Enzymatic recovery and in vitro culture of bovine primary oocytes
by Henry Charles Connor

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

Abstract:
Non-pregnant cows and heifers were ovariectomized under local anesthesia, The ovaries were placed in an insulated carrier containing Earle's Salts or Ham's F-10 warmed to 38°C and transported immediately to the laboratory. Under a sterile hood the cortex was separated from the medulla and diced into small fragments. The cortical fragments were then subjected to enzymatic digestion in either a trypsin or a collagenase solution. The digested tissue was then subjected to two cycles of washing and centrifugation. The dispersed cells were then aliquoted to culture dishes and flasks containing Ham's F-10 combined with calf serum and antibiotics.

Culture was carried out in an incubator at 37-38°C in a 5 percent COg 95 percent air humidified atmosphere. Attempts were made to further isolate oocytes from other ovarian cells by using sucrose density gradient centrifugation. The density gradient was made by layering successively lighter layers of 10, 20, 30 and 40 percent sucrose dissolved in Earle's Salt, in centrifuge tubes. Following digestion the dispersed cells were resuspended in Earle's Salts, distributed to the top of the centrifuge tubes and centrifuged at low speed for five minutes. The 30 and 40 percent layers were taken, washed and centrifuged twice. The cells were then aliquoted to dishes and flasks for culture.

In one experiment, media change schedules of 12, 24, 48, 72 and 96 hours were employed. Media samples were taken and frozen for later progesterone RIA analysis. In the one successful attempt, the use of the sucrose density gradient reduced the number of ovarian cells in culture by over sixty percent without affecting the number of oocytes isolated. The reduction in cell population as a result of sucrose density gradient centrifugation did not persist for the duration of the experiment. The cells which passed through the gradient exhibited extensive mitotic activity and by the end of the experiment the cell populations between isolation groups were equivalent. Subsequent attempts to utilize the sucrose density gradient were unsuccessful. Bovine ovarian cells exhibit steroidogenic activity in culture. The media change interval and total cell population appear to have some influence on the dynamics of this steroidogenic activity. Trypsin or collagenase are suitable for the enzymatic isolation of primary oocytes. However, the use of trypsin seems to result in more incidences of tissue clumping and oocyte overdigestion. Estimated yields of isolated oocytes varied between zero and eleven thousand.

The primary oocytes varied from 27 to 31 μ in diameter. Attempts to stimulate oocyte growth hormonally were unsuccessful, however, ooctye viability was maintained for up to thirteen days in vitro.

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DEDICATION

This is dedicated to Dr. Edward L. Moody for encouraging and supporting free thinking and for setting a fine example to follow.

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by

HENRY CHARLES CONNOR

A thesis submitted in partial fulfillment
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ABSTRACT

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Chapter 1

INTRODUCTION

Embryo transfer is a recognized technique for increasing the productivity of the genetically superior female. Presently, the typical cow is limited to a maximum of one calf per year, however, utilization of embryo transfer procedures can increase the reproductive level of the cow fifty to sixty fold. Refinements in embryo transfer techniques have led to non-surgical embryo recovery procedures which reduce the trauma to the cow and extend her useful lifetime as an embryo donor. In addition, chemicals and drugs which make the manipulation of the estrus cycle of the recipient cows more convenient, help to enhance the success rate of embryo transfer procedures. Clearly, embryo transfer can be a valuable tool for increasing the production potential and value of the livestock herd more rapidly than would be possible with conventional management methods.

Despite the improvement of embryo transfer procedures and the increased success rates being recorded, embryo transfers are still relatively expensive and are not feasible for wide-spread use in the typical commercial livestock herd. For embryo transfer to become an economical management tool, large numbers of embryos must be easily obtainable from a particular animal. The superovulation treatments associated with embryo transfer procedures are effective for increasing the number of oocytes available for fertilization during each of

the cow's estrus periods, however, even repeated administration of the hormonal treatments over a prolonged period of time does not effectively tap the ovarian oocyte resource which has been estimated conservatively at between ten and one hundred thousand oocytes. This ooctye resource, a majority of which are small primary oocytes, represents a large pool for increasing the reproductive potential of domestic livestock.

The objectives of this research were to develop procedures for the recovery of large numbers of bovine primary oocytes and determine how long they remain viable when subjected to in vitro culture.

