Peluso et al., 1977a). Examination of ova taken from the degenerating follicles showed atretic changes by 96 h after PMSG, yet functional viability was impaired much earlier (Peluso et al., 1977a).
STATEMENT OF PURPOSE

Understanding of the changes which initiate the process of atresia has been hampered by the inability to accurately determine which follicles in the adult ovary are destined to degenerate. However, in this PMSG-primed immature rat system, a large number of preovulatory follicles mature simultaneously but do not ovulate. Thus, sequential changes that occur as the follicles degenerate can be described.

The purpose of the present study is to elucidate the ultrastructural and histochemical changes which lead to atresia of preovulatory follicles of PMSG-primed rats, and to relate these changes to steroidogenesis within the ovary. By examining the early alterations that lead to atresia, the progress of atresia and its causes can be better understood.
MATERIALS AND METHODS

Experiment I - Ultrastructural Study

Twelve 24-day old immature Wistar rats, housed under controlled conditions of temperature (24°C), humidity (45%) and photoperiod (10 h light/24 h), received an i.p. injection of 5 IU pregnant mare serum gonadotropin (PMSG) at 9:30 AM. Four animals were killed by ether at 48, 72 and 96 h after PMSG injection. The ovaries were removed, trimmed of fat, diced into 4-8 pieces and fixed for 24 h at 4°C in 2.5% glutaraldehyde in cacodylate buffer (pH 7.4). The samples were washed 48-72 h in cacodylate buffer at 4°C, then post-fixed in 1.0% osmium tetroxide for 1 hour and rinsed in distilled water. After dehydration in a graded series of acetone, the specimens were embedded in Spurr's low viscosity media. Thick sections (approximately 0.5 μm) of follicles ≤500 μm in diameter were stained with 1.0% toluidine blue in 1.0% borax and examined under the light microscope. Those follicles with more than two pyknotic nuclei were classified as atretic. Thin sections (600-800 Å) were triple-stained with 0.9% potassium permanganate in phosphate buffer (pH 7.4), uranyl acetate and lead citrate, and examined with an Hitachi HU 11B electron microscope.

Experiment II - Ovarian Histochemistry and Steroid Content

Sixteen 24-day old immature Wistar rats were injected i.p. with 5 IU PMSG at 9:30 AM. Five animals were killed at
48 and 72 h, and six at 96 h after PMSG. Both ovaries from each animal were frozen in OCT embedding material (Miles Laboratories, Elkhart, IN), and stored at \(-20^\circ\text{C}\) until used for localization of 3-\(\beta\)-HSD activity and lipid, or assayed for progesterone, 17-\(\beta\)-estradiol and testosterone content.

**Histochemistry:** Frozen sections from one ovary of each animal were cut 10 \(\mu\text{m}\) thick on a cryostat, and placed on slides for 3-\(\beta\)-HSD localization. The sections were extracted in cold acetone (\(4^\circ\text{C}\)) for 30 minutes. The sections were then incubated for 60 minutes at \(37^\circ\text{C}\) in a solution containing 20 ml 0.2 M Tris-HCl buffer (pH \(7.4\)), 10 mg nitro-blue tetrazolium, 20 mg NAD\(^+\) and 2 mg of either pregnenolone or dihydroepiandrosterone (Steger et al., 1976). The sections were rinsed in distilled water and mounted in glycerol-gelatin. Control sections were incubated in media without substrate to insure that all endogenous substrate was removed by acetone. 3-\(\beta\)-HSD activity was detected as a blue-purple stain.

Ovarian cells that contained lipid droplets were identified in unextracted frozen sections using the modified Herxheimer method (Drury and Wallington, 1967). Sections were rinsed in 70% alcohol and then immersed for 5-10 minutes in a solution consisting of equal parts saturated Sudan III in acetone and 70% alcohol. Sections were differentiated in 70% alcohol to diffuse out excess stain, and rinsed in tap water. Lipid was revealed by a red-orange color.
Steroid Radioimmunoassay: The remaining ovaries were homogenized in 1.0 ml of PBS-gelatin with a ground glass homogenizer and by sonification. A 500 µl aliquot was extracted with 10.0 ml of anhydrous ethyl ether. The ether extract was evaporated to dryness, and the steroids were resuspended in 1.0 ml of PBS-gelatin. The efficiency of extraction was estimated by the addition of $^3$H-estradiol, $^3$H-testosterone or $^3$H-progesterone (New England Nuclear) to a series of ovarian homogenates prior to ether extraction. The extraction efficiency for estradiol and progesterone was 93% and for testosterone, 91%. As a result, none of the values were corrected for extraction efficiency.

