Dispersal by enzymatic digestion and recovery of bovine oocytes from whole ovaries
by James Dailey Strickland

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Animal Science
Montana State University

Abstract:
Regularly cycling beef cows (n=5) were bilaterally ovariectomized and the ovaries were transported to the laboratory in an insulated flask, containing Earle's Salts at 38° C. Once in a sterile unit, the ovarian stalk was trimmed off and the ovary sectioned transversely into 1-3 mm thick discs. The outer edge of the cortex, containing the stroma, tunica albuginea, germinal epithelium and primary oocytes, were removed in a ribbon about 0.5 mm thick with iridectomy scissors. The ribbon was diced finely,yielding cubes (0.5mm3) which were tryp-sinized (0.25%) in Hanks Ca++ & Mg++ free medium at 38.5°C for 1 hour. . The tissue suspension was strained through a 200 mesh screen into a centrifuge tube and spun to form a pellet, which was washed in Earle's Salts. The resuspended pellet was distributed equally into control & experimental portions. The experimental portion was placed in a petri dish and the oocytes with attached granulosa cells were isolated by the aid of a 10 µl micropipette and an inverted microscope. Recovered tissues were fixed and imbedded in agar and sectioned at 10µ, followed by staining with hematoxylin and eosin. A total of 2256 oocytes (control and experimental) were released from the digested tissue, with 513 (46%) being individually isolated from the 1151 in the experimental portion. The number of granulosa cells around the recovered oocytes ranged from 0-12 layers, with the average being 4-6 layers. Oocyte (n=633) diameters were measured with the size groupings of <40, 50,60,70,80,90,100 and >110. The percentages in each size grouping were 28,40,15,5.0,4.9,3.9,2.4, and 0.3, respectively. Trypsin digestion of the ovary is an effective way to recover larger numbers of oocytes for in vitro investigations.

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Signature James D. Strickland

Date May 28, 1976

DEDICATION

The author wishes to dedicate this thesis to the memory of his father Benjamin D. Strickland (1924-1970), whose hours of philosophical and intellectual discussions stimulated a young man to think and desire to become savant. His gratitude is also expressed to his mother, Lola May Beckman Strickland, for her perceptive advice and guidance which assisted him through many difficult times. Thank you.

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Approved:

Edward J. Moody
Chairperson, Graduate Committee

D. V. Blackwell
Head, Major Department

Henry L. Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

May, 1976

ACKNOWLEDGEMENTS

To my cherished wife Mary, I wish to express my deepest appreciation for her encouragement, patience, understanding and sacrifices made to help me fulfill my goal.

To Dr. Ed Moody, I must extent a most sincere thanks for his advice, guidance and support in allowing me to try most of my ideas out on my research. His assistance throughout my entire graduate program has been invaluable. My gratitude is expressed to Drs. Bill Dorgan, Jim McMillan and Larry Jackson for their advice and assistance in attempting to solve various problems. I would also like to thank Dr. Bob Moore for consenting to be on my graduate committee.

A very special thank you to Dr. James T. Bradbury for the assistance on oocyte histology, morphology and the thought stimulating questions and discussions as well as Dr. Jay F. Kirkpatrick of Eastern Montana College whose close friendship and thoughtful advice have been instrumental in the development of my scientific abilities.

My thanks are extended to my fellow graduate students for their companionship and assistance throughout my program, especially Dave Griswold, Greg Poncelet, Vern La Voie and Chris Stallings.

My gratitude is also expressed to Wava Goetz for the preparation of this manuscript.