Chapter 2

REVIEW OF LITERATURE

OOCYTES

Anatomy of the Bovine Ovary

The bovine ovary is an almond-shaped organ weighing 10-20g in the mature animal (Hafez 1974) and is morphologically separated into two major regions.

The central tissue, the medulla, consists of loose fibroelastic connective tissue, numerous large blood vessels, lymphatics, nerves and scattered strands of smooth muscle fibers. Enveloping the medulla is the cortex, consisting of a compact cellular stroma composed of networks of reticular fibers and spindle shaped cells which exhibit characteristics of both fibroblasts and smooth muscle cells (Leeson and Leeson 1976). Included in the cortical region are two other cell layers which envelop the cortex proper. These are: the tunica albuginea, a layer of dense connective tissue and the germinal epithelium, a layer of low cuboidal cells which constitutes the epithelial surface of the ovary.

The ovarian cortex is of special interest because it is the region in which the oocyte population is located and is also the site of the production of the major ovarian hormones.

Oocyte Population

Primary oocytes are formed by the mitotic division of the primordial germ cell derived oogonia. The primordial germ cells migrate from the yolk sac to the presumptive gonad during the period of organogenesis. The primary oocytes enter prophase of meiosis I and become arrested at the dictyate stage. Ohno and Smith (1964) observed that primary oocytes which became successfully arrested at the dictyate stage had become surrounded by fetal follicular cells. From these observations they concluded that the fetal follicular cells were important in the initiation and maintenance of the dictyate stage. Primary oocytes remain in the dictyate stage through their growth phase until just prior to ovulation (Shea et al. 1976b).

Large numbers of primary oocytes are formed during the period of fetal development. Baker (1972) reported the presence of six hundred thousand primary oocytes at the second month and seven million primary oocytes at the fifth month of human fetal development. After this point, the oocyte population declines sharply until, at birth, two million oocytes are present. Estimates of the oocyte populations for other species include: 160,000 in the rat, 700,000 in the dog (Austin 1961) and 60,000 to 100,000 in domestic livestock, i.e. cows, sow, ewe, depending on the species and breed (Hafez 1974). The oocyte population in terms of oocyte numbers, may vary between species, but the pattern of oocyte formation and population decline seems to

be the same in all species.

Oocyte size distribution

Primary oocytes are approximately 20u in diameter and become enclosed in a single layer of flattened granulosa cells. This complex of primary oocyte and accompanying follicular cells is known as a primordial follicle.

As the animal matures, cyclic ovarian activity is established, characterized by a number of growing oocytes and follicles. The development of ovarian follicles is often classified histologically by: 1) size, 2) number of layers of granulosa cells, 3) development of the theca, and 4) position of the occyte within its cumulus oophorus (Hafez 1974). A classification system which has received widespread use is that proposed by Pederson (1970) illustrated in Figure 1.

Cyclic activity appears early and, in the mouse, evidence of follicular growth is noted at seven days following birth (Peters 1969), however, the largest follicle present at this time is usually type 3b. As the animal matures, progressively larger follicle types are present. In any case, large preovulatory follicles (type 8) are not in evidence until the onset of puberty. It is only at puberty that a follicle reaches sufficient size and maturity that a successful ovulation occurs. The process of oocyte and follicular growth

