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.

STATEMENT OF PERMISSION TO COPY

In presenting this thesis in partial fulfillment of the requirements for an advanced degree at Montana State University, I agree that the Library shall make it freely available for inspection. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by my major professor, or, in his absence, by the Director of Libraries. It is understood that any copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Signature Amy C. Carr

Date 3/24/91

DEDICATION

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

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

Approved:

Peter J. Bryfogle
Chairperson, Graduate Committee

William J. Dorgan
Co-chairperson, Graduate Committee

Head, Major Department

Michael Malone
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

December, 1981

ACKNOWLEDGEMENTS

I would like to express my since gratitude to Dr. E. L. Moody for providing guidance and encouragement throughout my graduate program and for providing opportunities to expand my experiences in the many facets of experimental research as well as the applied aspects of animal reproduction.

Thanks to Drs. Dorgan, Jackson, Burfening and Bradbury for advice, counseling and the sustenance of my morale.

I would like to express my thanks also to my laboratory assistants, Karen King, Liz Frazer and Vicki Bokum.

I am grateful to Drs. J. Catlin and M. H. Smith for providing assistance on the surgical aspects of this research.

Muchas gracias to my fellow graduate students and good friends for providing moral support and for many good times.

Thanks also to Evelyn Richard for typing this manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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 CO_2 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*.

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

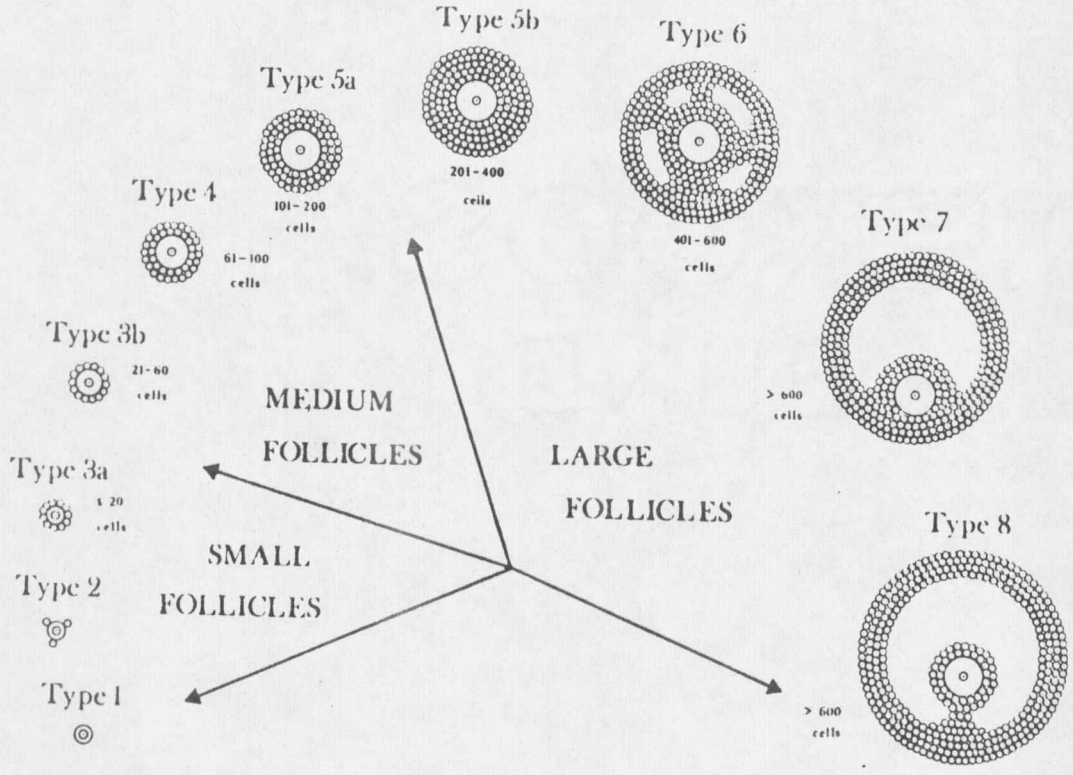

Figure 1. Classification of the different stages of follicle development in the mouse according to number of granulosa cells in the largest cross section. (Pederson, 1970)

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

a hooded appearance which increases their surface area and may also provide a specific micro-environment to facilitate exchange of metabolic intermediates with the endoplasmic reticulum (Cran et al. 1980). Such mitochondrial configurations have been noted in the oocytes of the cow, goat and sheep (Senger and Saake 1970, Cran et al. 1980). It was suggested that such a modification may be critical to a cell which depends, in part, on previously accumulated energy sources (Senger and Saake 1970). The oocyte represents such a cell since it is the predecessor to the embryo. The early embryo relies on stored nutrients to ensure its survival. This is evidenced by the restricted energy substrate requirements of early embryos (Biggers et al. 1967, Biggers 1972), and a reduction in the protein content of the early embryo by some twenty percent in the cow (Hafez 1974) and the mouse (Brinster 1974).

The endoplasmic reticulum is closely associated with the mitochondria of the growing oocyte, leading to the speculation that this association provides the endoplasmic reticulum with the high energy required for synthetic activity (Adams and Hertig 1964, Szollosi 1972). Later in the growth phase, the very extensive network of endoplasmic reticulum disappears, giving rise to numerous free vesicles, some of which are associated with the mitochondria (Wassarman and Josefowicz 1978).

The Golgi complex first appears as a single aggregate of smooth

membraned vesicles and short broad channels (Adams and Hertig 1964). In later stages of growth, the Golgi consists of a large conglomerate of vacuoles, granules and lamellae. The dimunition of the smooth endoplasmic reticulum suggests that it may function as a precursor for other membrane systems, while the changes of the Golgi suggest its increasing activity, involving possibly the concentration of secretory products, formation of cortical granules (Wassarman and Josefowicz 1978), adjusting the fluid reserves of the oocyte (Adams and Hertig 1964), and maybe a role in zona pellucida formation (Baker 1971).

The formation of the zona pellucida occurs during the growth phase of the oocyte. Baker (1971) implicates both oocyte and follicular cell involvement in the elaboration of the mucopolysaccharide components of the zona pellucida and particularly the Golgi complex which has been shown to synthesize mucopolysaccharides. The work of Adams and Hertig (1964) supports this idea. They noticed that the Golgi becomes peripherally located near the oolemma at the onset of zona formation.