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ABSTRACT

Regularly cycling beef cows (n=5) were bilaterally ovariectomized and the ovaries were transported to the laboratory in an insulated flask, containing Earle's Salts at 38°C. Once in a sterile unit, the ovarian stalk was trimmed off and the ovary sectioned transversely into 1-3 mm thick discs. The outer edge of the cortex, containing the stroma, tunica albuginea, germinal epithelium and primary oocytes, were removed in a ribbon about 0.5 mm thick with iridectomy scissors. The ribbon was diced finely, yielding cubes ($0.5mm^3$) which were trypsinized (0.25%) in Hanks Ca^{++} & Mg^{++} free medium at 38.5°C for 1 hour. The tissue suspension was strained through a 200 mesh screen into a centrifuge tube and spun to form a pellet, which was washed in Earle's Salts. The resuspended pellet was distributed equally into control & experimental portions. The experimental portion was placed in a petri dish and the oocytes with attached granulosa cells were isolated by the aid of a 10 µl micropipette and an inverted microscope. Recovered tissues were fixed and imbedded in agar and sectioned at 10µ, followed by staining with hematoxylin and eosin. A total of 2256 oocytes (control and experimental) were released from the digested tissue, with 513 (46%) being individually isolated from the 1151 in the experimental portion. The number of granulosa cells around the recovered oocytes ranged from 0-12 layers, with the average being 4-6 layers. Oocyte (n=633) diameters were measured with the size groupings of $\leq$40, 50,60,70,80,90,100 and $\geq$110. The percentages in each size grouping were 28,40,15,5.0,4.9,3.9,2.4, and 0.3, respectively. Trypsin digestion of the ovary is an effective way to recover larger numbers of oocytes for *in vitro* investigations.

CHAPTER 1

INTRODUCTION

The embryo transfer of bovine blastocysts has become a method of utilizing the female genetic qualities to a greater degree. In the last few years, the superior female is no longer limited to one calf per year, but has the potential of producing up to fifty offspring in any one year through embryo transfer.

The present method of obtaining embryos involved the artificial insemination of a super ovulated donor with semen from the desired sire. Following a wait of 4-6 days, the donor undergoes a surgical operation to remove an average of 10-20 fertilized embryos. The morphologically normal embryos are surgically transfered to synchronized recipients. The use of embryo transplantation has the potential of greatly improving the genetic quality of a herd in a relatively short time and provides a method for twinning.

Presently there are two limiting factors associated with embryo transplant programs. One is the development of a non-surgical method of transplantation which is being explored but no reliable procedure is available at this time. The second factor is the availability of a large number of fertilizable eggs from donor cows.

This research was undertaken to establish a procedure to obtain a large number of oocytes from an *in vitro* treatment of bovine ovaries. Such a procedure could then be used to develop techniques to permit

maturation of the recovered oocytes *in vitro*. The development of a method of recovery of primordial oocytes would facilitate expansion of embryo transplant programs and provide a readily available supply of oocytes for biochemical investigation.

CHAPTER 2

LITERATURE REVIEW

2.1. Anatomy of the Female Reproductive Tract

2.1.1. Embryonic Development

The primordial germ cells are initially located in the yolk-sac entoderm of the pre-somite embryo (Zamboni, 1972). These cells are readily recognizable as they are more spherical and larger in size than the somatic cells which surround them. Witschi (1948) proposed the most widely accepted theory of germ cell migration. In his classic paper he described the migration of germ cells to the germinal ridge as an ameboid movement through the mesentry. Once at the germinal ridge, the primordial germ cells rapidly multiply to produce oogonia which undergo mitotic divisions in the cortex of the gonad but degenerate in the medullary region (Brambell, 1962). At the end of mitotic proliferation the oogonia begin mitotic prophase and differentiate into oocytes where, at the end of diplotene they are arrested. Finally a growth process occurs and terminates with an ovulatory surge in the adult ovary.

2.1.2. Gross Anatomy in the Bovine

The internal genitalia of the cow are attached to the broad ligament which is connected dorsolaterally in the region to the ilium. The hylus of the ovary is attached to the mesoovarium and suspended near the brim of the pelvis. Although the size varies

greatly with the age and stage of the estrous cycle (Hafez, 1975) the almond shaped ovaries generally weigh from 10-20 grams.

2.1.3. Ovarian Anatomy

a. Medulla

The medulla is continuous with the mesoovarium, making up the central position of the ovary (DiFore, 1973) and contains primarily connective tissue and blood vessels. The medulla provides a capillary bed into which the developing follicles grow to receive an adequate blood supply to nourish the developing oocyte and its supportive tissue. The medulla, which is derived from the extra gonadal undifferentiated cells related to the mesonepheric rudiment (Zuckerman, 1962), seems to be of slightly different origin than the cortex.

b. Cortex

The outer portion of the ovary contains the germinal epithelium, tunica albuginea and cortical stroma which are derived from the first proliferation of the germinal epithelium. The tunica albuginea and the outer cell layers in the cortical stroma contain the primary oocytes in the cow (personal observation). The tunica albuginea is a dense connective tissue which holds the oocytes in late diplotene until maturation is initiated. As maturation progresses the oocyte and developing follicle are pushed into the stroma, while an increase in follicular liquor volume causes the follicle to protrude from the

stroma and form a bulge in the ovarian surface. Espey (1976) demonstrated by new staining technique that the follicle wall has a very high concentration of collagen; thereby providing an explanation for the rigidity of the pre-ovulatory follicle and the effect of collagenase on ovulation.