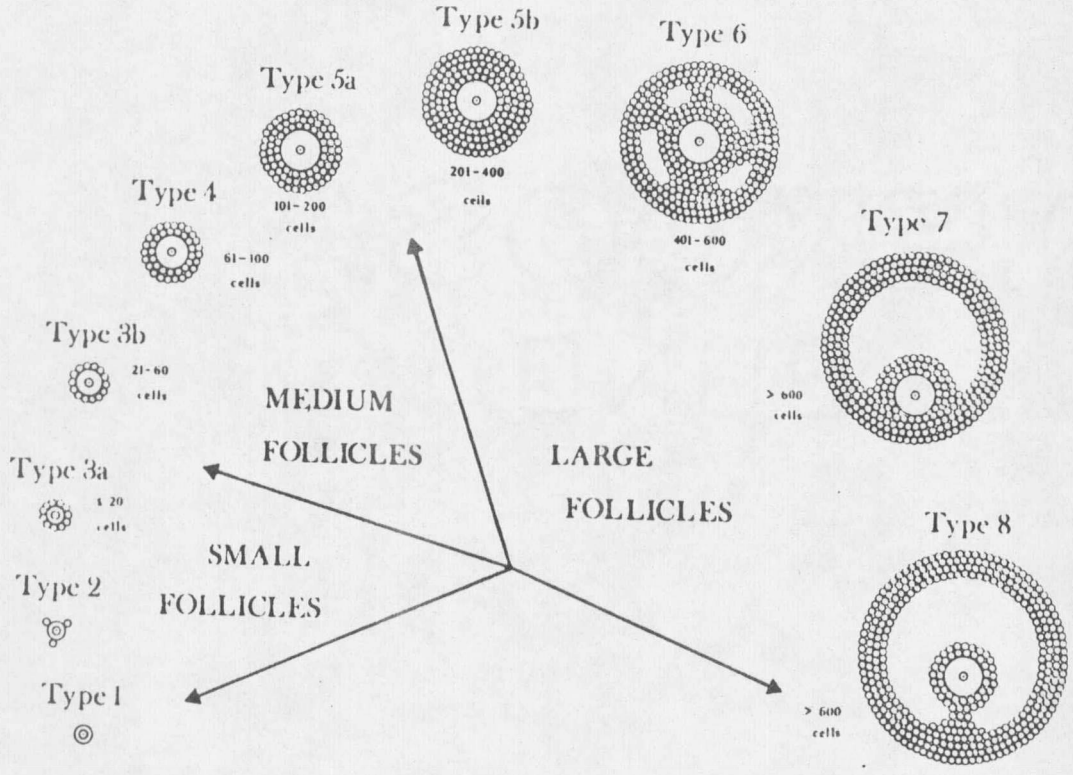

Figure 1. Classification of the different stages of follicle development in the mouse according to number of granulosa cells in the largest cross section. (Pederson, 1970)

is irreversible, that is, once the oocyte and follicle have entered the growth phase, it is fated to either ovulate successfully or succumb to the degenerative processes of atresia (Oakberg 1979). The number of follicles present in each particular stage of growth, at any point in time, depends partly upon escaping atresia and partly upon the number of oocytes which enter the growth phase during each cycle of ovarian activity. Evidence exists that this quantity is under intraovarian regulation, in that, the number of oocytes entering the growth phase is a function of the pool of non-growing oocytes (Krarup et al. 1969). This seems to indicate that as the oocyte population is depleted, as a result of age, fewer oocytes enter the growth phase during each estrous cycle. The number of oocytes in different stages of growth may vary, as well as the number of oocytes which initiate growth processes during each cycle, however, the non-growing pool of oocytes comprises approximately ninety percent of the oocyte population at any one time (Peters 1969, Baker 1971), clearly constituting the majority of the oocyte resource.

Oocyte isolation

A major proportion of past oocyte research was performed with fully grown oocytes obtained from antral follicles. Gonadotropin pretreatment was usually employed to enhance the number of oocytes

available for recovery (Hafez and Ishibashi 1969, Thibault et al. 1975, Magnusson et al. 1977). The oocytes were recovered by puncturing the follicle and expressing the follicular contents or by aspiration of the follicle with a syringe. However, such techniques did not permit the recovery of primary oocytes or oocytes in the intermediate stages of growth.

Recovery of growing oocytes from small and medium sized follicles was successfully performed through the utilization of enzyme digestion techniques. A variety of enzymes have been used, either singly, or in combination to isolate oocytes including: trypsin (Sorensen and Wassarman 1976, Strickland 1976), combinations of hyaluronidase, collagenase and egg white lysozyme (Mangia and Epstein 1975, Wassarman and Josefowicz 1978), and collagenase type III combined with DNase I (Eppig 1976). Varying degrees of success have been achieved with enzyme isolation techniques but, as yet, no particular enzyme or combination of enzymes has proven superior for oocyte isolation. Enzyme digestion techniques have permitted detailed investigation of the biochemical processes associated with oocyte growth (Schultz and Wassarman 1977, Mangia and Epstein 1975) and the development of meiotic competence in growing oocytes (Sorenson and Wassarman 1976). Research of this type requires the use of culture techniques which will support oocytes in vitro for moderate periods of time.