Even with the cessation of oocyte growth and the formation of the zona pellucida, the oocyte and the surrounding follicular cells remain in close contact. Concurrent with zona formation, microvilli develop over the oocyte accompanied by cytoplasmic projections of the granulosa cells (Adams and Hertig 1964; Zamboni 1970) insuring the

close functional relationship.

Biochemically, the growing oocyte is actively synthesizing RNA at a high rate (Oakberg 1968, Moore et al. 1974). A significant aspect of the dynamics of this RNA synthesis is that it decreases sharply in type 5b follicles and is at very low levels in type 6 and 7 follicles. The initial decline in the RNA synthesis occurs at the time that the oocyte is fully grown and coincides with antrum formation (Moore and Lintern-Moore 1978). This intense RNA synthesis represents transcription of the oocyte nucleolar genes (Moore and Lintern-Moore 1978, Lintern-Moore and Moore 1979). The RNA synthesized makes its way into the cytoplasm of the cell (Oakberg 1968, Moore et al. 1974), probably through the process of nuclear blebbing (Baker 1971). Both ribosomal and heterogeneous RNA species are being synthesized. Biochemical analyses show that eighty-five percent or more of the RNA in fully grown oocytes is ribosomal or RNA (Wassarman and Josefowicz 1978).

Protein synthesis patterns of growing oocytes, which probably reflect, in part, the transcriptional activity, present the same trends. Schultz and Wassarman (1977) demonstrated significant qualitative changes in the size classes of proteins synthesized during the oocyte growth period.

Enzyme system activities also increase during oocyte growth. Mangia and Epstein (1975) demonstrated that the levels of glucose-

6-phosphate dehydrogenase (G6PD) and lactate dehydrogenase (LDH) activity increase during oocyte growth and this activity declines after the oocyte has reached its maximum growth, suggesting that the synthesis of these enzymes is almost complete when the oocyte reaches its maximum size.

Eppig (1976) demonstrated that growing oocytes utilized pyruvate more efficiently than glucose, lactate or succinate as an energy source. Other research has shown that pyruvate serves as an important energy substrate in fully grown oocytes and early embryos (Biggers et al. 1967, Rushmer and Brinster 1973).

The end product of oocyte growth is a cell which is capable of resuming meiosis and, upon fertilization, has the ability to develop into a normal individual. Sorensen and Wassarman (1976), demonstrating the importance of growth, noted that the incidence of spontaneous initiation of meiotic maturation, of mouse oocytes, was related to the size or stage of growth of the oocyte. These observations have been corroborated by Schultz and Wassarman (1977) and McGaughey (1977) who demonstrated that oocytes isolated from larger follicles resumed meiosis more readily than oocytes from small follicles. These findings distinctly support the contention that oocyte growth represents the accumulation of "information" needed for subsequent development (Baker 1971, Smith 1972, Moore and Lintern-Moore 1978).

Growing oocytes have been cultured in vitro for short periods

of time (Sorensen and Wassarman 1976, Eppig 1976, Schultz and Wassarman 1977). Eppig (1979) cultured growing oocytes *in vitro* for seven days and concluded that the close junctional complex between the oocyte and the follicular cells must be maintained in order to facilitate growth. At present, there is a paucity of information concerning the recovery and *in vitro* culture of the large population of oocytes which comprises the non-growing pool. The physiological mechanisms which initiate oocyte growth are not known, but the work of Lintern-Moore and Moore (1979) indicates the *in vivo* requirement for a predominantly cuboidal follicular epithelium before oocyte growth commences. This, along with the work of Biggers (1972) and Eppig (1979), who concluded that the follicular cells and the oocyte reciprocally influence each other, indicates a major role for the follicular cells in the initiation and support, as well as the coordination of oocyte growth.

Meiotic maturation and *in vitro* fertilization

For some time following the completion of oocyte growth, the follicle continues to enlarge, with the formation of the antrum and the appearance of the structures of the classic Graafian follicle, i.e. corona radiata, cumulus oophorus. Shortly after ovulation, the oocyte resumes meiosis with the consequent emission of the first polar body. This event, referred to as meiotic maturation, has been

an area of intense study in mammals and other vertebrates (Schuetz 1964, Edwards 1966, Masui and Market 1971, Bae and Foote 1975).

The time scale and sequence of events of oocyte maturation are well chronicled (Sorensen 1973, Lopata et al. 1977). Oocytes of a number of species will mature spontaneously in vitro (mouse; Cross and Brinster 1970, Sorensen 1973, Bae and Foote 1975, Schultz and Wassarman 1977, cow; Hafez and Ishibashi 1964, Shea et al. 1976b, Thibault et al. 1976, Thibault 1977, pig; McGaughey 1977, rat; Magnusson et al. 1977, rabbit; Shea et al. 1976a).

Protein synthesis occurs during oocyte maturation (Smith 1972, Golbus and Stein 1976). Similar to oocyte growth, there are significant qualitative and quantitative changes in the size classes of the proteins synthesized, but it is not clear whether they are different from those proteins synthesized during the growth period (Schultz and Wassarman 1977). However, protein synthesis does not change in vitro in the same way it does intrafollicularly (Thibault 1977).

Metabolically, oxygen consumption increases during maturation with a concurrent increase in pyruvate utilization (Magnusson et al. 1977). Zeilmaker et al. (1972) and Zeilmaker and Verhamme (1974) concluded that oxygen (oxidation-reduction potentials) was important in the initation and support of meiotic maturation of mouse and rat oocytes in vitro.

Hormones are involved in the induction of meiotic maturation of oocytes of lower vertebrates (Schuetz 1964) and hormones have been used to stimulate meiotic maturation of follicle enclosed oocytes of mammalian species (Tsafriri et al. 1972, Thibault et al. 1975). While this information indicates a role for hormones in the initiation of meiotic maturation in vivo, research implicating the follicular cells in the maintenance of the dictyate stage (Thibault 1972, Foote et al. 1970, Hillensjo et al. 1979) and the partial characterization of meiosis inhibitors present in follicular fluid (Tsafriri et. al. 1976, Stone et al. 1978) supports the contention that fully grown oocytes are poised to resume meiosis and no new proteins need to be synthesized for initiating meiosis (Schultz and Wassarman 1977). In other words, hormones are not involved and final oocyte nuclear maturation is not follicle dependent (Thibault 1977). Research with meiosis inhibitors (Tsafriri et al. 1976, Stone et al. 1978) has shown that LH reverses the inhibition, indicating that, in vivo, the preovulatory surge of LH serves a permissive role in oocyte maturation.