Anti-progesterone-11-BSA, GDN#337, 1:2500; anti-estradiol-6-BSA, GDN#244, 1:20,000 and anti-testosterone-11-BSA, GDN#35250, 1:40,000 were provided through the courtesy of Dr. G. D. Niswender of Colorado State University. Specificity of the estradiol antiserum was determined by Campbell et al. (1977), for progesterone antiserum by Gibori et al. (1977) and for testosterone antiserum by Gay and Kerhan (1978). Estradiol was assayed in duplicate in 50 and 100 µl aliquots, progesterone in duplicate 20 and 50 µl aliquots and testosterone in duplicate 50 and 100 µl aliquots. Estradiol samples were corrected for distilled water blanks. Progesterone and testosterone levels in the water blanks were below the sensitivity of the assay.

All data were analyzed statistically using either the Student T test or Chi Square analysis.
RESULTS:

Ultrastructure of Follicles

All follicles collected 48 h after PMSG were non-atretic. Processes from the cells of the corona radiata projected through the zona pellucida, and interacted with the oolemma (Fig. 1). Also, numerous microvilli projected from the oolemma into the zona pellucida. The cells of the cumulus oophorus, cumulus hill and membrana granulosa were polyhedral in shape. Their cytoplasm contained evenly distributed ribosomes, oblong mitochondria with lamellar cristae, vesicles and cisternae of smooth and rough endoplasmic reticulum and Golgi complexes (Figs. 2 & 3). Occasionally, lysosome-like (dense) bodies and annular nexi were observed. The basal granulosa cells were columnar in appearance (Fig. 3). The cells of the basal and membrana granulosa were closely apposed, joined together by desmosomes and gap junctions (Fig. 4).

A distinct basement membrane separated the basal granulosa from the theca interna (Fig. 3). The theca interna contained steroidogenic cells interspersed between spindle-shaped fibroblasts and endothelial cells of capillaries. The steroid-secreting cells contained spherical mitochondria with tubular or vesicular cristae, endoplasmic reticulum and lipid droplets. Capillaries were generally small and narrow, and found throughout the theca interna (Fig. 3).
Figures 1 and 2: The oocyte isolated from a non-atretic follicle has numerous microvilli projecting into the perivitelline space. Cytoplasmic projections from the cumulus cells traverse the zona pellucida and come in close contact with the microvilli (Fig. 1). Antral granulosa cells are closely apposed, and contain large nuclei, dispersed mitochondria, endoplasmic reticulum and Golgi apparati. A cell undergoing mitotic division can be observed (Fig. 2).

(Fig. 1 x4300; Fig. 2 x3900)
Figures 3 and 4: Thecal and granulosa cells in a non-atretic follicle. Steroidogenic cells in the theca interna (arrows) contain lipid droplets and vesicles of smooth endoplasmic reticulum. Small capillary vessels (C) containing red blood cells, and fibroblasts are located near the distinct basement membrane. The granulosa cells in the lower right-hand side of the micrograph are columnar in shape (Fig. 3) and are joined by tight junctions (nexi) to adjacent follicle cells (Fig. 4). (Fig. 3 x4250; Fig. 4 x63,000)
All of the follicles collected 96 h after PMSG contained pyknotic nuclei (>2), and were considered to be in Stage I of atresia. In these atretic follicles, few pro-
Of the follicles collected 72 h after PMSG (n = 25), 28% of the follicles were in Stage I of atresia, while 72% did not possess pyknotic nuclei. This latter group was considered to be at an intermediate stage, between non-atretic and Stage I of atresia. In these intermediate follicles, the cumulus cell-oocyte contact was not altered (Fig. 5). However, many of these follicles contained areas where the granulosa cells were highly vacuolated and apposed only at junctional regions. The membrana granulosa cells on either side of these areas did not show the degenerative characteristics. The degenerating regions were often associated with the cells of the cumulus hill (Fig. 6).

Blebs of cytoplasm were observed in the apical membrane of antral granulosa cells. In addition, the membrana granulosa cells near the antrum were separated and irregular in shape. Organelles in many of these cells were clumped either around the nucleus or to one side of the cell. Lipid droplets, which are normally seen only in the basal area, were scattered in all layers of the membrana granulosa. Occasional autophagic vacuoles were also observed in cells close to the basement membrane. The cells closest to the basal lamina were still columnar but less uniform in shape. The basement membrane and the theca interna appeared unchanged.