Oocyte culture

A number of different nutrient media have been employed for the *in vitro* culture of oocytes and ovarian tissue including: modifed Krebs-Ringer bicarbonate (Biggers *et al.* 1967), Brinster's Medium for Ovarian Culture (BMOC-2) (Cross and Brinster 1970), Tissue Culture Medium 199 (TCM-199) (Hillensjo *et al.* 1979), Eagle's Medium (Tsafriri *et al.* 1972), Minimum Essential Medium (MEM) (Eppig 1979) and Ham's F-10 (Shea *et al.* 1976b). In most cases the media were supplemented with various substances such as: Calf serum, bovine serum albumin, buffers, carbohydrates (i.e. glucose, pyruvate), amino acids, cyclic 3', 5' adenoisine monophosphate (cAMP), steriods, gonadotropins and radioisotopes.

Culture vehicles included the various standard culture dishes and flasks. One unique approach to oocyte culture was that performed by Tsafriri *et al.* (1972). This involved excising the follicle from the ovary and utilizing organ culture procedures to maintain the oocyte inside of the complete intact follicular structure, closely mimicking the *in vivo* environment.

Standard conditions throughout the culture periods were maintained in most, if not all, of the oocyte experiments. These conditions were: Standard temperature of 37-38° C and a humidified atmosphere of five percent CO_2 in 95 percent air.

Duration of the culture period has been varied, but overall, was relatively short term. The reason for such short culture periods lies in the fact that much of the past oocyte research was conducted for the study of meiotic maturation. Such studies utilized fully grown oocytes and the events under study manifested themselves after short periods of in vitro culture. Oocytes have been successfully cultured for 12-13 hr (Cross and Brinster 1970), 18 hr (Bae and Foote 1975), 22-30 hr (Shea et al. 1976), 44 hr (Hunter et al. 1972) and 72 hr (Hillensjo et al. 1979). The culture period for oocytes inside intact follicles has also been relatively short term, i.e. 12-18 hr (Tasfriri et al. 1972).

Long term culture of oocytes and ovarian fragments has not been a major emphasis of experimental research, but Blandau's (1969) work, demonstrating that some human fetal ovarian fragments were viable following 79 days of in vitro culture lends support to the idea that long term oocyte culture may be possible.

Oocyte preservation

Although long term in vitro culture of oocytes has not received much attention, attempts have been made for long term storage of oocytes through utilization of low temperature preservation procedures. These attempts have not met with great success and, at present, embryos have proven more amenable to freezing than unfertilized

oocytes. Whittingham et al. (1972) have shown that mouse embryos could survive storage at - 196° C. Maurer (1976), in his review, states that mouse and rabbit embryos frozen at - 196° C or lower remained viable for at least 200 days. Also pointed out was that when mouse oocytes were rapidly cooled (60° C/min) to - 21° C, they could be subsequently fertilized with normal fetal development, if they were not held at this temperature. Further in the review, it was stated that mouse oocytes stored for 3.5 hr at -10° C retained their fertilizability, but only twelve percent developed into viable offspring, whereas, when stored for 6 hr at -10° C, none of the oocytes, when fertilized, developed into viable young.

FOLLICLES AND FOLLICLES CELLS

Follicle cells represent a group of specialized, multi-potential cells which function in a highly coordinated manner. They are intimately involved in the support of oocyte growth and function to produce the major ovarian hormones. They are important not only in the events leading up to ovulation, but also in the period following ovulation and the early stages of pregnancy.

Follicle formation and growth

During the period of organogenesis, the primary oocytes become enclosed by a layer of flattened follicular cells. These first

follicular cells are embryologically derived from the rete ovarii (Byskov and Lintern-Moore 1973). This complex, the primordial follicle, represents the basic unit of ovarian function.

Follicle growth involves a number of phases: 1) growth of the oocyte, 2) proliferation of the granulosa cells, 3) formation of the theca, and 4) formation of a cavity, or antrum with the accumulation of follicular fluid. All of these processes may culminate in ovulation, or release of a fertilizable oocyte. Following ovulation, the follicular cells form a corpus luteum for the support of early pregnancy. If no pregnancy occurs, the corpus luteum regresses and another cycle of follicular activity ensues.

The factor or factors initiating follicle growth remain obscure. At present, whether the initiation of follicular growth is a response to stimulation or the removal or inactivation of an inhibitor is unknown, however, it is doubtful that gonadotropins or estrogens are involved in the growth initiation process (Peters 1979).