2.2. Mechanism of Action of Peptide Hormones

Peptide hormones involved with oocyte maturation and ovulation include Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). The mechanism of action seems to be the same for both of these gonadotrophins with only the receptor site and the response varied. Much of the investigation involving the mechanism of peptide hormone action was carried out with insulin by P. Cuatrecasas, which he reviewed in 1974. The similarities in the physical structure of the peptide hormones and insulin allow the application of insulin work to be correlated to the bichained peptide hormones.

The many effects of the peptide hormones are mediated through a series of biochemical events. Hormones released by the adenohypophysis attach to the randomly positioned lipoprotein receptor sites (Lenninger, 1975) of the plasma membrane (Crofford and Okayama, 1970). It appears that the chain is most important in the bonding of the receptor-hormone complex. Kahn (1972) proposed evidence for high-affinity receptor sites for each peptide hormone. Once the hormone

has complexed with the receptor, the activation of the second messenger system has started. The adenylate cyclase is reported to carry the message through the cell membrane (Cuatrecasas, 1974). There are several theories on how this interaction takes place. Perkins (1973) and Cuatrecasas (1974) proposed the receptors are separate and discrete structures which complex by lateral diffusion along the plane of the cell membrane with random encounters leading to interaction and activation of the enzyme only when the receptor has complexed with the hormone. Rodbell *et al.* (1975) suggested there are at least 3 activation sites needed to activate the enzyme: the hormone-receptor site, a catalytic site which reacts within ATP chelated to magnesium, and a nucleotide regulatory site which preferably reacts with guanyl nucleotides.

Robison, Butcher and Sutherland (1967) proposed the original 2 component model from which the others were developed. Swislocki and Turney (1975) demonstrated that the nucleotide regulation of enzyme activity is independent of membrane structure. Activation of adenylate cyclase and production of cyclic AMP are important steps in the mechanism of action of the tropic hormones upon the target tissues. Upon activation, this enzyme acts on the ATP converting it to 3' 5' cyclic AMP. This reaction yields pyrophosphate which is converted into 2 inorganic phosphates by pyrophosphatase.

A rise in cyclic AMP has been demonstrated in many species by several gonadotrophins (as reviewed by Catt and Dufau, 1976). There is evidence that more receptors are present than are needed to elicit maximal response from the steroids and peptides thereby making more efficient use of the low concentrations of systemic hormones (Catt and Dufau, 1973; Mendelson *et al.*, 1975).

Cyclic AMP has been shown to activate protein phosphokinase in the hormone-stimulated cell (Krebs, 1972, 1973). This cytoplasmic enzyme is composed of 2 conjugated subunits when in the inactive form. The 2 subunits, regulatory and catalytic, form the inactive holoenzyme which can be stimulated by the cyclic AMP. When the cyclic AMP binds the regulatory subunit, the holoenzyme is dissociated, allowing the catalytic subunit to initiate the phosphorylation of the protein substrates.

Cyclic AMP's action upon translation processes suggests that phosphorylation of ribosomal proteins may represent an important action of protein kinase (Eil and Wool, 1971; Bardon and Labrine, 1973). This function has not been shown to alter ribosomal function suggesting no regulatory effect of the protein kinase.

The principal role of the protein kinase is to initiate the transformation of cholesterol to the various steroid hormones depending upon the type of protein hormone complexing with a receptor.

2.3. Peptide Hormones Involved in Reproduction

2.3.1. Gonadotrophin-Releasing Hormone (GN-RH)

The hypophyseal regulatory hormone responsible for the release of both LH-RH and FSH-RH was found by Matsuo *et al.* (1972) to be a simple decapeptide with the proposed structure being: Pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. Although there may be additional LH and FSH releasing hormones, it has been shown that the two activities are inseparable (Schally *et al.*, 1971). The LH-RH and FSH-RH are released from glandular secretory cells in the anterior hypothalmic area of the hypothalmus. These releasing hormones travel by way of the hypophyseal portal system to the anterior hypophysis, where the LH and FSH are stored for release in the Δ_1 and Δ_2 basophils (Turner and Bagnara, 1971).