All of the follicles collected 96 h after PMSG contained pyknotic nuclei (>2), and were considered to be in Stage I of atresia. In these atretic follicles, few pro-
Figures 5 and 6: An intermediate (pre-atretic) stage follicle isolated 72 h after PMSG. The cumulus cell-oocyte association is still intact with numerous microvilli from the oolemma and cumulus cell projections into the zona pellucida (Fig. 5). Focal areas of granulosa cells are highly vacuolated. Adjacent to this area of degenerative cells are normal-looking granulosa cells. These disrupted cellular regions are frequently found within the cumulus hill area (Fig. 6).
(Fig. 5 x4500; Fig. 6 x3900)
cesses originating from the cumulus cells were evident in
the zona pellucida. The number of microvilli also appeared
to diminish from that of oocytes in the non-atretic and in-
termated groups. The intercellular spaces also
appeared to be more irreglar and, some were large
and others were irregular and, some were
sterile. In Table 1, a high degree of autolysis
was shown in the microvilli (50%) (p<0.01) (Fig. 14). The largest follicles showed the
most intense staining.

Lipid was located within the thecal cells and inter-
cesses originating from the cumulus cells were evident in the zona pellucida. The number of microvilli also appeared to diminish from that of oocytes in the non-atretic and intermediate (pre-atretic) follicles (Fig. 7). The other alterations observed in the intermediate follicles were also present in the Stage I atretic follicles but appeared to be more numerous (Figs. 8 & 9).

Also in this group, the basement membrane was irregular and, in some follicles, disrupted. Blood vessels were large and often adjacent to the basal lamina. Leukocytes were attached to the endothelial wall in some vessels. Some steroid-secreting cells in the theca interna also contained autophagic vacuoles (Figs. 10 & 11).

The sequential ultrastructural alterations are shown in Table 1. The granulosa layer is the first follicular component to show degenerative changes, which are potentiated as atresia progresses.

Enzyme Localization and Steroid Levels

3-B-Hydroxysteroid dehydrogenase activity was present in the interstitial and thecal compartments of ovaries from all three groups. However, 3-B-HSD activity was observed more often in the granulosa cell layers of follicles 500 microns or more in diameter (38%) than in smaller follicles (50%) (p < 0.01) (Fig. 14). The largest follicles showed the most intense staining.

Lipid was located within the thecal cells and inter-
Figures 7 and 8: A Stage I atretic follicle 96 h after PMSG. The cumulus cell projections in the zona pellucida are absent and the number of microvilli on the surface of the oocyte reduced, indicating a dissociation of the cumulus cell-oocyte complex (Fig. 7). Antral granulosa cells are irregular, and blebbing of the cell membrane occurs. Note lipid (arrow) and the clumped appearance of cell organelles (Fig. 8).

(Fig. 7 x4300; Fig. 8 x3900)
Figure 9: Cells of the membrana granulosa in an atretic follicle. Numerous autophagic vacuoles (AV) and lipid droplets (L) are present. Tight junctions are also seen (arrow).

(x6500)
Figures 10 and 11: Thecal and granulosa cells of an atretic follicle. Note in Figure 10 the macrophage (M) invading the theca interna near the basement membrane. Figure 11 illustrates an autophagic vacuole (AV) in the theca interna. The steroidogenic cells and basement membrane appear disrupted. Large amounts of lipid are present in the basal granulosa cells in the lower right corners of the micrographs. (Figs. 10 and 11 x9000)
Table 1: Sequential Ultrastructural Alterations Associated with Atresia of Pre-Antral Follicles

<table>
<thead>
<tr>
<th>Follicular State</th>
<th>Stage I Atresia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicles</td>
<td>+</td>
</tr>
<tr>
<td>Cumulus Cells</td>
<td>+</td>
</tr>
<tr>
<td>Relationship</td>
<td>++</td>
</tr>
<tr>
<td>Antral Granulosa (including Membrana)</td>
<td>++</td>
</tr>
<tr>
<td>Basal Granulosa</td>
<td>++</td>
</tr>
<tr>
<td>Basal Laminar Cells</td>
<td>++</td>
</tr>
<tr>
<td>Thecal Cells</td>
<td>++</td>
</tr>
</tbody>
</table>

- Not seen in any follicles
+ Seen in all follicles
++ Very frequently observed in all follicles
Table 1

Sequential Ultrastructural Alterations Associated with Atresia of Preovulatory Follicles