Early follicular growth consists mainly of an increase in the number of granulosa cells. Some controversy exists concerning the origin of these new follicular cells. Whether the increase in the number of follicle cells is a result of mitotic division of existing cells (Oakberg 1979) or the accumulation and differentiation of surrounding stromal cells (Lintern-Moore and Moore 1979) has not been

resolved. As the number of follicular cells increases, a change occurs in the conformation of the follicular epithelium. There is a transition from a flattened, squamous layer to one which is cuboidal in appearance. The next discernable event in the follicular growth process is the appearance of the theca which has been suggested to be a response to a theca cell organizer produced by the granulosa cells (Midgely et al. 1974). Thecal development is an important event since follicles in which the theca layer is absent do not exhibit further development. The theca layer is sensitive to and dependent on a fine interplay of hormones to succeed in its full development (Peters 1979).

Later stages of follicular growth are characterized by increasing follicular size and the formation of an antrum. Gonadotropins are necessary for the support of follicular growth (Peters 1979), and their action on the follicle is modulated by an adenylyl cyclase system (Vaitukaitus and Albertson 1979, Hunzicker-Dunn et al. 1979), possessing a guanine nucleotide regulatory site (Lindner et al. 1977). Recent information (Anderson 1979, Fletcher 1979) has disclosed that follicular cells are attached by gap junctions which develop about the same time as the appearance of gonadotropin receptors. Gap junctions function to electrically and metabolically couple cells in many different tissue types. Therefore, gap junctions have been implicated in coordinating many of the aspects of follicular development and may be involved in propagating hormone initiated signals (Amsterdam and

Linder 1979).

Follicular cells, especially those from large follicles, actively synthesize steroids, most notably, progesterone, 20 α dihydroprogesterone, testosterone, androstenedione, 17 β estradiol and estrone (Mills 1979). Steroidogenesis is a coordinated and complicated process. For example, both thecal and granulosa cells are necessary in the biosynthesis of estrogen (Younglai 1979). The thecal cells are thought to be responsible for androgen production since they are embryologically derived from the rete ovarii. The rete ovarii iteself, represents a part of the mesonephric tubules, which in the male, develop into the epididymis. The epididymis is known to have the ability to transform steroid precursors such as acetate and cholesterol into testosterone. Therefore, analogous derivatives of the mesonephric tubules might also be implicated in androgen production in the ovary (Peters 1979).

Steroidogenic activity is dependent upon the stage of follicular development, that is, the rate of steroid production and the steroids produced are related to the size of the follicle. Cells from larger follicles are more apt to synthesize progesterone than granulosa cells from smaller follicles (Nakano et al. 1975, Thanki and Channing 1979). In addition, steroidogenic activities are influenced by hormonal stimulation. Luteinizing hormone (LH) has the capability to stimulate follicular androgen production, while another pituitary

hormone, follicle stimulating hormone (FSH), tends to stimulate the aromatization of androgens into estrogens (Armstrong et al. 1979).

Some of the products of follicular cell synthesis accumulate in the follicular fluid which, aside from steroids, also contains several proteins, amino acids, sugars, enzymes, mucopolysaccharides and salts (Kang et al. 1979). Some of the proteins are: Steroid binding proteins (Cook et al. 1977), a granulosa cell luteinizing inhibitor (Channing 1979), and an oocyte meiosis inhibitor (Tsafriri et al. 1976, Stone et al. 1978, Channing 1979). The components of follicular fluid do not remain stable but vary in relation to the stage of follicular development. For example, the luteinization inhibitor is present in follicular fluid obtained from small follicles but is absent in fluid from large follicles (Alexander et al. 1978, Channing 1979). Likewise, the meiosis inhibitor is more potent in fluid from small follicles than that of large follicles (Stone et al. 1978). The significance of the fluctuations of the various components in follicular fluid is not understood, but it is tempting to presume that these changes reflect the physiological status and may characterize the degree of developmental maturity of the follicle.

Atresia

Once a follicle begins to grow, it will either successfully

reach ovulation or be lost through degenerative atretic processes. The majority of follicles which enter their growth phase succumb to atresia, which affects follicles of all sizes and occurs continuously throughout the life of the female. There is no uniform pattern and atretic processes vary with the size or stage of differentiation of the follicle (Byskov 1970). Oakberg (1979) reported that in the mouse, degeneration of type 1 through 4 follicles is rare, but in type 5a, 78 percent of the follicles were classified as degenerating, regardless of the stage of estrous. Only in larger follicles, i.e. type 6, did stage of estrous show any effect on the number of atretic follicles, with fewer present at estrus than any other stage.