2.3.2. LH and FSH

The adenohypophyseal gonadotrophins have a molecular weight of about 30,000 being glycoproteins containing about 20% carbohydrate. They are formed of α and β subunits with the α chain the same as TSH. The β chain appears to vary and is the active component of the molecule (Schreiber, 1974) and exhibits amino acid variations.

The feedback controls of the gonadotrophins are very complex compared to the other adenohypophyseal hormones. Progesterone and estrogen exhibit both a positive and negative feedback on the hypo-

thalmic hormones. Estrogen inhibits FSH more than LH in negative feedback, then stimulates LH more than FSH in the LH surge (Yen *et al.*, 1971). Progesterone will inhibit the gonadotrophins in conjunction with estrogen, but can induce ovulation when estrogen is not present. There is evidence that species variation exists in the biological effect of FSH (Gemzell and Roos, 1966). In their comprehensive study, Channing and Kammerman (1974) found that FSH binds almost exclusively to the granulosa cells of medium and large follicles. LH and HCG bind primarily to luteal cells and thecal and granulosa cells in larger follicles. They also determined that the maturity of the follicle determines the number of LH receptors on the granulosa cells.

2.4. Female Sex Steroids

2.4.1. Physical Characteristics

Figure 1 represents the probable biosynthetic and metabolic pathways of progesterone and estrogen, the primary female sex steroids. These are highly specialized in their function and do not produce direct general or systemic effects on metabolism. They produce an effect only in those cells that contain cytoplasmic receptors for that particular steroid.