<table>
<thead>
<tr>
<th>Follicular Component</th>
<th>Characteristic Alteration</th>
<th>Non-Atretic</th>
<th>Intermediate</th>
<th>Stage I Atresia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cumulus Cell-Oocyte Relationship</strong></td>
<td>Absence of Cumulus Cell-Ova Contact</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Antral Granulosa Cells</strong></td>
<td>Pyknosis</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Blebbing of Cytoplasm</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Loss of Cellular Contacts</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>Granulosa Cell Layer (including Antral &amp; Membrana Granulosa)</strong></td>
<td>Decreased # of Cell Layers</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Clumping of Organelles</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lipid Droplets</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Alteration in Cell Shape</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Autophagic Vacuoles</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Dense Bodies</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>Basal Granulosa Cells</strong></td>
<td>Lack of Low Columnar Shape</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Basal Lamina</strong></td>
<td>Disrupted and Irregular</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>Thecal Cell Layers</strong></td>
<td>Autophagic Vacuoles</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Vascularity</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

- Not seen in any follicles  
+ Seen in all follicles  
++ Very frequently observed in all follicles
stitial tissue in the preovulatory follicles collected 48 h after PMSG, but was only occasionally observed in the granulosa compartment. If lipid was observed, it was in the basal granulosa (Fig. 3). While lipid-staining in the thecal and interstitial cells remained regardless of hour after PMSG treatment, the percent of follicles with detectable lipid in the granulosa layer increased from 0% at 48 h to 33% at 72 h and 64% by 96 h after PMSG (Figs. 12 & 13).

There was no difference among the groups in ovarian progesterone content, but 17-β-estradiol and testosterone content decreased significantly after 48 h (p<0.05) (Table 2). Although there appears to be a slight increase in mean steroid levels at 96 h, no significant differences were seen when compared to the levels at 72 h.
Figure 12: Light micrograph of follicles within the ovary obtained 48 h after PMSG injection. The theca and interstitial tissue show presence of lipid. The granulosa cells of the large follicles, however, contain very little lipid. (x330)

Figure 13: Follicles in an ovary obtained 96 h after PMSG injection showing presence of lipid in the thecal and interstitial tissue, and in the granulosa cells of large follicles (left side of micrograph). (x330)
Figure 14: The effect of PMSG and follicular diameter on the localization of 3-β-hydroxysteroid dehydrogenase activity within the granulosa cells using either pregnenolone or dehydroepiandrosterone as substrates. Activity was expressed as + for intense staining, ± for moderate staining and - for no staining reaction.
Pregnenolone

Dehydroepiandrosterone

Relative Activity

hr. after PMSG
- 48
- 72
- 96

Follicular Diameter

(μm × 10^2)
Table 2

The Effect of PMSG on Ovarian Steroid Concentration

hour after PMSG

<table>
<thead>
<tr>
<th>Steroid Concentration$^+$ (pg/mg ovary)</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>$298.7 \pm 110.1$</td>
<td>$77.0 \pm 31.6$</td>
<td>$178.6 \pm 57.0$</td>
</tr>
<tr>
<td>17-$\beta$-Estradiol</td>
<td>$93.9 \pm 25.6$</td>
<td>$19.9 \pm 5.8^*$</td>
<td>$36.3 \pm 7.8^*$</td>
</tr>
<tr>
<td>Testosterone</td>
<td>$41.5 \pm 8.2$</td>
<td>$14.1 \pm 4.4^*$</td>
<td>$22.7 \pm 4.5^*$</td>
</tr>
</tbody>
</table>

$^+$Values expressed as mean ± standard error

*Significantly different from 48 h group ($p<0.05$)
DISCUSSION

By 72 h after PMSG, LH serum levels and androgen content in the ovaries are reduced (Peluso et al., 1979c). This is coupled with an increase in acid phosphatase activity and a decrease in LH binding in the granulosa cell layer (Peluso et al., 1977a). Although LH binding in the theca interna remains unaltered through 96 h after PMSG administration (Peluso et al., 1977a), the decrease in circulating LH could account for a reduction in ovarian testosterone synthesis. Treatment of PMSG-primed immature rats with cyproterone acetate, a potent anti-androgen, has been shown to rapidly increase the rate of atresia and to enhance the development of ovarian cysts in preovulatory follicles (Peluso et al., 1979c). Thus, androgens may prevent the development of acid phosphatase activity and degeneration of preovulatory follicles (Peluso et al., 1979c). As androgen levels decrease, degeneration of the follicle begins. The data of the present study support this concept. The significant decrease in ovarian testosterone levels, reported in this study, may also account for the declining estrogen levels seen at the same time, since androgens are obligatory intermediate substrates for estrogen synthesis.