Masui and Market (1971) demonstrated, in the frog, that the cytoplasm played a major role in the control of meiotic maturation. Balakier and Czolowska (1977) had similar results using mouse oocytes, concluding that meiotic maturation is induced by a cytoplasmic factor which is produced or unmasked independently of the nucleus. Schultz et al. (1978) confirmed these results and suggested that meiotic

maturation is under the control of cytoplasmic factors: that a change in quality of the cytoplasm rather than quantity is responsible for the acquisition of meiotic competence during oocyte growth.

Although ooctyes can be matured *in vitro*, fertilizability remains in question. Normal young have been born to mice (Cross and Brister 1970) when follicular oocytes were matured and fertilized *in vitro* and in rabbits and sheep (Betteridge 1977) when matured oocytes were transferred and fertilized in the recipient. Similar work with oocytes of the calf (Menezo *et al*. 1976, Thibault *et al*. 1976) have proven ddisappointing. *In vitro* matured oocytes of rabbits, pigs, cows and women can be penetrated by spermatazoa but, the male nucleus does not dissociate and swell immediately as it does in those oocytes matured *in vivo* (Thibault 1977). The preliminary report of Soupart (1979) indicates that fusion of two oocytes can be successfully performed, providing an alternative to *in vitro* fertilization with spermatazoa.

Chapter 3

MATERIALS AND METHODS

The experimental procedure can be separated into four major phases: 1) surgery, 2) tissue preparation, 3) enzymatic digestion and 4) plating and culture.

SURGERY

Non-pregnant, cycling cows and heifers from the Montana State University beef herd were ovariectomized, the surgery being performed under local anesthetia (Lidocaine). Upon removal, the ovaries were placed in an insulated carrier containing either Earle's Salts[1] or Ham's F-10[1] nutrient mixture which had been warmed to 38°C. The ovaries were then transported to the laboratory for tissue preparation.

TISSUE PREPARATION

The ovaries were removed from the carrier under a sterile hood, where all subsequent preparatory work was performed. The first step in preparing the tissue involved washing the ovaries with warmed Earle's Salts and removing extraneous connective tissue. The ovaries were then placed in a new petri dish containing Earle's Salts and sliced transversely into strips 1-3 mm in width. With the aid of tissue forceps and iridectomy scissors the medullary tissue was separated from the

---

[1]Grand Island Biological Company, Santa Clara, California.

cortex. Any antral follicles, which had not been previously punctured, were excised. This was done to ensure a more homogeneous population of oocytes following enzyme digestion. The remaining cortical tissue was placed into a clean petri dish containing a small (5 ml) amount of fresh Earle's Salts and diced into small (1 mm^3) fragments with a scissor-type action of two scalpels with # 11 blades. Once dicing was complete, the Earle's Salts solution was decanted and the tissue subjected to enzymatic digestion.

ENZYME DIGESTION

The enzyme digestion apparatus consists of a standard trypsinizing flask containing a magnetic stirring bar. The open sidearm and top are covered with aluminum foil and the whole apparatus is sterilized. The digestion solution consisted of the required amount of enzyme dissolved in warmed (38°C) Hank's Ca^{++} and Mg^{++} free Balanced Salt Solution (HBSS)[1]. The initial experiments involved simultaneous, side-by-side digestions using trypsin and collagenase. The trypsin solution was prepared to a strength of .25 percent by mixing 4 ml of 2.5 percent Trypsin Solution[1] in 36 ml HBSS. The strength of the collagenase solution was 1 percent and prepared by dissolving .4 g collagense Type II powder[2] into 40 ml HBSS. Both enzymes satisfactorily dispersed the ovarian tissue, but since the majority of the connective tissue of animals is composed mainly of collagen, it

---

[2]Sigma Chemical Company, St. Louis, Missouri.

was decided to use collagenase exclusively. Subsequent experiments involved variation of the strength of the collagenase solution. These concentrations varied from .25 percent to 1 percent, the best results coming from .5 percent collagenase solutions, prepared by dissolving .25 g of collagenase in 50 ml of warmed HBSS.

Immediately following the preparation of the enzyme solution, the prepared tissue was then added to the flask and the flask was swirled by hand to remove any tissue fragments stuck to the side. The flask was then placed on a magnetic stirrer in an incubator that maintained the temperature at 38°C. The mixture was agitated at low speed and monitored periodically to determine the extent of cell dispersion. Samples of the digestion mixture were also taken and examined under an inverted microscope in an attempt to ascertain the degree of cell dispersion and determine the time at which the optimum number of primary oocytes had been liberated.

Duration of the digestion varied from 45 minutes to 2.5 hours. Because the strength of the enzyme solution varied, as well as the specific activity of the enzyme, the decision to terminate the digestion was made from the previously mentioned observational techniques rather than a set time. Upon the decision to terminate the digestion, the supernatant was decanted into four sterile 15 ml centrifuge tubes. These were spun for five minutes at moderate speed in a table-top clinical centrifuge. The centrifugation creates a tissue pellet in

the bottom of the tube. Following centrifugation, the supernatant was aspirated and fresh, warmed Earle's Salts (5 ml) was added to each tube. The pellet was broken up by sucking and expelling the fluid, by mouth, with a sterile pipette. When the pellet was sufficiently broken up, the tubes were recentrifuged for five minutes, followed by another cycle of washing and centrifugation. After the final washing and centrifugation, the supernatant was aspirated and the tissue pellet was resuspended in culture media and aliquoted to the culture flasks and dishes.

PLATING AND CULTURE

All cultures were carried out under similar conditions of controlled temperature (38°C) in a 5 percent CO_2, 95 percent air humidified atmosphere.

Initially, two culture media were used. These were: Brinster's (BMOC-3) Culture Medium[1] and Ham's F-10 Nutrient Mixture[1]. Both were normally supplemented with: calf serum[1] to a concentration of 10 percent and a Pencillin-Streptomycin-Fungizone mixture[3] to a 1 percent concentration.