2.4.2. The Mechanism of Action of Estrogen

The estrogens, 17 β-Estradiol, Estrone and 17 α-Estradiol have

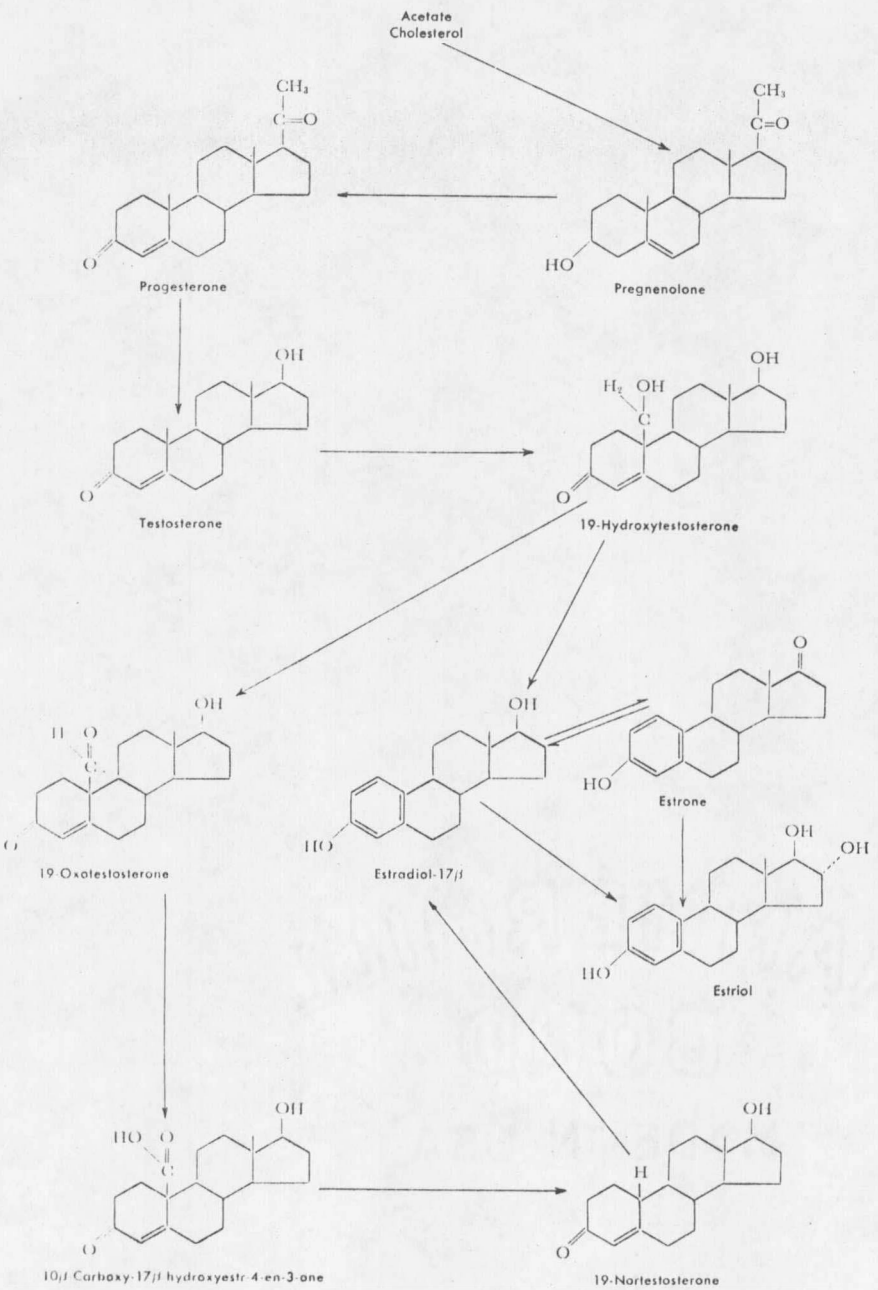

Figure 1. Probable pathways in the biosynthesis and metabolism of estrogens. (Turner and Bagnera, 1971).

been shown by *in vitro* studies to arise in the bovine adrenal, ovary and placental tissue from: 1) acetate, 2) cholesterol, 3) progesterone, or 4) neutral C_{19}- and C_{18}- steroids (Mellin and Erb, 1965). Estrogens have their primary effect on the uterus (Baulieu, 1971), vagina (Lenninger, 1975) and anterior pituitary (Jensen and Jacobsen, 1966). They are reported to increase granulosa cell sensitivity to FSH (Swain, 1969). According to Mercur *et al.* (1966) testosterone and estrogen are carried by the same plasma protein, the sex steroid binding protein, to the target cells. The exact mechanism by which it goes through the plasma membrane is not known; however, it is known that the steroid readily passes through the membrane and is quickly bound to a 4S or 8S proteinaceous carrier to form an estrogen-receptor complex. The carrier changes to a 5S complex at the nucleus, which is the only form to stimulate uterine-RNA synthesis and work specifically on nucleolar-RNA Polymerase I (Arnaud *et al.*, 1971). In a few minutes one sees an increased uptake of RNA and protein precursors, an increase in nuclear ribosomal and messenger RNA precursors and an increase in RNA polymerase activity. About 30 minutes later there is an increase in DNA-dependent RNA synthesis. Within 3-6 hours following estrogen entry into the cell there is a considerable growth noted in the uterus, probably due to water retention due to protein and RNA synthesis (Swain, 1969; Jackson, 1975). In the vagina both estrone and estradiol are bound, stimulating an increased cornification of the

vaginal epithelium (Swain, 1969). The primary estrogen effect upon the anterior pituitary is a feedback inhibition on the gonadotrophins. Estrogen can produce more receptors there by magnifying its own action (Jackson, 1975).

2.4.3. The Mechanism of Action of Progestins

Progesterone appears to be responsible for transformation of uterine endometrial cells in order for implantation of the developing blastocyst to take place (O'Malley and Means, 1975). Other functions seem to be: 1) support of mammary growth, 2) direct antagonization of estrogen, and 3) appear to alter a number of physiological parameters with no relationship to pregnancy or reproduction, i.e. it alters hepatic function, increases conversion of amino acids to urea and influences renal permeability to sodium (Landau and Lugibihl, 1961). The primary cyclic production site of progesterone is in the corpus luteum; while some is also secreted by the granulosa cells in the late follicular stage (Swain, 1969).

As with estrogen, progesterone and its principal metabolite, 5 α-pregnane-3,20-dione, bind with a "receptor" macromolecule within the cytoplasm. This receptor seems to be a physiological receptor as it is found only in tissues sensitive to progesterone (O'Malley and Means, 1975) where they appear to interact with the genome at specific DNA sites, determined by the chromatin acidic proteins (Spelsberg

et al., 1971). Evidence suggests that progesterone works at the transcription level increasing RNA polymerase and stimulating m-RNA synthesis, which stimulates protein synthesis to cause a protein induced cell transformation (O'Malley and Means, 1975).