A decreased content of estrogen and androgen in the ovary could initiate degenerative ultrastructural and histochemical changes in follicular cells. The results of this
study support this possibility and demonstrate that the first degenerative changes occur in the granulosa cells. The observed clumping of organelles in the granulosa cells suggests a disruption of intercellular organization. Also, it appears that some granulosa cells may be more susceptible to atresia than others, since normal-looking cells are found in close association with cells exhibiting a more advanced degree of deterioration. A similar situation has been reported in atretic ovine follicles (Hay and Cran, 1978). These initial areas of degeneration seen at 72 h after PMSG in this study are characterized by highly vacuolated, disrupted cells, and are often seen in the cumulus hill vicinity, indicating a possible early interruption of communication between the oocyte and the follicle. Oocytes collected 72 h after PMSG have already begun to degenerate, as defined by the rate of germinal vesicle breakdown and polar body formation in vitro (Peluso et al., 1979a), although no ultrastructural signs of degeneration were detected in this study or in others (Peluso et al., 1979a). Therefore, focal degeneration in the area of the cumulus oophorus may be the initial site at which the follicle becomes atretic, leading to the eventual deterioration of the oocyte.

The presence of numerous microvilli on cells from healthy preovulatory follicles has been correlated with increasing numbers of LH receptors (Chang et al., 1977). In the present study, granulosa cells appear flattened and loosely attached by 72 h after PMSG. The loss of intercellular contacts and
cohesion, along with alterations in cell shape, are characteristic of atretic follicles (Byskov, 1977; Peluso et al., 1977a), and were observed in follicles beginning to undergo atresia in this study. This may reflect a decrease in the number of gonadotropin receptors seen at this time (Peluso et al., 1977b), and a reduction in gap junctions, probably due to decreasing estrogen levels observed in this study, since estrogen maintains gap junctions (Merk et al., 1972).

These data also show an increase in lipid droplets in the ovarian granulosa cells, which may indicate an excess of steroid hormone precursors (Deane, 1952). If a decrease in cellular phospholipids occurs very early in atresia (Guraya and Greenwald, 1964), intracellular steroid transport may be impaired. This view is based on evidence that phospholipid micelles provide a vehicle for solubilizing cholesterol and other neutral molecules (Fleischer and Brierly, 1961), thus possibly serving as carriers for these substances in the cytoplasm.

Our results additionally support previous observations that 3-β-hydroxysteroid dehydrogenase activity is detectable in thecal and granulosa cells of preovulatory follicles and that it remains high throughout the atretic process (Pupkin et al., 1966; Motta and Bourneva, 1970). This is consistent with our observations that ovarian progesterone levels remain unaltered, despite decreased secretion of other steroids. It has been shown the FSH stimulates 3-β-HSD activity in granulosa cells of mature follicles (Zeleznik et al.,
1974; Zeleznik, 1979). Also, since FSH levels are reported to remain unaltered through 96 h after PMSG (Peluso and Steger, 1978), there appears to be a sufficient number of FSH receptors to maintain progesterone production in the granulosa layer as shown in the present study.

All follicles at 96 h after PMSG are in Stage I atresia in our study, exhibiting pyknosis and further disruption of the granulosa layer. Ultrastructural changes detected in the theca interna by 96 h include autophagic vacuoles, phagocyte-like cells adhering to capillary walls and disruption of the basement membrane. The appearance of autophagic vacuoles associated with steroidogenic cells suggests cellular degeneration rather than differentiation into interstitial tissue (Peluso et al., 1979b). The invasion of leukocytes in the thecal and granulosa layers seen in this study, may enhance the deterioration of the follicle wall and may eventually lead to the formation of cysts.

Decreasing steroid secretion appears to be an initial event associated with atresia. Whether this decline is a cause of result of atresia is the premise of an on-going investigation employing antisteroids to estrogen and testosterone. Preliminary findings indicate that initial degenerative changes in the proovulatory follicle may be caused by alterations in the ratio of androgen and estrogen in the ovarian environment.
REFERENCES


The thesis submitted by Mary Cristine England-Charlesworth has been read and approved by the following committee:

Dr. John Peluso, Director  
Assistant Professor, Biology, Loyola

Dr. Genaro Lopez  
Associate Professor, Biology, Loyola

Dr. Albert Rotermund  
Associate Professor, Biology, Loyola

The final copies have been examined by the director of the thesis, and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Sciences.

4-15-70  
Date  
John Peluso  
Director's Signature