The cultures, in the beginning, were carried out in sterile plastic 75 cm^2 culture flasks[4]. Flasks allowed sufficient observation of the

---

[3]Microbiological Associates, San Francisco, California.

[4]Falcon Plastics - VWR Scientific, Seattle, Washington

cultured cells but reliably counting the cells and attempts at manipulation were not feasible with this system. Subsequent experiments utilized a 60 x 15 mm Integrid dish[4] which had a 2 mm grid system. Twenty-five mm glass coverslips were added to each dish. During culture the coverslips could be removed, the attached cells fixed in Formalin-Acetic Acid (FAA) and stained by routine Hematoxylin-Eosin staining procedures. Sudan Black-B lipid staining procedures were also occasionally employed. Attempts were also made to isolate oocytes for fixation and staining by using a mouth aspirated 10 ul pipette[5]. The technique involved observing the dish with the inverted microscope, picking up the located oocytes with the pipette and transferring them to a standard glass slide. The transferred cells were fixed to the slide with FAA and stained with Hematoxylin and Eosin. Fixation and staining for histological examination permitted the gathering of a data base on primary oocytes in culture and lent confidence to visual observations using the inverted microscope which would not provide the resolution required for definite analysis.

Once the pellet of digested tissue was resuspended in culture media, it was aliquoted to the flasks and dishes which had been previously prepared by addition of culture media and placement in the

---

[5]Kimble Owen-Corning, Toledo, Ohio.

incubator to allow equilibration. Following addition of the digested tissue, the final volume of the flasks and dishes was approximately 15 ml and 3 ml respectively. After one night of culture, the dishes were examined and an oocyte count performed. This was done by counting the number of oocytes in ten randomly selected squares in each dish. The number of oocytes observed was totaled and divided by the number of dishes examined, arriving at a figure for the average number of oocytes observed per dish. Since the ten observations per dish represented 2 percent of the total area of each dish, calculations, using the formula $0.2x$ = average number of oocytes observed per dish, allowed an estimate of an average number of oocytes per dish. When this figure was multiplied by the number of dishes in the experiment, which was either 50 or 60, then an estimate of the total number of oocytes isolated was attained. Twice it was possible to take tissue weights and the number of oocytes per mg of digested tissue was calculated.

Media changes were performed at 48 or 72 hour intervals by aspiration of the old media and pipetting in fresh media. Various attempts were made to stimulate growth through media supplementation. These included supplementing the media with: 1 mM concentrations of pyruvate; PMS (1000 IU/ml), HCG (1200 IU/ml) and combinations of PMS and HCG (1000 IU/ml and 800 IW/ml respectively); and cAMP in concentrations of 10, 100, and 1000 µg/ml. In all cases, visual observations of the

flasks and dishes were made at various intervals and coverslips were taken, fixed and stained for histological examination.

Attempts were made to selectively isolate oocytes by using a sucrose density gradient. The absence of follicular and stromal cells in the cultures would permit easier observation of the oocytes as well as allow comparisons between attempts to stimulate oocyte growth in the presence and absence of other ovarian cells. The preparation of the sucrose density gradient consisted of mixed 10, 20, 30 and 40 percent sucrose solutions. This required dissolving the necessary amount of sucrose in Earle's Salt Solution. The prepared mixtures were autoclaved and stored in the refrigerator, but were warmed before use. Preparation of the gradient itself required layering of the solutions in a sterile centrifuge tube with the 40 percent solution at the bottom and the decreasing density solutions successively layered on top of that. The first attempt used 15 ml centrifuge tubes with each layer being a volume of 2 ml. Subsequent attempts involved 50 ml centrifuge tubes with each sucrose layer being a volume of 10 ml. The sucrose density gradient procedure is inserted toward the end of the tissue digestion process, following the two cycles of washing and centrifugation. At this point, the tissue pellet was resuspended in fresh, warmed Earle's Salts and pipetted carefully to two centrifuge tubes containing the sucrose density gradient. The tubes were spun on the centrifuge at the lowest speed setting for five minutes. Following

centrifugation, each successive layer, in one tube was examined under the microscope for the presence of oocytes, which were found in the 30 and 40 percent layers. These, and the 30 and 40 percent layers of the second tube, were taken and subjected to washing with Earle's Salts and centrifugation to remove the sucrose and lessen the osmotic shock which could be attributed to the sucrose. After two cycles of washing and centrifugation, the pellets were resuspended in culture media and aliquoted to dishes by methods previously described. Subsequent attempts utilizing the sucrose density gradient, did not involve screening, in an attempt to reduce osmotic shock. Evaluation of the sucrose density gradient was made in terms of oocyte recovery and possible effects on maintenance of oocytes in culture. To do this, one-half of the digested tissue was plated and cultured normally, while the other one-half went through the sucrose density gradient, followed by standard plating and culture. The experiment involved 50 dishes distributed among five media change schedules: 12, 24, 48, 72 and 96 hours. Ten dishes were in each treatment, with five taken from the group of cells from the standard isolation procedure and the remaining five from the digested tissue which had been put through the sucrose density gradient. Just before the media was changed, a 1 ml sample was taken from a randomly selected dish in each respective isolation treatment, i.e. one sample from a regular isolation dish, one from a sucrose isolation. The media samples were stored in a labeled vial

and frozen for subsequent progesterone radioimmunoassay (RIA). Coverslips were also taken, the attached cells stained and mounted for use in determining, in conjunction with the RIA results, the cell density and metabolic activity, to evaluate the efficacy of the sucrose density gradient for isolating oocytes and to try to evaluate the effects of the follicular cells on oocytes in culture. Standard counting procedures were performed to evaluate if the sucrose density gradient had any effect on the number of oocytes isolated.

The use of the gridded dishes permitted attempts to locate and follow specific oocytes over the course of the experiments. This was done by marking a reference point on the dish and dividing it into nine quadrants. Ten areas were delineated for observation on the basis that they could be consistently found. Each dish was observed daily in these ten areas and the presence and size of oocytes was scored. If successful, this method would permit observation and evaluation of particular oocytes, providing better data for drawing conclusions about oocyte response to various attempts to stimulate oocyte growth.

In two instances, it was possible to utilize fetal ovaries, but unfortunately, contamination ruined the experiments. However, these short-lived experiments will be discussed.