Atresia in small follicles occurs mainly by lysis or phagocytosis of the oocyte. Byskov (1979) observed that a small fraction of medium sized follicles become atretic but most do not exhibit luteinization. In comparison, large follicles, a majority of which do become atretic, tend to luteinize and the granulosa cells exhibit an accumulation of lipid droplets or they tend to become pyknotic.

The causes of atresis have not been defined. Perusal of the literature in this area leads to the conclusion that many factors are involved, either singly, or in concert in the onset of atresia. Ingram's (1962) review noted that the greatest loss of oocytes occurs during the early postnatal period, inexplicably. He also implicated hormone levels and pituitary involvement in atresia. Channing (1979)

reported the presence of gonadotropin binding inhibitors, which along with the loss of hormone receptor sites, may lead to the onset of atresia. Ledwitz-Rigby (1979) proposed that the failure of the follicular fluid luteinization inhibitor to decline as the follicle grows may render the follicle unresponsive to LH stimulation and predispose the follicle to other factors initiating atresia.

It is known that there exists a close, junctional complex between the oocyte and its follicular cells (Zamboni 1970, Fletcher 1979). Biggers et al. (1967) demonstrated that the follicular cells may function to provide substrates for the support of oocyte metabolism. Therefore, it is conceivable that disruption of this close interaction may lead to atresia.

Follicle cell culture

Follicle cells can be collected and cultured in vitro relatively easily. Granulosa cell cultures tend to luteinize and secrete progesterone (Channing and Tsafriri 1977) and the presence of Δ5-3β, 3α, 17β and 20α hydroxysteroid dehydrogenases has been demonstrated (Fischer and Kahn 1972).

In the presence of gonadotropins, cultured cells undergo morphological changes associated with luteinization (Thanki and Channing 1979). Isolated granulosa cells from hypophysectomized rats secrete estradiol 17β in the presence of FSH and testosterone

(Fortune and Armstrong 1979). In general, it appears that gonadotropins stimulate steroidogenic activity of follicular cells *in vitro* as well as *in vivo*.

Granulosa cells *in vitro*, exhibit fibroblast type growth (Stoklosowa *et al.* 1978, Hillensjo *et al.* 1979), and accumulate droplets of cytoplasmic lipid (Thanki and Channing 1979). The presence of the oocyte does not necessarily inhibit the luteinization of granulosa cells *in vitro* (Channing and Tsafriri 1977).

OOCYTE METABOLISM AND GROWTH

Oocyte formation, as mentioned previously, occurs during the period of organogenesis. Meiotic activity begins during fetal life and the diplotene stage is reached shortly before or after birth. At this point, meiotic activity ceases and the primary oocytes enter the dictyate or "resting" stage. This meiotic arrest persists until shortly before the oocyte is ovulated. The duration of the dictyate period varies with the reproductive lifespan of the species. For example, human oocytes may remain in the dictyate stage for several decades (Zamboni 1970). In the interval between meiotic arrest and the resumption of meiosis, the oocyte undergoes a period of growth which is closely associated with the follicular activity discussed previously.

The most immature primary oocyte is a relatively simple cell

consisting of a large, slightly eccentric nucleus, a few small peripheral mitochondria, a profusion of granular cytoplasmic vesicles and free ribonucleoprotein (RNP) particles. The majority of the organelles are concentrated in a crescent shaped region close to the nucleus (Adams and Hertig 1964).

The oocyte commences its growth phase when it is surrounded by nine to ten follicle cells (type 3a) (Lintern-Moore and Moore 1979). Histologically, oocyte growth is characterized by progressive changes in the nucleoli, ribosomes, mitochondria, endoplasmic reticulum, Golgi complex and other organelles which reflect extensive alterations in the metabolism of the oocyte (Wassarman and Josefowicz 1978).

During oocyte growth, nuclear activity is high and the nuclear envelope has a wavy appearance. Protrusions or blebs appear in the nuclear envelope, often occurring in close proximity to the pores of the nuclear envelope (Adams and Hertif 1964, Baker 1971). Structures, distributed throughout the cytoplasm, similar to the blebs of the nuclear envelope, indicate that the blebs are shed into the cytoplasm, functioning not only in the proliferation of the cellular organelles, but also contributing to the distribution of informational material, from the nucleus, needed for early embryonic development (Baker 1971).

The proliferating mitochondria are present in varying conformations which may represent stages of budding or transitional stages of metabolism (Baker 1971, Szollosi 1972). The mitochondria exhibit