2.5. Prostaglandins in Oocyte Maturation and Ovulation

At the present time there is a great deal of controversy as to the role and the mechanism of action that prostaglandins have in ovulation. The most recent review (Karim, 1975) brings up the following points: 1) levels of prostaglandins of the F series, but not the E series, increase most markedly as the time of follicular rupture approaches. Both series are synthesized by the follicule; 2) a prostaglandins inhibitor will prevent ovulation, however, its effect can be nullified by an increase in LH; 3) follicular prostaglandins have an effect on ovulation, but do not have an effect on luteinization. Tsfariri *et al.* (1972) demonstrated with an *in vitro* system of Graafian follicles that resumption of the first meiotic division could be induced with the addition of LH or prostaglandin E_2, but prostaglandin $F_{2\alpha}$ was only partially effective.

With these points in mind, one must hypothesize the yet to be elucidated action of prostaglandins. Does it work synergistically with LH to multiply its effect? Does it cause smooth muscle contraction in the follicular wall? Is it an end product of LH stimulation

with an effect of inducing ovulation via smooth muscle contraction or feeding back on LH to inhibit its action? These questions, along with many more must be investigated before an answer to the function of prostaglandins in ovulation can be determined.

2.6. Oocyte Maturation

2.6.1. Meiosis

Meiosis consists of a single chromosomal duplication during two cell divisions and is a phenomenon observed only in germ cells. The process has been broken down into several stages for identification purposes; however, there is no clear demarcation between the end of one stage and the beginning of another. The specific stages are useful in determining the approximate stage of maturation and age of the germ cells, specifically, the oogonia and the oocytes.

Experiments with microsporocytes have shown there is an irreversible commitment to meiosis after the stage of interphase, which involves DNA synthesis (Cohn, 1969). G_2(RNA synthesis and chromosomal condensation) must be completed for normal meiosis to occur. Following G_2 the cell enters meiosis I which contains essentially the same stages as mitosis.

2.6.1.1. Prophase I

Prophase I of meiosis is a relatively long period that is marked by a substantial increase in nuclear volume. The stage has been

further subdivided due to the variety of events that occur at this time into the stages leptotene, zygotene, pachytene and diplotene. Each individual stage will be discussed (DeRobertis *et al.*, 1970; Watson, 1970 and Herskowitz, 1973).

a) Leptotene

At the beginning of leptotene chromosomes are not coiled and are very long. Although the chromosomes are elongated, individual chromosomes are not recognizable: During leptotene, they develop a number of small coils; the tightest coils become major coils and are recognizable as chromosomes. There is at this time a functionally single kinetochore which keeps the individual chromatids associated. There is also an increase in RNA synthesis.

b) Zygotene

During the zygotene substage the two chromatids of each chromosome synapse, forming a synaptinemal complex with the homologous chromosomes yielding a tetrad. This pairing occurs part by part and may begin at the ends and progress toward the kinetochore region, at the kinetochore region and move to the ends or by pairing of various segments along the length of the tetrad. During this substage there is some DNA synthesis and structural protein synthesis. The chromosomal coiling and condensation continue with the major coils increasing in diameter and becoming shorter and thicker.

c) Pachytene

Pachytene begins once the pairings described above are complete. During this important substage the chromosomes grow shorter and thicker as a result of an increase in coil diameter. The individual bivalents become readily identifiable as does the genetic crossing over or chiasma. It appears that the longer chromatids can have more than one chiasma per bivalent which may or may not interfere with crossing over in other bivalents in adjacent areas. There is some DNA synthesis associated with the crossing over.

d) Diplotene

This substage is identifiable by an increased coiling with continued condensing and an apparent repulsion between homologous chromosomes in areas that had no crossing over. The number and position of the chiasmata indicate the configuration of the bivalents associated at the chiasmatic sites.