Chapter 4

RESULTS

ENZYME DIGESTION AND OOCYTE RECOVERY

Recovery of primary oocytes through enzymatic digestion of ovarian tissue is possible and both trypsin and collagenase are suitable for use in such procedures. Trypsin digestion seemed to result in more evidence of tissue clumping than collagenase digestion. In such cases, there were more aggregates of cells and many, if not most, of the primary oocytes tended to have large numbers of follicular cells still attached. Primary oocytes which had been completely stripped of granulosa cells sometimes showed signs of overdigestion, evidenced by disruption of the cellular membrane. In contrast, collagenase digestion resulted in fewer observed instances of tissue clumping. There were numerous primary oocytes which had been stripped of granulosa cells but showed no evidence of overdigestion.

Neither enzyme was clearly superior in its ability to liberate or denude primary oocytes. The number of oocytes liberated by either enzyme were probably equal, however; since the intial isolation experiments utilized culture flasks for the incubation of the digested tissue, no reliable oocyte counts could be made. The oocytes liberated by digestion with either enzyme varied from being completely stripped, or denuded, to oocytes which had a large number of follicular cells still attached.

The decision to use collagenase exclusively was made on the basis of its ability to successfully liberate primary oocytes in a denuded state without resulting in large oocyte loss due to overdigestion. Collagenase is highly selective for collagen as its substrate and possesses very little general proteolytic activity. Its use decreases the chances of overdigestion possible with more general proteolytic enzymes such as trypsin. Since the connective tissue of the ovary, as with most other animal tissues, is composed primarily of collagen, the use of collagenase matches more closely the tissue digestion agent with the tissue to be digested.

ISOLATED OOCYTES

The initial enzyme digestion trials resulted in the liberation of oocytes of all size classes, ranging from primary oocytes to fully grown preovulatory oocytes possessing well developed zona pellucidae. When antral follicles were punctured or excised prior to tissue digestion, a more homogeneous population of oocytes was obtained. This step was incorporated into all subsequent isolation trials.

The primary oocytes isolated varied in size from approximately 23u to 31u in diameter. Photomicrographs of primary oocytes stained with Hematoxylin and Eosin (H + E) during culture are shown in Figures 2 through 4. Primary oocytes isolated with the aid of a mouth aspirated 10 ul pippette and stained with H + E are presented in Figures 5 and 6. The size and appearance of these oocytes is similar to the stained

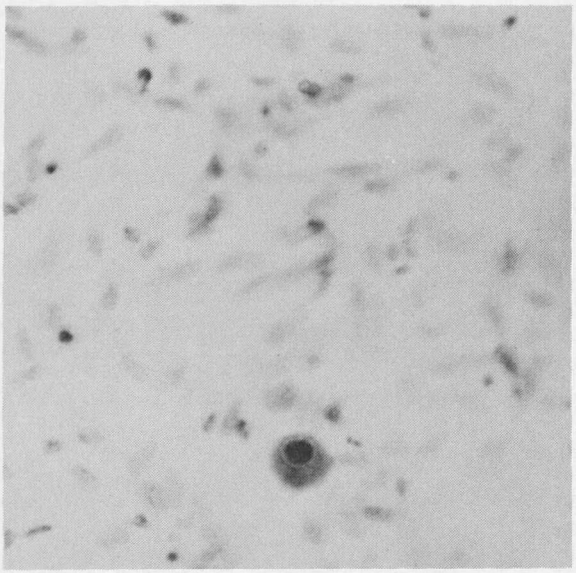

Figure 2. Primary oocyte after 1 day *in vitro* culture. Oocyte diameter approximately 31 μ. Magnification 300.

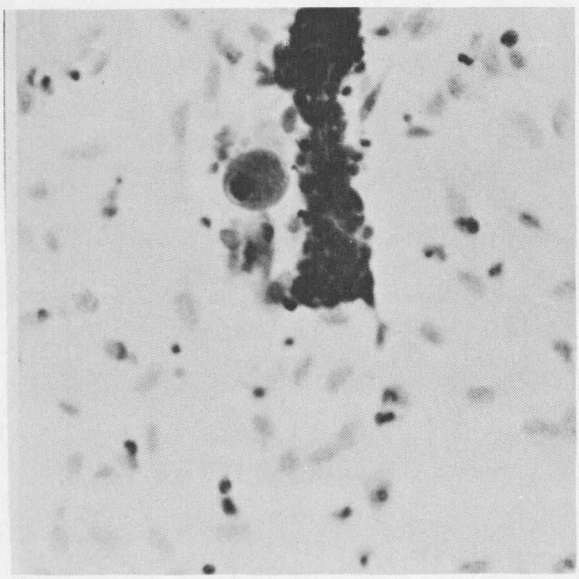

Figure 3. Primary oocyte after 1 day *in vitro* culture. Oocyte diameter approximately 28 μ. Magnification 300.

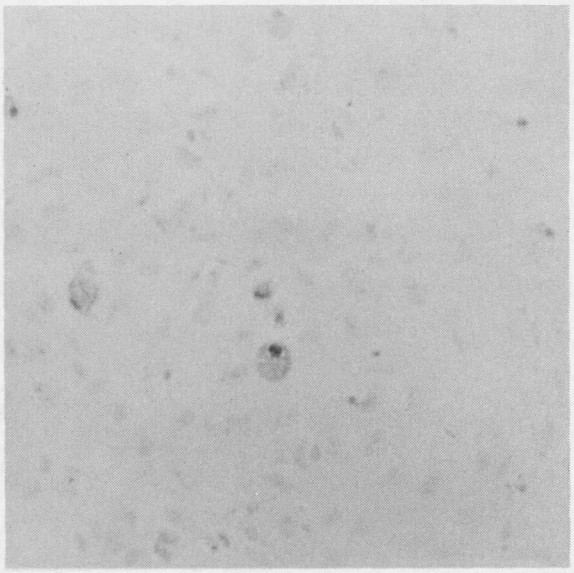

Figure 4. Primary oocyte after 13 days of *in vitro* culture. Magnification 300.

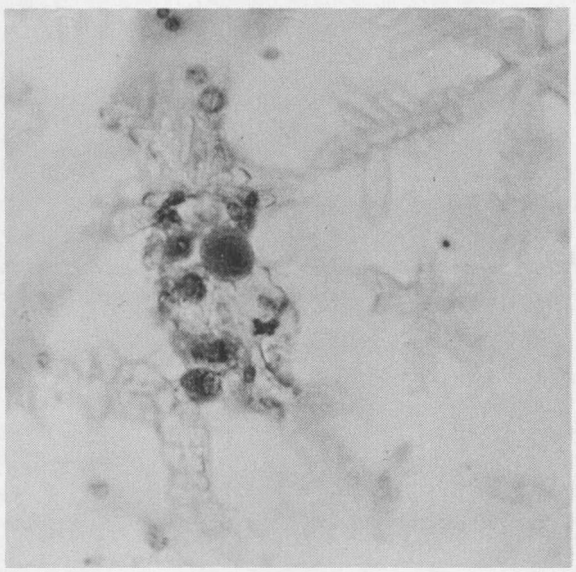

Figure 5. Primary oocyte aspirated with a capillary pipette after 11 days of *in vitro* culture. Oocyte diameter approximately 24 µ. Magnification 300.

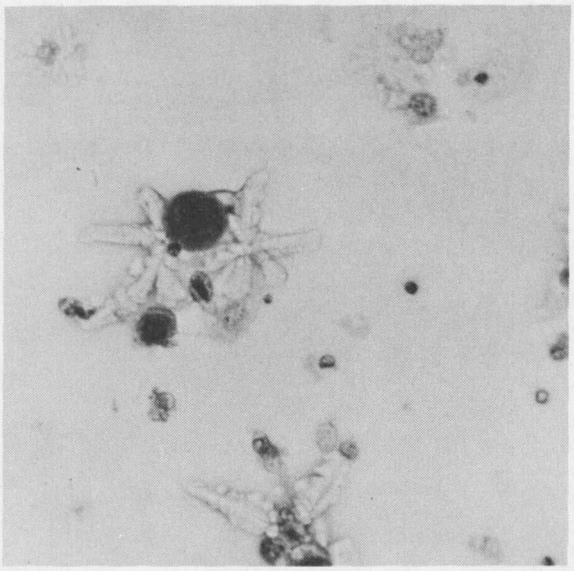

Figure 6. Primary oocyte aspirated with a capillary pipette after 11 days of *in vitro* culture. Oocyte diameter approximately 27 μ. Magnification 300 X.

sections of bovine ovaries made by Strickland (1976) and are similar to oocytes of other species (hamster - Adams and Hertig 1964; mouse - Sorensen and Wassarman 1976).

The number of oocytes isolated enzymatically ranged from zero to an estimated eleven thousand as shown in Table 1. Also shown is the one successful attempt utilizing sucrose density gradient centrifugation. In this trial, the sucrose density gradient had no effect on the number of oocytes isolated. Subsequent attempts utilizing the sucrose density gradient failed for, as yet, undetermined reasons. The first trial using the gradient was accompanied by quick visual observation of the successive sucrose layers. Since the oocytes were found in the 30 and 40 percent sucrose layers, the next trial was not accompanied by visual observation. Instead the 30 and 40 percent sucrose layers were extracted and used immediately in an attempt to reduce osmotic shock. When this failed to yield any results, visual observations were made during the third attempt. In the third trial no oocytes were observed and the sucrose density gradient was not used again.

PRIMARY OOCYTES IN CULTURE

Primary oocytes are easily identifiable during the initial phases of culture because they are the largest cell visible. They become difficult to discern after four to five days of culture because of the growth characteristics of the other cells in culture. These cells exhibit fibroblast-like growth patterns and in many instances the

Table 1

OOCYTES ISOLATED THROUGH COLLAGENASE DIGESTION OF OVARIAN TISSUE

Technique	No. of Oocytes	No. of Oocytes/ Mg Tissue
Regular Isolation	6230	10.4
Regular Isolation	11000	-
Regular Isolation	0	-
Combined	3166	1.8
A. Regular Isolation	1500	
B. Sucrose Density Gradient	1666	
Sucrose Density Gradient	0	-
Sucrose Density Gradient	0	-

cells spread to such an extent that the nuclei exceed 20u in diameter, roughly the diameter of a primary oocyte. In such cases, the resolution of the transmitted light inverted microscope was insufficient to distinguish a primary oocyte from an enlarged nucleus.

Initially many oocytes were observed to be floating or only weakly attached to the culture dish and only a small number of oocytes exhibited relatively strong attachment. This made following particular oocytes for the duration of the culture period impossible. The use of gridded culture dishes was not sufficient to permit this type of observation and other factors, i.e. coverslip movement, media changes, hindered attempts to follow particular oocytes. Time-lapse cinemicrography could effectively be used to rectify the situation.

The depletion of material during the experimental period as a result of gathering material for fixation and staining, negated any estimation of the survival rate of primary ooctyes during moderate periods of *in vitro* culture. Generally, the presence of oocytes became more difficult to detect as culture progressed. This decline in the number of observed oocytes was attributed primarily to death loss, however; mechanical manipulatory techniques, such as media changes, could break the oocytes' fragile attachment and contribute significantly to the decline in the number of observed oocytes. Figure 4 illustrates an oocyte which has survived thirteen days of culture, demonstrating the oocytes' ability to survive under relatively standard culture

conditions.

The Ham's F-10 nutrient mixture, supplemented with calf serum and antibiotics was satisfactory for the culture of primary oocytes, though during the initial experiments, Brinster's Medium (BMOC-3) was also used for the culture of oocytes. Neither medium was clearly superior, but Ham's F-10 contains a wider variety of nutrients. Since the nutritional requirements of primary oocytes have not been defined, it was thought that using Ham's F-10 might help to ensure the viability of primary oocytes in culture.

STIMULATION OF OOCYTE GROWTH

Attempts to hormonally stimulate growth of primary oocytes met with no success. Neither PMSG nor HCG alone or in combination stimulated oocyte growth when added to the media. Also unsuccessful was cAMP when added to the media in several concentrations. Table 2 summarizes the results of the stimulation trials. Evidently hormones, especially gonadotropins which can be used to support and enhance the growth of large oocytes *in vivo*, do not work under *in vitro* conditions, in the initiation of the growth of oocytes derived from the non-growing pool. However, these attempts were only preliminary and a role for hormones in the initiation of oocyte growth cannot be discounted.