2.6.2. Diakinesis

During diakinesis the bivalents separate and move to the periphery of the nucleus. The chromosomes have condensed with the coils tightened to their maximum. The tetrads at this time undergo terminalization which involves the separation of the tetrads so that one bivalent slides off the other. The nucleolus disappears with the last event of diakinesis and Prophase I, being the dispersal of the nuclear envelope.

a) Metaphase I

The chromosomes align along the equator of the cell, with their kinetochores directed toward the poles and their arms toward the equator. The kinetochores appear to repel each other and act as an individual unit. Since the chromosomes are not aligned along the equator, the number of chromosomes dividing at anaphase I will not be the same as the number present in the original cell. The kinetochore is structurally double even though it is functionally single. The spindle fibers attach only to the kinetochore of the chromosomes as chromosome fibers. Meiotic coils are apparent.

b) Anaphase I

The chromosomes of the bivalent move to opposite poles, with the kinetochores moving first and the arms following completing the terminalization. This reduces the chromosome number from diploid to haploid.

c) Telophase I

The chromosomes elongate through loosening of their coils, the nucleolus reappears and a nuclear envelope forms around each polar group. This is the final step in meiosis I, resulting in the formation of daughter nuclei. During the interphase between meiosis I and II, there is an absence of DNA synthesis.

2.6.3. Meiosis II

a) Prophase II

Prophase II resembles prophase I with the following exceptions: The chromatid arms are widely separated and there is no coiling as the chromosomes are still coiled from meiosis I. Spindle fibers appear and the nuclear envelope disappears at the end of prophase II.

b) Metaphase II

The chromosomes align along the equator with the kinetochore at the equator and the arms extending outward. As soon as the kinetochore is functionally double, the chromosomes begin to move to opposite poles with each chromatid having its own kinetochore. Ovulation in cattle occurs at this time.

c) Anaphase II and Telophase II

The daughter chromosomes move to opposite poles in anaphase II and uncoil in telophase II. The nucleolus reappears and a nuclear envelope forms around each group.

Meiotic maturation in the mouse begins when oogonia are in prophase, about 8 days before birth. By approximately 5 days post parturition primary oocytes have reached diplotene and maturation is arrested at this point until puberty. At dictyate the chromatin mass has become less condensed and a large nucleolus is present. This stage remains until the oocyte is stimulated to continue maturation to metaphase II.

In most mammalian ovaries the first four stages of meiosis, leptotene, zygotene, pachytene and most of diplotene are carried out in the foetal ovary. In late diplotene the germinal vesicle forms, as do the first layers of granulosa cells, and meiosis is halted until puberty. The lag period from birth to puberty is recognizable and is known as the dictyate stage by most researchers.

As the female approaches puberty there is a large reduction in oocyte numbers due to atresia. These oocytes are reabsorbed; the remaining oocytes await the resumption of meiosis, which can be anywhere from a few weeks to many years. It has been estimated by Hafez (1975) that there are approximately 200,000 oocytes in the mature bovine ovary.

The oocytes need gonadotrophin stimulation for the resumption of meiosis. FSH stimulates the Graafian follicle to enlarge and LH stimulates the oocyte to resume meiosis, completing its meiotic division. The effect of LH is enhanced by the FSH and estrogen stimulation.

2.7. Follicular Genesis

As mentioned earlier, mitosis of the oocyte stops at or near birth; while meiosis is not completed until ovulation. During the early mitotic phase of growth the oocyte becomes surrounded by other types of ovarian cells forming the primordial follicle (Pederson, 1969, 1970). These follicles are at their maximum number at the end

of mitosis and decline throughout the reproductive lifespan of the female. The decline in numbers results from atresia or ovulation. Figure 2 represents the sequence of follicular maturation beginning neonatally and advancing to the most mature stage (ovulatory stage). The smallest follicles would be found in the pre-pubertal ovary. The follicles may become atretic early in the neonatal life or remain quiescent for the life span of the ovary. Figure 3 and Table 1 (Schwartz, 1974) present a theoretical description of the follicle population at any given time within the ovary.

2.7.1. The Action of FSH and LH on Follicular Growth and Maturation

Data on exogenous hormone treatment of hypophysectomized pre-pubertal rats and mice have suggested that the follicular growth is independent of pituitary secretion or exogenous treatment (Schwartz, 1974). At the same time, gonadotrophin treatment produces an increase in estrogen concentration with the associated estrogen effects (Price and Ortz, 1974). In the pre-pubertal mouse, Ryle (1969, 1971, 1972) showed with *in vitro* experiments that ^{3}H-Thymidine uptake was greatly increased in the presence of FSH but not LH; while LH would work only on the larger follicles. This is significant as the thymidine uptake is an indication of granulosa cell division and increased follicular size. Lostroth and Johnson (1966) very clearly demonstrated the result of highly purified FSH stimulation to be antrum formation and ovarian