OTHER ASPECTS OF CULTURE

Although oocytes can be liberated through enzyme digestion

Table 2

EFFECT OF HORMONE ADDITION TO THE CULTURE MEDIA FOR STIMULATION OF OOCYTE GROWTH

Hormone	Concentration	No. of Cultures Treated	Response[a]
PMSG	1000 IU/ml	5	-
HCG	1200 IU/ml	3	-
PMSG + HCG	1000 + 800 IU/ml	3	-
cAMP	10 ug/ml	10	-
cAMP	100 ug/ml	10	-
cAMP	1000 ug/ml	10	-

[a] + indicates stimulation of oocyte growth.
- indicates no effect.

techniques, the cultures initiated with such procedures also contain follicular and other ovarian cells derived from the cortex of the ovary. These cells are quite amenable to culture, exhibiting rapid attachment to the surface of the culture dish and fibroblast growth patterns. Mitotic figures are prevalent during the early stages of culture and the cells remain viable during extended culture periods.

The synthetic activity of ovarian cells in culture has been well documented (Mills 1979, Thanki and Channing 1979). However, the effects of this synthetic activity on primary ooctyes in culture has not been determined. By using the sucrose density gradient, it was hoped that the effects of the cultured ovarian cells on the primary oocyte would be diminished or eliminated. Progesterone synthesis was measured by radioimmunoassay (RIA) and reflected the viability and activity of the cells in culture. When the sucrose density gradient was used successfully, it had no effect on the number of oocytes isolated, but it reduced the number of other ovarian cells in culture by 60 percent. Figures 7 through 14 are paired micrographs which show the population of ovarian cells during the beginning, middle and latter stages of culture. There is a dramatic difference in cell numbers between the isolation groups at the beginning of the culture period (figures 7-8). However, as culture progresses, the cells which passed through the gradient multiply rapidly and by the end of the culture period there is no difference between the isolation groups in

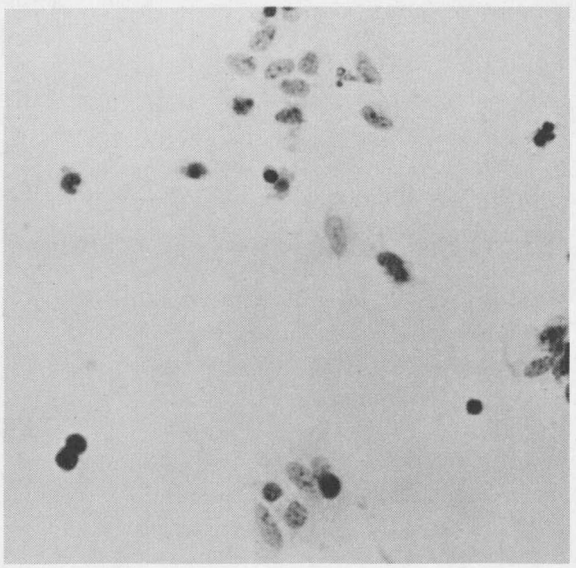

Figure 7. Cell population of sucrose density gradient isolation following 1 day of *in vitro* culture. Magnification 400 X.

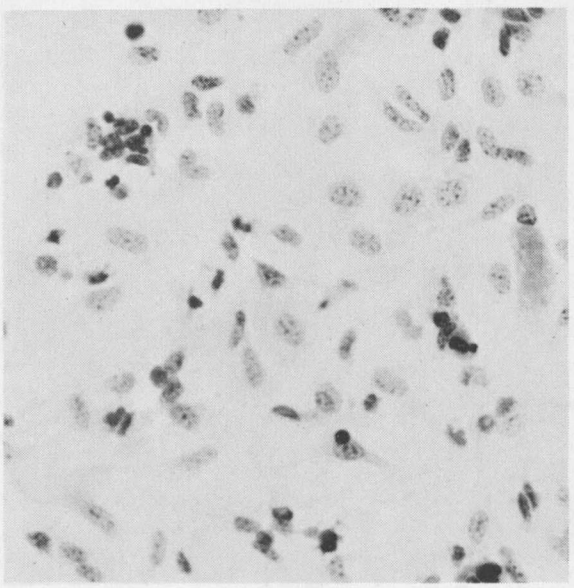

Figure 8. Cell population of normal collagenase isolation following 1 day of *in vitro* culture. Magnification 400 X.

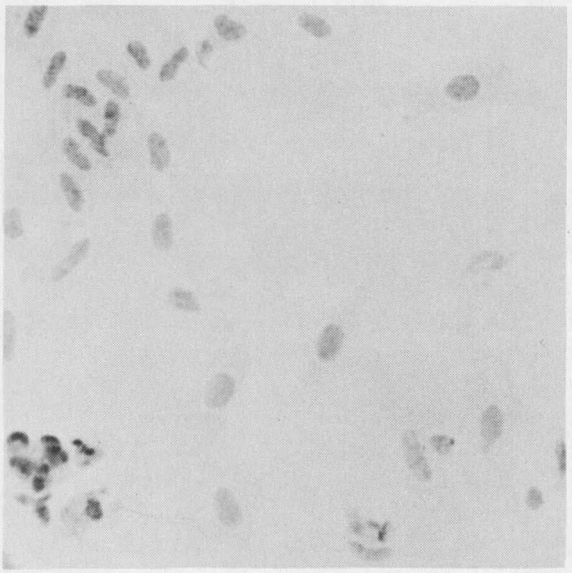

Figure 9. Cell population of sucrose density gradient isolation after 36 hours of *in vitro* culture. Magnification 400 X.

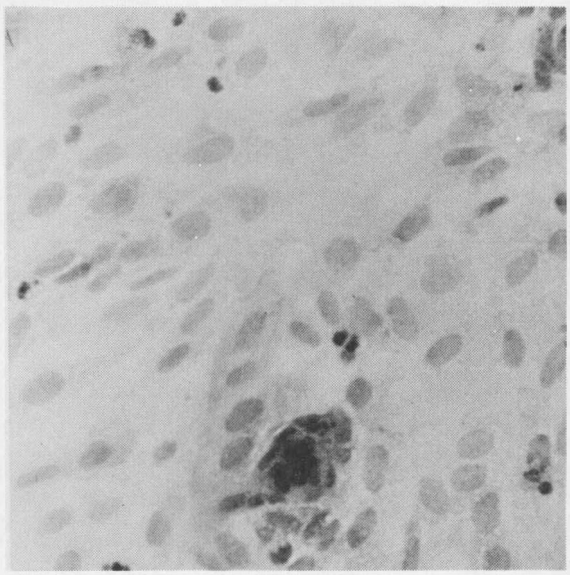

Figure 10. Cell population of normal collagenase isolation following 36 hours of *in vitro* culture. Magnification 400 X.

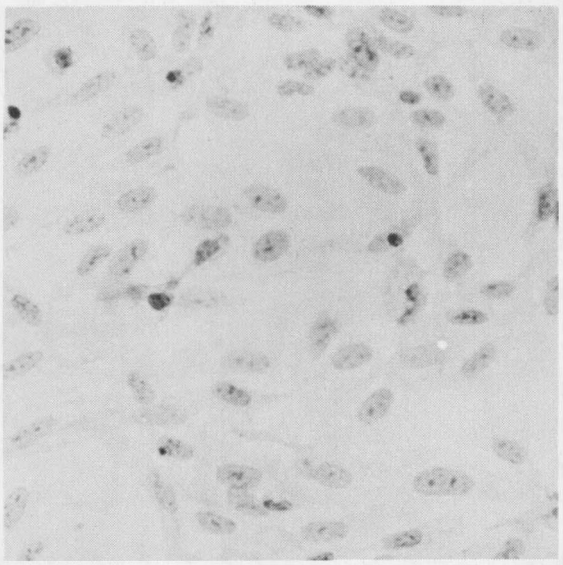

Figure 11. Cell population of sucrose density gradient isolation following 12 days of in vitro culture. Magnification 400 X.

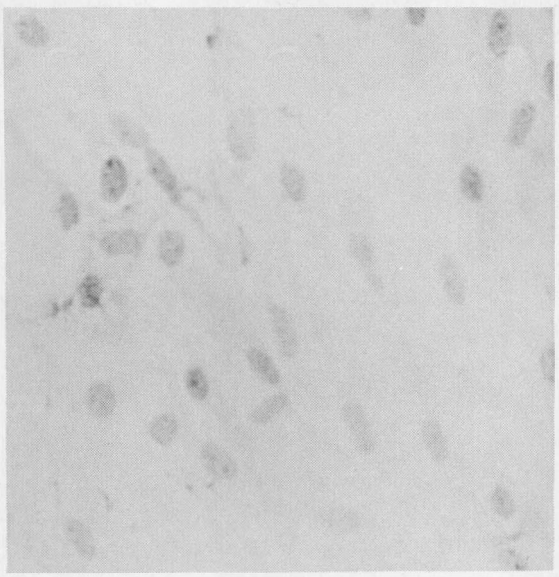

Figure 12. Cell population of normal collagenase isolation following 12 days of in vitro culture. Magnification 400 X.

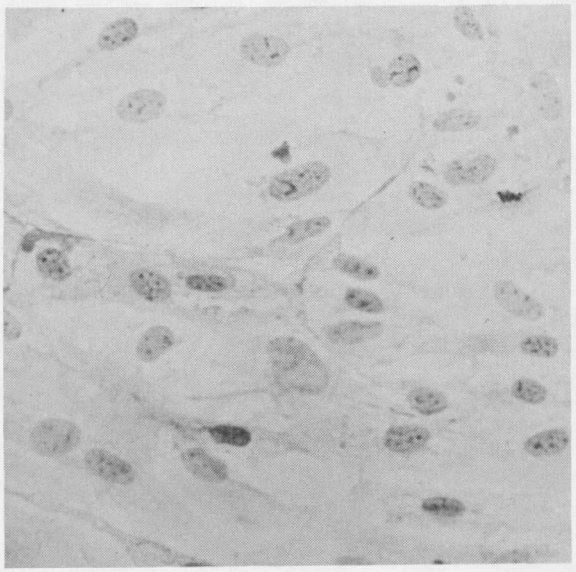

Figure 13. Cell population of sucrose density gradient isolation following 13 days of *in vitro* culture. Magnification 400 X.

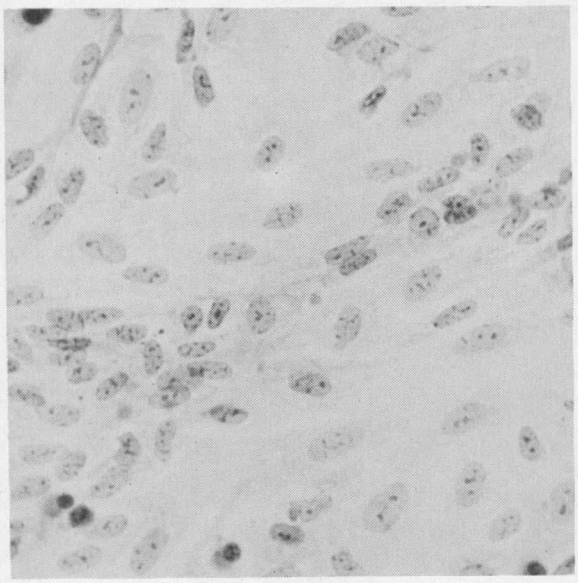

Figure 14. Cell population of normal collagenase isolation following 13 days of *in vitro* culture. Magnification 400 X.

terms of cell numbers (figures 13-14).

The summarizations of the progesterone RIA are shown in figures 15 through 19, illustrating that the use of the sucrose gradient effectively decreased the amount of progesterone present during the initial stages of culture. This disparity in the level of progesterone production could easily be attributed to the smaller cell population of the sucrose density isolation groups. As culture progressed, the cells in this group became more numerous. Consequently, as the cell population increased, the production of progesterone increased, surpassing the progesterone production of the regular isolation group by 4 to 6 days of culture. The regular isolation group generally exhibited high initial progesterone production and a decline in production as culture progressed.

Though this experiment was never successfully repeated, it is indicative of the processes that have been occurring in previous experiments. The cells in other experiments have demonstrated similar characteristics and the progesterone data lend credence to the assumption that isolation of bovine primary oocytes also liberates a population of multipotential ovarian cells which become steroidogenic during *in vitro* culture. These cells develop stores of cytoplasmic lipids (figures 20 and 21) which can be mobilized for synthesis of steroids and other, as yet, undetermined products.